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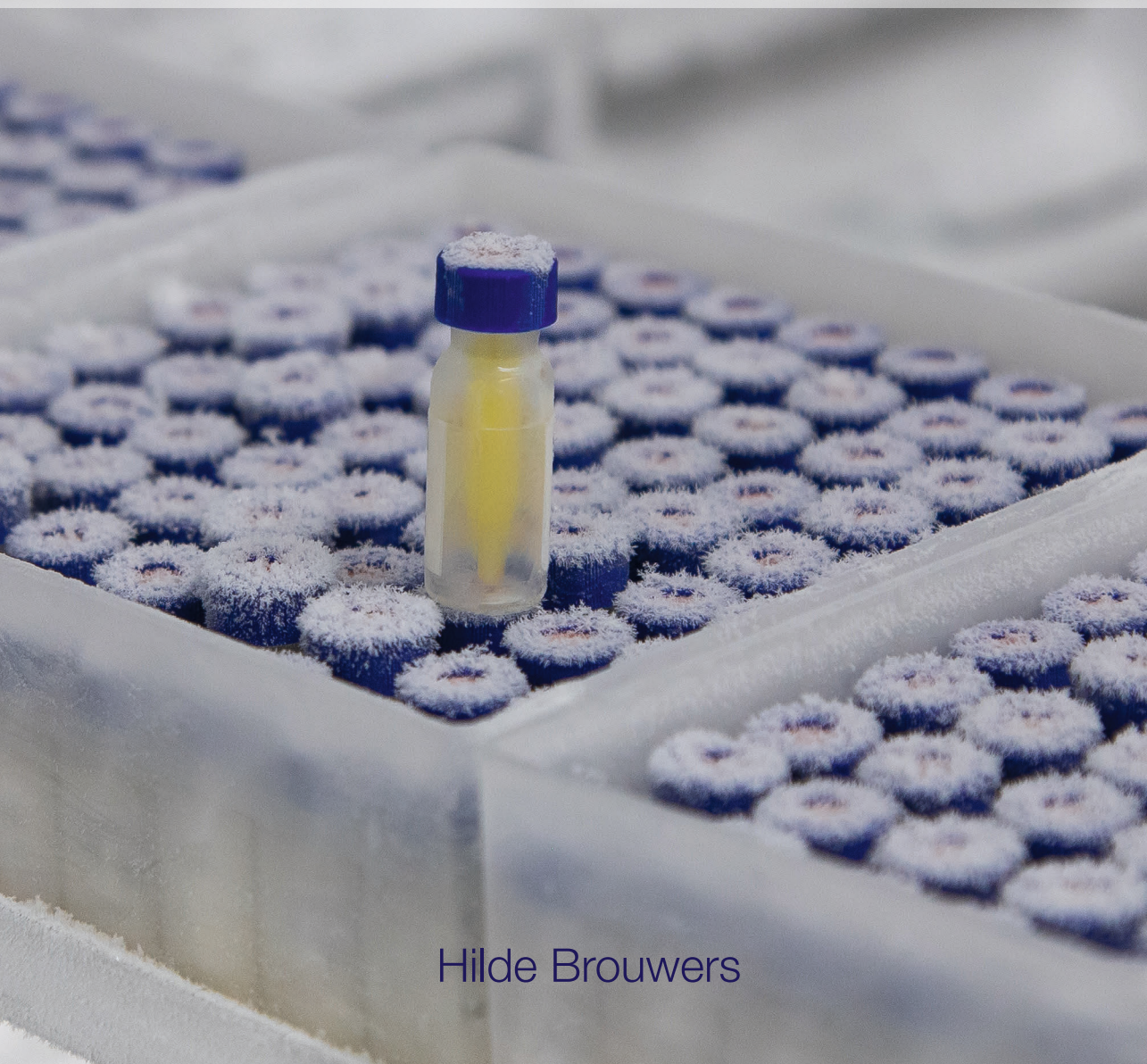

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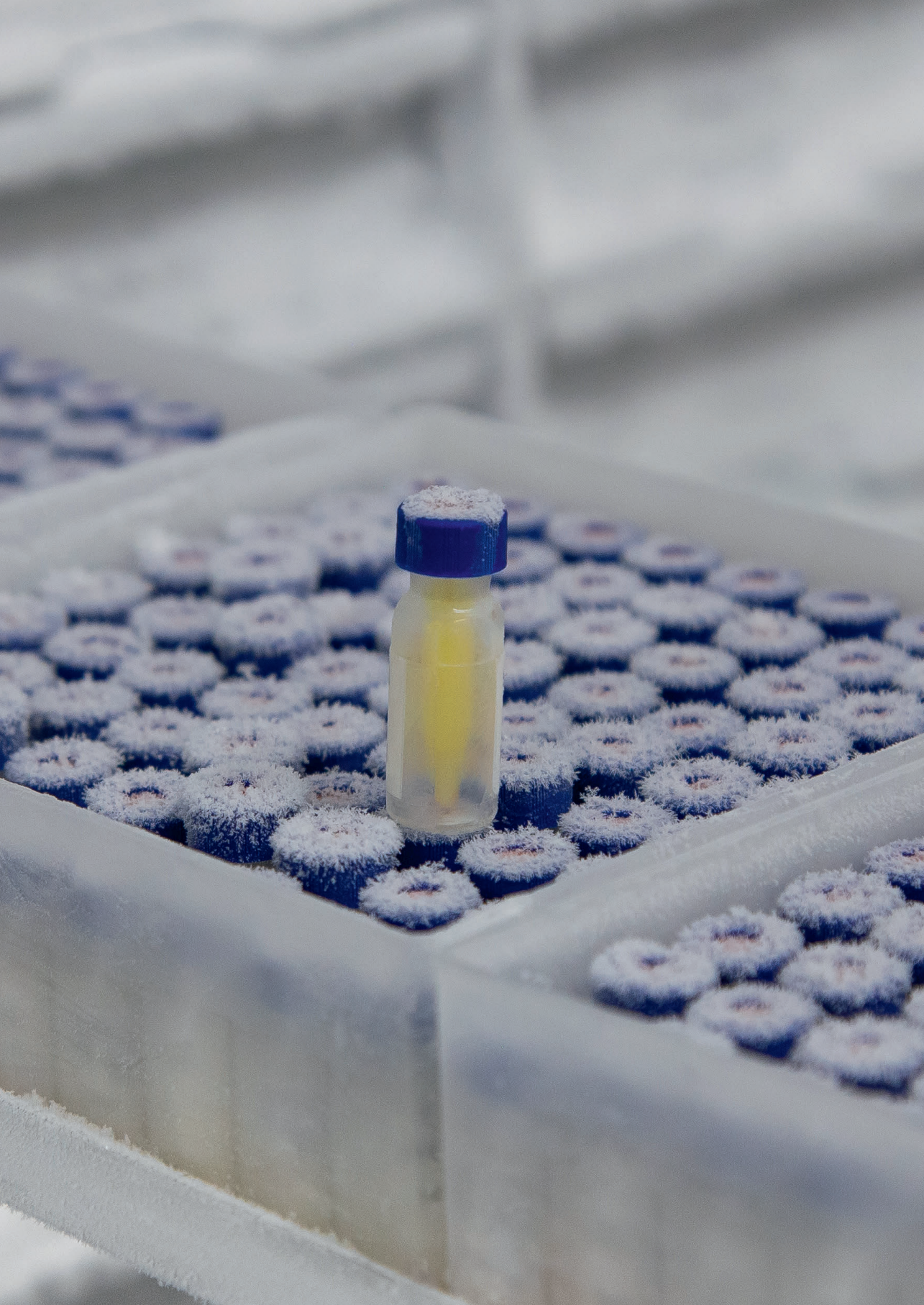


1

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.

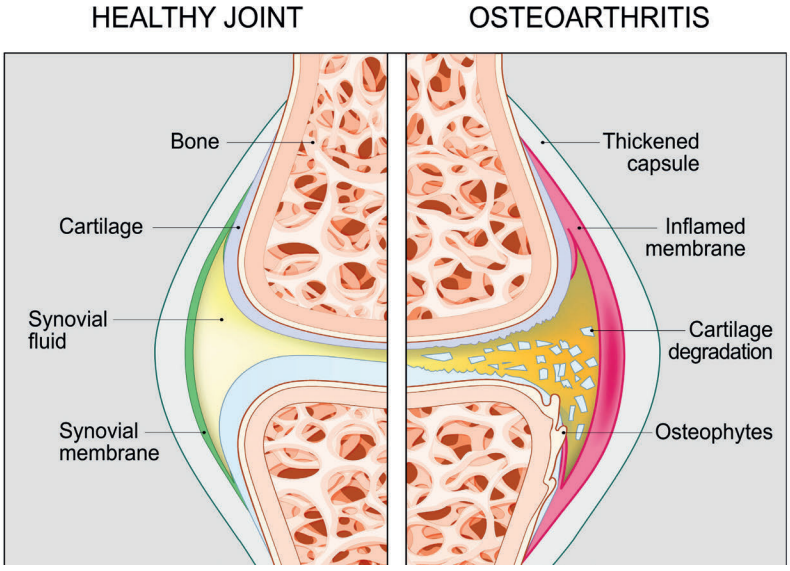


Figure 1. Healthy and OA joint

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.

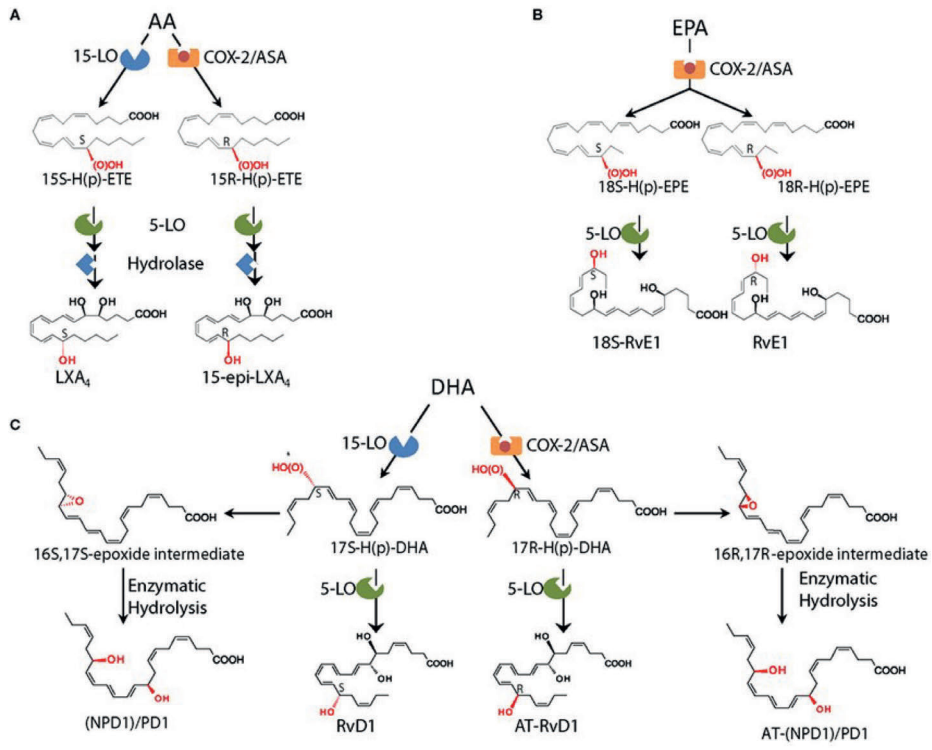


Figure 2. Biosynthesis of SPM. Reprinted from(61). AT: Aspirin-triggered; COX-2/ASA: Aspirin acetylated COX-2; H(p)-ETE: hydro(per)oxyeicosatetraenoic acid; H(p)-EPE: hydro(per) oxyeicosapentaenoic acid; H(p)-DHA: hydro(per)oxydocosahexaenoic acid; LO: lipoxygenase

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.

References

1. Artrose | Cijfers & Context | Huidige situatie | Volksgezondheidenzorg.info. https://www.volksgezondheidenzorg.info/onderwerp/artrose/cijfers-context/huidige-situatie#!node-prevalentie-en-aantal-nieuwe-gevallen-van-artrose-huisartsenpraktijk. Accessed May 12, 2020.
2. Kolasinski SL, Neogi T, Hochberg MC, et al. 2019 American College of Rheumatology/Arthritis Foundation Guideline for the Management of Osteoarthritis of the Hand, Hip, and Knee. *Arthritis Rheumatol*. 2020;72(2):220-233. doi:10.1002/art.41142
3. Kloppenburg M, Ramonda R, Bobacz K, et al. Etanercept in patients with inflammatory hand osteoarthritis (EHOA): A multicentre, randomised, double-blind, placebo-controlled trial. *Ann Rheum Dis*. 2018;77(12):1757-1764. doi:10.1136/annrheumdis-2018-213202
4. Kloppenburg M, Peterfy C, Haugen IK, et al. Phase IIa, placebo-controlled, randomised study of lutikizumab, an anti-interleukin-1α and anti-interleukin-1β dual variable domain immunoglobulin, in patients with erosive hand osteoarthritis. *Ann Rheum Dis*. 2019;78(3):413-420. doi:10.1136/annrheumdis-2018-213336
5. Fleischmann RM, Bliddal H, Blanco FJ, et al. A Phase II Trial of Lutikizumab, an Anti–Interleukin-1α/β Dual Variable Domain Immunoglobulin, in Knee Osteoarthritis Patients With Synovitis. *Arthritis Rheumatol*. 2019;71(7):1056-1069. doi:10.1002/art.40840
6. Gato-Calvo L, Magalhaes J, Ruiz-Romero C, Blanco FJ, Burguera EF. Platelet-rich plasma in osteoarthritis treatment: Review of current evidence. *Ther Adv Chronic Dis*. 2019;10:1-18. doi:10.1177/2040622319825567
7. Whitney KE, Liebowitz A, Bolia IK, et al. Current perspectives on biological approaches for osteoarthritis. *Ann N Y Acad Sci*. 2017;1410(1):26-43. doi:10.1111/nyas.13554
8. Scott DL, Wolfe F, Huizinga TWJ. Rheumatoid arthritis. In: *The Lancet*. Vol 376. Lancet Publishing Group; 2010:1094-1108. doi:10.1016/S0140-6736(10)60826-4
9. van Delft MAM, van Beest S, Kloppenburg M, Trouw LA, Ioan-Facsinay A. Presence of autoantibodies in erosive hand osteoarthritis and association with clinical presentation. *J Rheumatol*. 2019;46(1):101-105. doi:10.3899/jrheum.180256
10. Brown TD, Johnston RC, Saltzman CL, Marsh JL, Buckwalter JA. Posttraumatic Osteoarthritis: A First Estimate of Incidence, Prevalence, and Burden of Disease. *J Orthop Trauma*. 2006;20(10):739-744. doi:10.1097/01.bot.0000246468.80635.ef
11. Kraus VB, Blanco FJ, Englund M, Karsdal MA, Lohmander LS. Call for Standardized Definitions of Osteoarthritis and Risk Stratification for Clinical Trials and Clinical Use. *Osteoarthritis Cartilage*. 2015;23(8):1233. doi:10.1016/J.JOCA.2015.03.036
12. Reyes C, Leyland KM, Peat G, Cooper C, Arden NK, Prieto-Alhambra D. Association Between Overweight and Obesity and Risk of Clinically Diagnosed Knee, Hip, and Hand Osteoarthritis: A Population-Based Cohort Study. *Arthritis Rheumatol*. 2016;68(8):1869-1875. doi:10.1002/art.39707
13. Goldring MB, Otero M. Inflammation in osteoarthritis. *Curr Opin Rheumatol*. 2011;23(5):471-478. doi:10.1097/BOR.0b013e328349c2b1
14. Guilak F. Biomechanical factors in osteoarthritis. *Best Pract Res Clin Rheumatol*. 2011;25(6):815-823. doi:10.1016/j.berh.2011.11.013
15. Ioan-Facsinay A, Kloppenburg M. Bioactive lipids in osteoarthritis. *Curr Opin Rheumatol*. 2018;30(1):108-113. doi:10.1097/BOR.0000000000000463

16. Heidari B. Knee osteoarthritis prevalence, risk factors, pathogenesis and features: Part I. *Casp J Intern Med*. 2011;2(2):205-212. /pmc/articles/PMC3766936/?report=abstract. Accessed July 22, 2020.

17. Palazzo C, Nguyen C, Lefevre-Colau MM, Rannou F, Poiraudeau S. Risk factors and burden of osteoarthritis. *Ann Phys Rehabil Med*. 2016;59(3):134-138. doi:10.1016/j.rehab.2016.01.006

18. Deane KD, Demoruelle MK, Kelmenson LB, Kuhn KA, Norris JM, Holers VM. Genetic and environmental risk factors for rheumatoid arthritis. *Best Pract Res Clin Rheumatol*. 2017;31(1):3-18. doi:10.1016/j.berh.2017.08.003

19. Altman R, Asch E, Bloch D, et al. Development of criteria for the classification and reporting of osteoarthritis: Classification of osteoarthritis of the knee. *Arthritis Rheum*. 1986;29(8):1039-1049. doi:10.1002/art.1780290816

20. Arnett FC, Edworthy SM, Bloch DA, et al. The american rheumatism association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum*. 1988;31(3):315-324. doi:10.1002/art.1780310302

21. De Lange-Brokaar BJE, Ioan-Facsinay A, Yusuf E, et al. Association of pain in knee osteoarthritis with distinct patterns of synovitis. *Arthritis Rheumatol*. 2015;67(3):733-740. doi:10.1002/art.38965

22. Krasnokutsky S, Belitskaya-Lévy I, Bencardino J, et al. Quantitative magnetic resonance imaging evidence of synovial proliferation is associated with radiographic severity of knee osteoarthritis. *Arthritis Rheum*. 2011;63(10):2983-2991. doi:10.1002/art.30471

23. Kortekaas MC, Kwok WY, Reijnierse M, Watt I, Huizinga TWJ, Kloppenburg M. Pain in hand osteoarthritis is associated with inflammation: The value of ultrasound. *Ann Rheum Dis*. 2010;69(7):1367-1369. doi:10.1136/ard.2009.124875

24. de Lange-Brokaar BJE, Ioan-Facsinay A, Yusuf E, et al. Evolution of synovitis in osteoarthritic knees and its association with clinical features. *Osteoarthr Cartil*. 2016;24(11):1867-1874. doi:10.1016/j.joca.2016.05.021

25. Kroon FPB, Kortekaas MC, Boonen A, et al. Results of a 6-week treatment with 10 mg prednisolone in patients with hand osteoarthritis (HOPE): a double-blind, randomised, placebo-controlled trial. *Lancet*. 2019;394(10213):1993-2001. doi:10.1016/S0140-6736(19)32489-4

26. Kortekaas MC, Kwok WY, Reijnierse M, Kloppenburg M. Inflammatory ultrasound features show independent associations with progression of structural damage after over 2 years of follow-up in patients with hand osteoarthritis. *Ann Rheum Dis*. 2015;74(9):1720-1724. doi:10.1136/annrheumdis-2013-205003

27. Kortekaas MC, Kwok WY, Reijnierse M, Stijnen T, Kloppenburg M. Brief Report: Association of Inflammation with Development of Erosions in Patients with Hand Osteoarthritis: A Prospective Ultrasonography Study. *Arthritis Rheumatol*. 2016;68(2):392-397. doi:10.1002/art.39438

28. Damman W, Liu R, Bloem JL, Rosendaal FR, Reijnierse M, Kloppenburg M. Bone marrow lesions and synovitis on MRI associate with radiographic progression after 2 years in hand osteoarthritis. *Ann Rheum Dis*. 2017;76(1):214-217. doi:10.1136/annrheumdis-2015-209036

29. Scanzello CR, Goldring SR. The role of synovitis in osteoarthritis pathogenesis. *Bone*. 2012;51(2):249-257. doi:10.1016/j.bone.2012.02.012

30. Griffin TM, Scanzello CR. Innate inflammation and synovial macrophages in osteoarthritis pathophysiology. *Clin Exp Rheumatol*. 2019;37(5):57-63. /pmc/articles/PMC6842324/?report=abstract. Accessed November 21, 2020.

31. Blom AB, van Lent PLEM, Holthuysen AEM, et al. Synovial lining macrophages mediate osteophyte formation during experimental osteoarthritis. *Osteoarthr Cartil*. 2004;12(8):627-635. doi:10.1016/j.joca.2004.03.003

32. Bondeson J, Wainwright SD, Lauder S, Amos N, Hughes CE. The role of synovial macrophages and macrophage-produced cytokines in driving aggrecanases, matrix metalloproteinases, and other destructive and inflammatory responses in osteoarthritis. *Arthritis Res Ther*. 2006;8(6):R187. doi:10.1186/ar2099

33. Kraus VB, McDaniel G, Huebner JL, et al. Direct in vivo evidence of activated macrophages in human osteoarthritis. *Osteoarthr Cartil*. 2016;24(9):1613-1621. doi:10.1016/j.joca.2016.04.010

34. Wood MJ, Leckenby A, Reynolds G, et al. Macrophage proliferation distinguishes 2 subgroups of knee osteoarthritis patients. *JCI Insight*. 2019;4(2). doi:10.1172/jci.insight.125325

35. Culemann S, Grüneboom A, Nicolás-Ávila JÁ, et al. Locally renewing resident synovial macrophages provide a protective barrier for the joint. *Nature*. 2019;572(7771):670-675. doi:10.1038/s41586-019-1471-1

36. Berenbaum F. Osteoarthritis as an inflammatory disease (osteoarthritis is not osteoarthrosis!). *Osteoarthr Cartil*. 2013;21(1):16-21. doi:10.1016/j.joca.2012.11.012

37. Scott DL, Coulton BL, Symmons DPM, Popert AJ. LONG-TERM OUTCOME OF TREATING RHEUMATOID ARTHRITIS: RESULTS AFTER 20 YEARS. *Lancet*. 1987;329(8542):1108-1111. doi:10.1016/S0140-6736(87)91672-2

38. Elliott MJ, Maini RN, Feldmann M, et al. Treatment of rheumatoid arthritis with chimeric monoclonal antibodies to tumor necrosis factor α. *Arthritis Rheum*. 1993;36(12):1681-1690. doi:10.1002/art.1780361206

39. Fullerton JN, Gilroy DW. Resolution of inflammation: a new therapeutic frontier. *Nat Rev Drug Discov 2016 158*. 2016;15(8):551-567. doi:10.1038/nrd.2016.39

40. Serhan CN, Savill J. Resolution of inflammation: The beginning programs the end. *Nat Immunol*. 2005;6(12):1191-1197. doi:10.1038/ni1276

41. Serhan CN, Hamberg M, Samuelsson B. Trihydroxytetraenes: A novel series of compounds formed from arachidonic acid in human leukocytes. *Biochem Biophys Res Commun*. 1984;118(3):943-949. doi:10.1016/0006-291X(84)91486-4

42. Serhan CN, Hamberg M, Samuelsson B. Lipoxins: novel series of biologically active compounds formed from arachidonic acid in human leukocytes. *Proc Natl Acad Sci U S A*. 1984;81(17):5335-5339. http://www.ncbi.nlm.nih.gov/pubmed/6089195. Accessed June 1, 2018.

43. Serhan CN, Clish CB, Brannon J, Colgan SP, Chiang N, Gronert K. Novel functional sets of lipid-derived mediators with antiinflammatory actions generated from omega-3 fatty acids via cyclooxygenase 2-nonsteroidal antiinflammatory drugs and transcellular processing. *J Exp Med*. 2000;192(8):1197-1204. doi:10.1084/jem.192.8.1197

44. Serhan CN, Hong S, Gronert K, et al. Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals. *J Exp Med*. 2002;196(8):1025-1037. http://www.ncbi.nlm.nih.gov/pubmed/12391014. Accessed June 1, 2018.

45. Marcheselli VL, Hong S, Lukiw WJ, et al. Novel Docosanoids Inhibit Brain Ischemia-Reperfusion-mediated Leukocyte Infiltration and Pro-inflammatory Gene Expression. *J Biol Chem*. 2003;278(44):43807-43817. doi:10.1074/jbc.M305841200

46. Serhan CN, Gotlinger K, Hong S, et al. Anti-inflammatory actions of neuroprotectin D1/protectin D1 and its natural stereoisomers: assignments of dihydroxy-containing docosatrienes. *J Immunol*. 2006;176(6):3843.2-3843. doi:10.4049/jimmunol.176.6.3843-a

47. Serhan CN, Yang R, Martinod K, et al. Maresins: Novel macrophage mediators with potent antiinflammatory and proresolving actions. *J Exp Med*. 2009;206(1):15-23. doi:10.1084/jem.20081880

48. Deng B, Wang CW, Arnardottir HH, et al. Maresin biosynthesis and identification of maresin 2, a new anti-inflammatory and pro-resolving mediator from human macrophages. *PLoS One*. 2014;9(7). doi:10.1371/journal.pone.0102362

49. Lee TH, Horton CE, Kyan-Aung U, Haskard D, Crea AEG, Spur BW. Lipoxin A4 and lipoxin B4 inhibit chemotactic responses of human neutrophils stimulated by leukotriene B4 and N-formyl-L-methionyl-L-leucyl-L-phenylalanine. *Clin Sci*. 1989;77(2):195-203. doi:10.1042/cs0770195

50. Godson C, Mitchell S, Harvey K, Petasis NA, Hogg N, Brady HR. Cutting Edge: Lipoxins Rapidly Stimulate Nonphlogistic Phagocytosis of Apoptotic Neutrophils by Monocyte-Derived Macrophages. *J Immunol*. 2000;164(4):1663-1667. doi:10.4049/jimmunol.164.4.1663

51. Chandrasekharan JA, Sharma-Wali N. Lipoxins: Nature's way to resolve inflammation. *J Inflamm Res*. 2015;8:181-192. doi:10.2147/JIR.S90380

52. Flak MB, Colas RA, Muñoz-Atienza E, Curtis MA, Dalli J, Pitzalis C. Inflammatory arthritis disrupts gut resolution mechanisms, promoting barrier breakdown by Porphyromonas gingivalis. *JCI Insight*. 2019;4(13). doi:10.1172/jci.insight.125191

53. Serhan CN, Dalli J, Karamnov S, et al. Macrophage proresolving mediator maresin 1 stimulates tissue regeneration and controls pain. *FASEB J*. 2012;26(4):1755-1765. doi:10.1096/fj.11-201442

54. Wang CW, Yu SH, Fretwurst T, et al. Maresin 1 Promotes Wound Healing and Socket Bone Regeneration for Alveolar Ridge Preservation. *J Dent Res*. May 2020:22034520917903. doi:10.1177/0022034520917903

55. Ariel A, Chiang N, Arita M, Petasis NA, Serhan CN. Aspirin-Triggered Lipoxin A 4 and B 4 Analogs Block Extracellular Signal-Regulated Kinase-Dependent TNF-α Secretion from Human T Cells . *J Immunol*. 2003;170(12):6266-6272. doi:10.4049/jimmunol.170.12.6266

56. Chiurchiù V, Leuti A, Dalli J, et al. Proresolving lipid mediators resolvin D1, resolvin D2, and maresin 1 are critical in modulating T cell responses. *Sci Transl Med*. 2016;8(353):353ra111. doi:10.1126/scitranslmed.aaf7483

57. Krishnamoorthy N, Burkett PR, Dalli J, et al. Cutting Edge: Maresin-1 Engages Regulatory T Cells To Limit Type 2 Innate Lymphoid Cell Activation and Promote Resolution of Lung Inflammation. *J Immunol*. 2015;194(3):863-867. doi:10.4049/jimmunol.1402534

58. Ramon S, Bancos S, Serhan CN, Phipps RP. Lipoxin A4 modulates adaptive immunity by decreasing memory B-cell responses via an ALX/FPR2-dependent mechanism. *Eur J Immunol*. 2014;44(2):357-369. doi:10.1002/eji.201343316

59. Wu S-H, Chen X-Q, Liu B, Wu H-J, Dong L. Efficacy and safety of 15(R/S)-methyl-lipoxin A_4 in topical treatment of infantile eczema. *Br J Dermatol*. 2013;168(1):172-178. doi:10.1111/j.1365-2133.2012.11177.x

60. Melissa Gabbs, Leng S, Devassy JG, Monirujjaman M, Aukema HM. Advances in Our Understanding of Oxylipins Derived from Dietary PUFAs. *Adv Nutr*. 2015;6(5):513-540. doi:10.3945/AN.114.007732

61. Recchiuti A, Serhan CN. Pro-resolving lipid mediators (SPMs) and their actions in regulating miRNA in novel resolution circuits in inflammation. *Front Immunol*. 2012;3(OCT). doi:10.3389/FIMMU.2012.00298

62. Richmond GS, Smith TK. Phospholipases A 1. *Int J Mol Sci*. 2011;12(1):588-612. doi:10.3390/ijms12010588

63. Gilroy DW, Newson J, Sawmynaden P, Willoughby DA, Croxtall JD. A novel role for phospholipase A $_2$ isoforms in the checkpoint control of acute inflammation. *FASEB J*. 2004;18(3):489-498. doi:10.1096/fj.03-0837com

64. Serhan CN, Sheppard KA. Lipoxin formation during human neutrophil-platelet interactions: Evidence for the transformation of leukotriene A4 by platelet 12-lipoxygenase in vitro. *J Clin Invest*. 1990;85(3):772-780. doi:10.1172/JCI114503

65. Norris PC, Gosselin D, Reichart D, Glass CK, Dennis EA. Phospholipase A2 regulates eicosanoid class switching during inflammasome activation. *Proc Natl Acad Sci U S A*. 2014;111(35):12746-12751. doi:10.1073/pnas.1404372111

66. Archambault AS, Turcotte C, Martin C, et al. Comparison of eight 15-lipoxygenase (LO) inhibitors on the biosynthesis of 15-LO metabolites by human neutrophils and eosinophils. *PLoS One*. 2018;13(8). doi:10.1371/journal.pone.0202424

67. St-Onge M, Flamand N, Biarc J, et al. Characterization of prostaglandin E2 generation through the cyclooxygenase (COX)-2 pathway in human neutrophils. *Biochim Biophys Acta - Mol Cell Biol Lipids*. 2007;1771(9):1235-1245. doi:10.1016/j.bbalip.2007.06.002

68. von Hegedus JH, Kahnt AS, Ebert R, et al. Toll-like receptor signaling induces a temporal switch towards a resolving lipid profile in monocyte-derived macrophages. *Biochim Biophys Acta - Mol Cell Biol Lipids*. 2020;1865(9):158740. doi:10.1016/j.bbalip.2020.158740

69. Levy BD, Clish CB, Schmidt B, Gronert K, Serhan CN. Lipid mediator class switching during acute inflammation: Signals in resolution. *Nat Immunol*. 2001;2(7):612-619. doi:10.1038/89759

70. Yin P, Peter A, Franken H, et al. Preanalytical aspects and sample quality assessment in metabolomics studies of human blood. *Clin Chem*. 2013;59(5):833-845. doi:10.1373/clinchem.2012.199257

71. Colas RA, Shinohara M, Dalli J, Chiang N, Serhan CN. Identification and signature profiles for pro-resolving and inflammatory lipid mediators in human tissue. *Am J Physiol - Cell Physiol*. 2014;307(1). doi:10.1152/ajpcell.00024.2014

72. De Jager W, Bourcier K, Rijkers GT, Prakken BJ, Seyfert-Margolis V. Prerequisites for cytokine measurements in clinical trials with multiplex immunoassays. *BMC Immunol*. 2009;10:52. doi:10.1186/1471-2172-10-52

73. Wagner-Golbs A, Neuber S, Kamlage B, et al. Effects of long-term storage at –80 °C on the human plasma metabolome. *Metabolites*. 2019;9(5). doi:10.3390/metabo9050099

74. Kamlage B, Maldonado SG, Bethan B, et al. Quality markers addressing preanalytical variations of blood and plasma processing identified by broad and targeted metabolite profiling. *Clin Chem*. 2014;60(2):399-412. doi:10.1373/clinchem.2013.211979

75. Kamlage B, Neuber S, Bethan B, et al. Impact of prolonged blood incubation and extended serum storage at room temperature on the human serum metabolome. *Metabolites*. 2018;8(1). doi:10.3390/metabo8010006

76. Brouwers H, Von Hegedus J, Toes R, Kloppenburg M, Ioan-Facsinay A. Lipid mediators of inflammation in rheumatoid arthritis and osteoarthritis. *Best Pract Res Clin Rheumatol*. 2015;29(6):741-755. doi:10.1016/j.berh.2016.02.003

77. Jónasdóttir HS, Brouwers H, Kwekkeboom JC, et al. Targeted lipidomics reveals activation of resolution pathways in knee osteoarthritis in humans. *Osteoarthr Cartil*. 2017;25(7):1150-1160. doi:10.1016/J.JOCA.2017.01.018

78. Giera M, Ioan-Facsinay A, Toes R, et al. Lipid and lipid mediator profiling of human synovial fluid in rheumatoid arthritis patients by means of LC–MS/MS. *Biochim Biophys Acta - Mol Cell Biol Lipids*. 2012;1821(11):1415-1424. doi:10.1016/J.BBALIP.2012.07.011

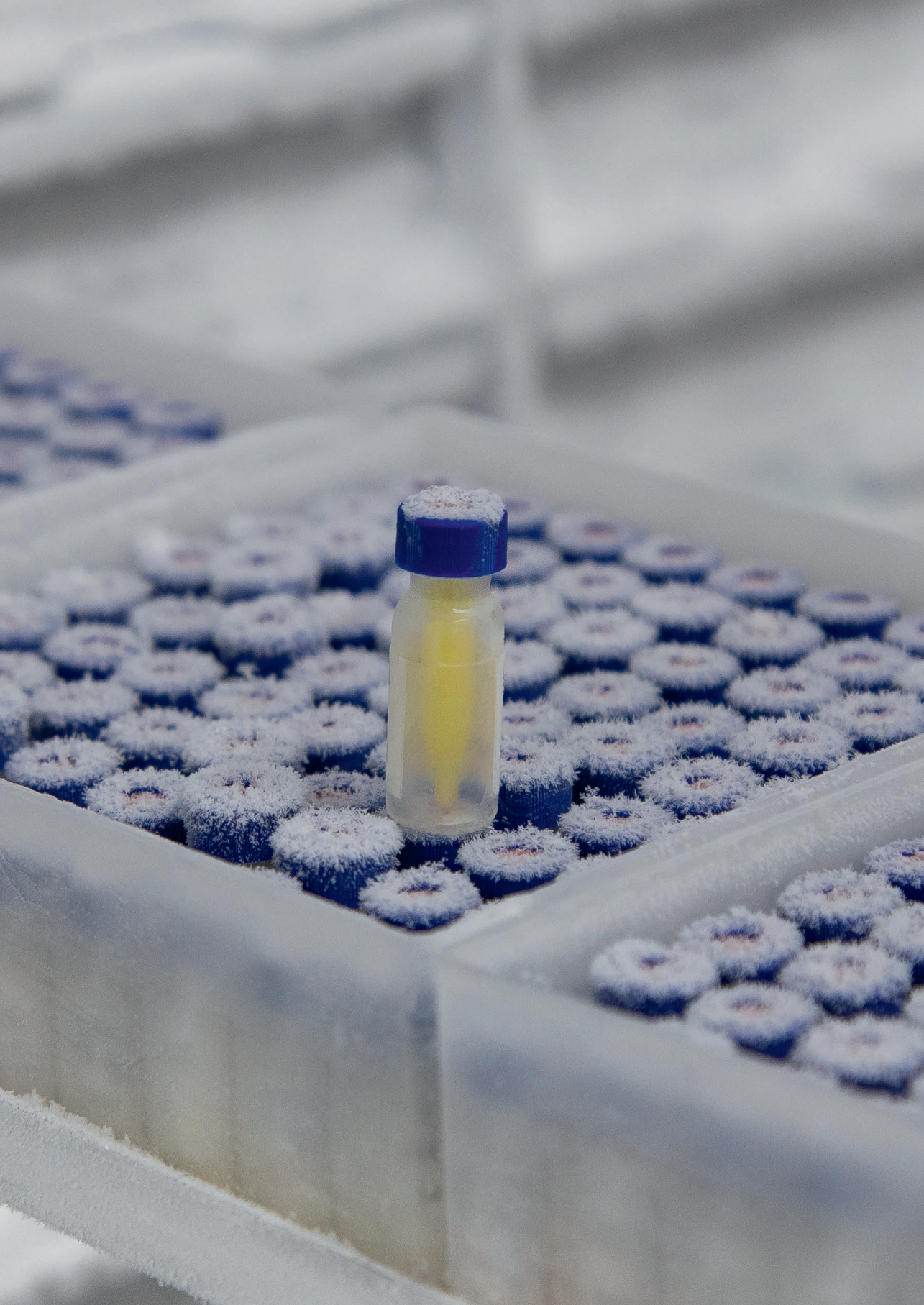
79. Griffin TM, Fermor B, Huebner JL, et al. Diet-induced obesity differentially regulates behavioral, biomechanical, and molecular risk factors for osteoarthritis in mice. *Arthritis Res Ther*. 2010;12(4):R130. doi:10.1186/ar3068

80. Sansone V, Applefield RC, de Luca P, et al. Does a high-fat diet affect the development and progression of osteoarthritis in mice?: A systematic review. *Bone Jt Res*. 2019;8(12):582-592. doi:10.1302/2046-3758.812.BJR-2019-0038.R1

81. Alarcon-Barrera JC, von Hegedus JH, Brouwers H, et al. Lipid metabolism of leukocytes in the unstimulated and activated states. *Anal Bioanal Chem*. 2020;412(10):2353-2363. doi:10.1007/s00216-020-02460-8

82. Jonasdottir HS, Brouwers H, Toes REM, Ioan-Facsinay A, Giera M. Effects of anticoagulants and storage conditions on clinical oxylipid levels in human plasma. *Biochim Biophys Acta - Mol Cell Biol Lipids*. 2018;1863(12):1511-1522. doi:10.1016/j.bbalip.2018.10.003

83. Brouwers H, von Hegedus JH, van der Linden E, et al. Hyaluronidase treatment of synovial fluid is required for accurate detection of inflammatory cells and soluble mediators. *Arthritis Res Ther*. 2022;24(1):1-10. doi:10.1186/S13075-021-02696-4/FIGURES/5



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Lipid mediators of inflammation in rheumatoid arthritis and osteoarthritis

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Abstract

Rheumatoid arthritis (RA) and osteoarthritis (OA) are inflammatory joint diseases, characterized by pain and structural damage. Besides prostaglandins, usually targeted by non-steroidal anti-inflammatory drugs, other lipids, including fatty acids, phospholipids and other bioactive lipid mediators derived from fatty acids could also contribute to RA and OA.

In this review, we will present evidence for a role of fatty acids and derivatives in RA and OA by summarizing findings related to their presence in serum and synovial fluid, as well as their association with clinical characteristics and effects on RA and OA tissues in vitro. Finally, a more direct evidence for their role in RA and OA derived from intervention studies in humans or mouse models of disease will be summarized. Based on the presented data, we will present a research agenda, in which some key unresolved questions regarding the role of lipids in RA and OA will be formulated.

Key words: Osteoarthritis, Rheumatoid arthritis, lipid mediators, lipids, inflammation, oxylipins

Introduction

Rheumatoid arthritis (RA) and osteoarthritis (OA) are joint diseases characterized by different pathophysiological mechanisms, but displaying common clinical characteristics, such as joint pain, functional impairment and structural damage which is hallmarked by bone erosions in RA and osteophytes in OA. Moreover, both diseases display joint space narrowing, reflecting cartilage loss. Another common feature of these diseases is the presence of inflammation in the majority of the patients. While inflammation is a long-established player in the pathogenesis of RA, its presence and possible role in OA has been only recently revealed. Several studies during the past 10 years have shown an association between synovial inflammation and pain on one hand and radiographic progression on the other hand, establishing inflammation as an important player also in OA (reviewed in (1,2)).

Fatty acids acquired through diet are usually transported through the body in triglycerides or phospholipids incorporated in lipoproteins, but can also be found in free form in blood. Moreover, they are present both in bound and in free form in cells, where they have various functions as energy source, membrane constituents or signalling molecules. They are essential building blocks for higher order lipids such as phospholipids, sphingolipids, glycerolipids and glycolipids. Moreover, they could be metabolized into bioactive lipid mediators such as oxylipins, including eicosanoids (prostaglandins, thromboxanes and leukotrienes) and other lipids with more anti-inflammatory and pro-resolving activity such as lipoxins, resolvins, maresins and protectins. Enzymes such as phospholipases (PLA) which release fatty acids from phospholipids, cyclooxygenases (COX) and lipoxygenases (LOX) that oxidize fatty acids are involved in generation of oxylipins. Fatty acids, higher order lipids and oxylipins can interact with inflammatory as well as tissue-resident cells, thereby contributing to various processes in the body, including inflammation, wound healing, pain, etc, and potentially playing a role in RA and OA. In general, it is believed that saturated fatty acids, n-6 polyunsaturated fatty acids (PUFA), such as arachidonic acid (AA), and AA derivatives (prostaglandins and leukotrienes) have a pro-inflammatory effect. In contrast, unsaturated fatty acids, n-3 PUFA, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), and the oxylipins derived from them (resolvins, maresins, protectins) are believed to have an anti-inflammatory function. The latter are also believed to be pro-resolving, thereby actively helping wound healing and return to tissue homeostasis after an inflammatory response.

In this review, we will present data supporting a role of lipids in RA and OA. To this end, we will summarize findings from 3 lines of evidence. First, data related to the presence of various fatty acids and their derivatives (lipid mediators) in blood or synovial fluid (SF)

of RA and OA patients will be summarized. Next, the association of these lipids with clinical disease parameters will be presented, as well as in vivo intervention studies both in humans and mouse models of disease. Finally, in vitro studies indicating the effects of these lipids on human joint tissues will be presented (summarized in fig.1). The role of cholesterol and lipoprotein metabolism in RA and OA has been reviewed elsewhere (3,4) and will not be discussed in this review.

Lipids and lipid classes described in serum of healthy individuals

In an extensive study in which the major 6 lipid categories as defined by the Lipid Maps were measured in plasma of healthy individuals, more than 500 lipid species were identified (5). The measured sample was obtained from the National Institute of Standards and Technology (USA) and represents a pooled plasma sample, obtained and stored after overnight fasting in a standardized fashion, from 100 healthy individuals between 40-50 years of age including equal numbers of men and women whose ethnicity was representative of the US population. The most abundant (on a molar basis) were sterols (including cholesterol), followed by triglycerides (part of lipoproteins), glycerophospholipids, free fatty acyls, sphingolipids, diacylglycerols and prenols, which were the least abundant. In terms of free fatty acids, oleic acid, followed by palmitic acid and stearic acid were the most abundant and comprised approximately 78% of all free fatty acids after overnight fasting. The most abundant PUFA were linoleic acid (LA) and AA, but EPA and DHA, which are derived from fish oil and are known for their anti-inflammatory effects, were also detectable. Lipid mediators such as oxylipins were also detected in plasma, with 15-deoxy-PGD_2 being the major metabolite generated by COX, while 5-HETE was the most prominent eicosanoid of the LOX pathway found in plasma (5).

This review focuses on a selection of lipids in the 6 lipid classes: fatty acids either in free form or incorporated in higher order lipids (especially phospholipids), as well as their bioactive lipid mediators in RA and OA (Table 1).

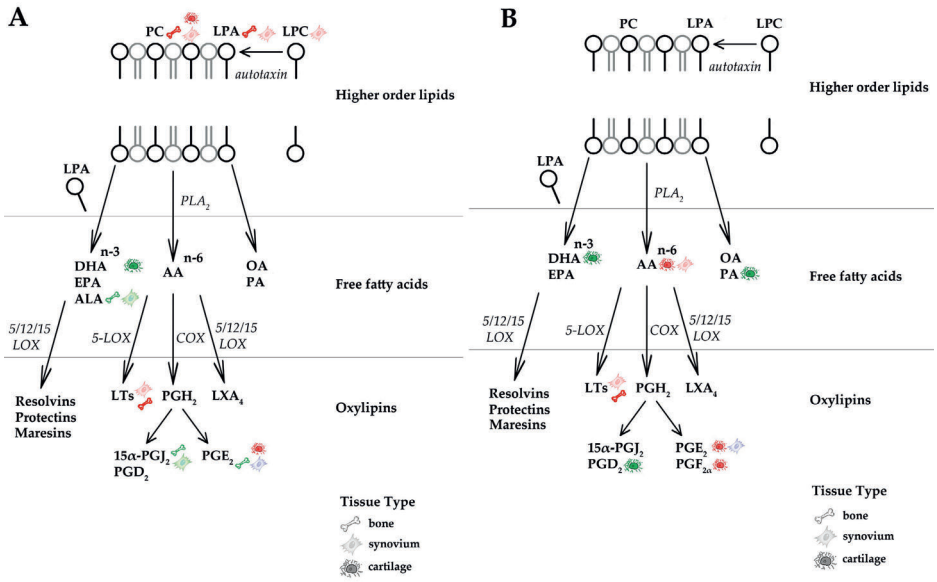

Figure 1. Summary showing lipids and relevant enzymes (in italic) in the context of rheumatoid arthritis (A) or osteoarthritis (B) discussed in this review. Lipids are incorporated in phospholipids in the membrane. The enzyme autotaxin can convert lysosphosphatidylcholine (LPC) to lysophospatidic acid (LPA), and phopholipases (PLA) can both generate LPC from phosphatidylcholine (PC) and release fatty acids from the membrane phospholipids. These fatty acids can be metabolized into oxylipins by different cyclooxygenases (COX) and lipoxygenases (LOX). Symbols represent different tissues that have been shown to be affected by lipids. The effects of lipids on tissues are indicated by colours: red = inflammatory, green = anti-inflammatory/resolving, blue = both inflammatory and anti-inflammatory/resolving.

Presence of fatty acids and derivatives in RA and OA patients

Lipids in serum of RA patients

Direct comparisons between different lipids in RA serum/plasma and healthy controls were only made in few studies and generally included relatively low numbers of patients (between 10 and 16). In a metabolomics study, it was found that the levels of lipids in general were lower in serum of newly presenting RA patients compared to healthy controls although a systematic investigation of different lipid classes was not performed. To exclude the effect of disease-modifying antirheumatic drugs (DMARD) on metabolic status, patients that were taking DMARD's were excluded from this analysis (6). In another study in 166 RA patients, the levels of free fatty acids were found to be similar in RA patients and healthy controls (7), indicating that free fatty are not quantitatively different in the diseased state. Fatty acids in phospholipids and sphingomyelins were also described in serum of healthy individuals and RA patients (8). Of the different phospholipids studied, only the ratio phosphatidylcholine (PC)/lysophosphatidylcholine (LPC, generated from

PC by the PLA enzyme) was found to be lower in serum of RA patients compared to healthy individuals (9), indicating a higher activation of PLA in RA patients. This could results in higher levels of free fatty acids that can further be metabolized into bioactive lipids. Indeed, lipid mediators such as prostaglandins, PGD_2 and PGE_2 (10), generated by COX enzymes, were shown to be present in serum of RA patients, indicating that not only precursor fatty acids might be higher in RA compared to healthy individuals, but also certain enzymes involved in generation of bioactive lipids. However, bioactive lipids were mainly studied in SF.

Lipid class (according to Lipid Maps)	Lipids included in this review
Fatty acyls	Fatty acids, oxylipins: eicosanoids (prostaglandins, leukotrienes, lipoxins), derivatives of DHA, EPA (resolvins, maresins, protectins)
Glycerolipids	-
Glycerophospholipids	Phospholipids (e.g. PC) and lysophospholipids (e.g. LPC, LPA)
Sphingolipids	Sphingomyelin
Sterol lipids	-
Prenol lipids	-

Table 1. Summary of lipids included in the present review and their corresponding lipid classes. DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; PC: phosphatidylcholine; LPC: lysophosphatidylcholine; LPA: lysophospatidic acid.

Lipids in SF of RA patients

Broad lipidomic studies in SF of RA patients were performed in which most of the lipid species described in serum/plasma could be detected (8,10-13); several phospholipid and fatty acid species were found. Similarly to plasma, PC were the most abundant phospholipids in SF, followed by LPC and sphingomyelins (12) and their levels were higher in RA SF than controls (12,13). The ratio of PC/LPC was higher in RA SF than in controls, in contrast to what was found in serum (12,13). A detailed analyses of the species of lipids revealed that RA SF was relatively enriched in LPC containing saturated fatty acids, while saturated PC were lower than in controls, indicating that saturated fatty acids were possibly more efficiently released from PC in RA. The length of fatty acids contained in PC species was not different between RA SF and controls, but RA SF contained relatively more short-chain fatty acids in LPC compared to controls (12,13). As a longer chain length and higher saturation grade are believed to be beneficial for the lubricating properties of phospholipids, these data could indicate that the phospholipids present in RA SF are less potent lubricators and protect less against mechanical damage than in healthy controls. All studies were small and included no more than 20 patients.

Eicosanoids such as prostaglandins and leukotrienes were intensively investigated in SF of RA patients and were recently reviewed (14). ProstaglandinE2 (PGE_2) (14,15), as well as PLA_2 and COX, enzymes involved in its generation, were found to be higher in SF or were higher expressed in synovium of RA patients (mPGES) (16-20). Likewise, the enzyme converting PGE_2 into the inactive 15-PGDH was higher in RA synovium, indicating that regulatory mechanisms targeting PGE_2 are also activated in this disease. Interestingly and in line with this observation, the more anti-inflammatory prostaglandins, PGD_2 and its metabolite 15-deoxy-PGJ_2 were also found in the RA joint. Expression of PGD_2 was detected in cells in RA joints, however no evidence was found for enhanced biosynthesis of 15-deoxy-PGJ_2. Next to prostaglandins, leukotrienes such as LTB_4, LTD_4 and LTE_4 were found in SF of RA patients (14).

Interestingly, anti-inflammatory products of lipoxygenases were also described in SF of RA patients. These included the anti-inflammatory and pro-resolving mediators lipoxin A4 (LXA_4) and the mediators derived from the n-3 PUFA DHA: maresin 1 (Mar1) and resolving D5 (RVD5) (11). Although this study was performed in only 5 patients and although the effects of pro-resolving lipids on RA development or progression was not yet investigated, their potent immune modulatory functions (21) identify them as promising therapeutic agents for chronic inflammatory diseases.

Besides the PLA, COX and LOX enzymes, autotaxin makes an important contribution to bioactive lipids in RA. It can convert membrane phospholipids such as LPC to lysophospatidic acid (LPA), as well as sphingosylphosphorylcholine to yield sphingosine-1-phosphate (S1P). S1P can act as an intracellular second messenger or extracellular lipid mediator via G-coupled receptors and affects pro-inflammatory pathways and cell migration. Both LPA and S1P, as well as autotaxin were shown to be present in SF of RA patients (22-24), while higher autotaxin levels were shown in RA SF (n = 16) and serum (n = 26) compared to controls (25).

Lipids in serum/SF of OA

Several lipidomics studies in OA patients have been performed over the recent years. Although most of them study SF, one study investigated the phospholipid profile of hip and knee OA patients (n = 59 females) in plasma (26). Similarly to RA, the LPC/PC ratio was higher in both mild and moderate OA (based on radiographic damage) compared to controls, which is the opposite to what was found in SF. In two studies with 30 and 48 OA patients, the levels of phospholipids were found to be elevated in SF of OA patients compared to post-mortem controls (12,13), similar to what was found for RA.

Few studies have investigated oxylipin levels in OA. In one study with 10 OA patients, 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro$PGF_{2\alpha}$ were detected in both serum and SF, but the levels did not correlate in these two fluids (27). This indicates that there might be local production of these lipids, rather than systemic diffusion. Noteworthy is that levels of both metabolites were higher in serum of OA patients compared to controls. Higher levels of these metabolites in serum of OA patients could indicate higher systemic oxidative damage and inflammatory responses. For some patients, the levels of both compounds, but especially 15-keto-dihydro$PGF_{2\alpha}$ tended to be somewhat higher in SF compared to serum. These data are, however, difficult to interpret due to the lack of healthy SF and low numbers of patients. More recently, a study investigated the 15-LOX product 15-HETE and the COX product PGE_2 in plasma of symptomatic OA patients and controls (28). Both metabolites were elevated in patients (three different cohorts with 291 patients in total) compared to controls, which could be a systemic reflection of local inflammation in the knee. Similarly, leukotriene B_4 (LTB_4) has also been described in SF of some OA patients, although no comparison with healthy controls was performed (29). Together, these data indicate that pro-inflammatory lipids are present in OA patients, although a more extensive investigation of the presence of other lipid classes, such as prostaglandins and anti-inflammatory oxylipins, in OA patients is warranted.

Fatty acids and phospholipids

Dietary fatty acids, such as the n-3 PUFA EPA and DHA derived from fish oil. These n-3 PUFA are believed to have anti-inflammatory properties, while the n-6 PUFA arachidonic acid (AA) is believed to be more pro-inflammatory. Long-chain fatty acids such as AA, EPA and DHA are incorporated in phospholipids and these are essential constituents of membranes. In healthy individuals on a typical Western diet, about 10-20% of phospholipids in membrane of leukocytes are composed of the AA, while 0,5-1% is EPA and 1,5-3% is DHA (30). Dietary intake of EPA and DHA leads to an increase of these fatty acids in cellular membranes and this occurs usually at the expense of AA. This incorporation begins within days and is dose-dependent (31,32) . Because erythrocytes have a life-span of 100-120 days, their membrane phospholipid composition is generally used to monitor dietary intake of lipids (32,33).

Association with clinical characteristics and intervention studies in RA

Most studies investigating types of fatty acids present in RA patients and association with clinical parameters focused on dietary fatty acids.

Several clinical trials have been performed with either fish oil or n-3 supplementation in RA patients. These randomized control trials have been recently summarized in a

systematic review by Miles and Calder (31). The authors analysed 23 studies, in which EPA and DHA doses varied largely, between <1 and > 7g/day and were administered mostly orally as fish oil supplements. The duration of the studies varied between 4 and 52 weeks and the placebo controls were usually other types of oils, such as corn, olive oil, paraffin oil, etc. The sample size of these studies was typically around 20-30 patients/group with few exception in which less or more patients participated. Many studies had methodological shortcomings and no meta-analysis was performed. In general, beneficial effects of n-3 PUFA were observed that were related to morning stiffness, number of tender/swollen joints, grip strength, pain or disease activity. The effects were overall modest.

Since this systematic review, one other study investigated the effect of intake of moderate amounts of n-3 PUFA (2,090g EPA and 1,165g DHA) in combination with regular anti-inflammatory therapy in 109 RA patients. High oleic acid sunflower oil was used as control. Although there was an increase in n-3 PUFA and a relative decrease in n-6 PUFA AA in erythrocyte membranes in the treatment group, there was no significant effect on clinical symptoms, NSAID usage, cytokines, eicosanoids and bone turnover markers in this group (34), which might be attributable to the low dose of n-3 PUFA administered. Similar results regarding the incorporation of n-3 PUFA in erythrocyte membranes at the expense of AA were found in another study with a relative low number of patients (54 RA and 6 psoriatic arthritis patients) in which patients were randomized into 3 groups: one treated with 3g n-3 PUFA/day, one with 3,2g g-linolenic acid (n-6 PUFA)/day and one with a combination of a low dose n-3 PUFA (1,6g) and g-linolenic acid (1,8g)/day. The treatment duration was 12 weeks. A relative decrease of AA/EPA ratio was observed in the n-3 PUFA treated group, similar to earlier studies. Additionally, an enrichment in g-linolenic acid in plasma lipids, cholesterol membranes and erythrocyte membranes was observed, and this enrichment was dependent on the dose indicating that this n-6 PUFA is also dose-dependently incorporated in cellular membranes upon intake (35).

Interestingly, several recent studies have investigated effects of n-3 PUFA on early RA patients or on development of RA and related features in pre-RA individuals. Early RA patients (n = 140) treated with DMARD therapy were additionally treated with 5,5g/day EPA+DHA or with 0,4g/day EPA and DHA (control group). Primary outcome was failure on triple DMARD after 3 months. Failure was lower and the rate of remission was higher in the n-3 PUFA treated group compared to the control group after 3 months of treatment (36). Remarkably, EPA in plasma phospholipids was inversely associated with time to remission and to DMARD failure, while similar results, albeit not significant, were also obtained for DHA, indicating that EPA might be more effective than DHA as disease modulator.

Several studies investigated the effect intake of fish containing n-3 PUFA on the risk of development of RA in healthy individuals. In a meta-analysis containing both prospective and case-control studies, the authors found that for each serving of fish per week the relative risk of RA became 0.96 (95%CI 0.91;1.01), while there was a 20-24% lower risk (0.76 (95%CI 0.57;1.02) of RA for 1-3 servings of fish/week compared to no servings (37). In an observational population-based cohort study (32,232 women aged 54–89 years), self-reported intake of more than 0,21g/day of n-3 PUFA led to a 35% (95% CI 10 to 52%) lower risk of developing RA than lower intake. Moreover, long-term consistent intake of > 0,21g/day of n-3 PUFA led to a 52% (95% CI 29 to 67%) lower risk of RA than lower intake, while long-term consistent intake of more than 1 serving of fish/week led to 29% lower risk (RR 0.71; 95% CI 0.48 to 1.04) compared to <1 serving/week (38). Although the intake was based on self-reported data and no measurements of n-3 PUFA in phospholipids were presented, these studies suggest that n-3 PUFA could be beneficial for lowering the risk of RA development in pre-disease individuals and this effect is probably dose-dependent. This would also be in line with a more recent study in which individuals at risk for developing RA (non-diseased first degree relatives of RA patients and HLA-DR4+ individuals) and positive for anti-CCP antibodies (n = 30) were compared to seronegative individuals (n = 47) for the amount of n-3 PUFA intake (self-reported) and the percentage of n-3 PUFA in erythrocyte membranes. The findings indicate that anti-CCP+ individuals were less likely to report n-3 PUFA intake and the anti-CCP positivity was inversely correlated to the percentage of n-3 PUFA in erythrocyte membranes (39). All together, these findings suggest that n-3 PUFA might affect the risk for development of RA, as well as the clinical parameters of the disease in RA patients.

Supporting these data, interventions with DHA and EPA have been also shown to reduce the onset, incidence and severity of collagen-induced arthritis (CIA) in mice (40,41).

Other fatty acids have only scarcely been studied. Although not yet investigated in relation to human RA, α-lipoic acid (ALA) showed promising results in mice, as it inhibited joint inflammation and bone destruction in the CIA model both when administered intra-peritoneally (42) and through diet (43). Although the mechanisms are unclear, ALA inhibited synovial inflammation in both studies, as well as human osteoclast differentiation in vitro (42) and in mice in vivo (43).

Supporting a possible role for LPA/autotaxin axis in arthritis, inhibition of the LPA receptor inhibits development of disease in the K/BxN serum transfer model through effects on cartilage destruction and bone erosions, possibly through inhibition of osteoclast differentiation and activity and promotion of osteoblast differentiation (44). Similarly, mice lacking autotaxin in the mesenchymal cell compartment had less arthritis on a

hTNFα transgenic background, as well as in the CIA model of arthritis and this effect was likely mediated by LPA effects on synovial fibroblasts (25). Interestingly, preventive oral administration of LPC precursor PC to CIA mice also inhibited severity of CIA, possibly through inhibition of leukocyte-endothelium interactions and nitric oxide (NO) production (45). These data indicate that the PC/LPC/LPA metabolic pathway might represent a promising target for intervention in RA patients.

Association with clinical characteristics and intervention studies in OA

Two studies have measured phospholipids in both early OA and late OA patients, with early and late being defined by the Outerbridge classification scale. One of these studies assessed the difference in lipids between early (n = 17) and late OA (n = 13) and showed that concentrations of 66 phospholipid species were different between these stages and that total lipid content was higher in late OA compared to early OA (12). In addition, the PC/LPC ratio was higher in late OA then early OA, possibly indicating a higher activation of PLA_2 in early compared to late disease. A metabolomic approach can also be used to classify OA patients based on their metabolite profile. Using this approach, it was found that especially levels of carnitines and its acyl esters acylcarnitines (involved in fatty acid metabolism) in knee SF, divided a group of 80 OA patients in two distinct groups (46). The group with lower acylcarnitine levels could be further divided into two subgroups based on their glycophospholipid and sphingomyelin(SM) levels. Although the groups were not correlated to any clinical OA characteristics, these data indicate that OA patients can be divided in metabolically distinct groups. An earlier study by Kosinska *et al* did correlate the levels of SM with relevant OA features (47). All measured SF SM species were 2.4-fold higher in early OA patients (n = 17) compared to controls and 2 fold higher in late (n =13) vs early OA patients. Early and late OA patients were classified using the Outerbridge score.

Most reports studying lipids and their association with clinical parameters have studied fatty acids, rather than phospholipids or other higher order lipids. To our best knowledge, only one study investigated the association of plasma fatty acid levels with clinical features in OA patients. This study measured fasting plasma levels of fatty acids in 472 individuals, including OA patients, as well as individuals at risk of developing knee OA (48). N-3 fatty acids, in particular DHA, were inversely correlated with patellofemoral cartilage loss, but not tibiofemoral cartilage loss or synovitis at 30 months follow-up, suggesting a protective effect of this n-3 fatty acid on selected structural findings. The levels of the n-6 fatty acid AA, which is believed to be more pro-inflammatory, was positively correlated with synovitis. In line with this result, in another study in knee OA patients, the AA levels were found to correlate with histologic disease severity (49).

While fish oil or n-3 PUFA supplementation has been intensively studied in RA patients, only a few intervention studies have been performed in OA patient groups. Most of these studies aimed at reducing pain. Fourty-seven patients with knee or hip OA taking 1200 mg fish oil (total n-3 18% EPA and 12% DHA) a day for 12 weeks in a randomised trial, showed no improvement on either Visual Analogue Scale (VAS) for patient self-assessment of pain or the Health Assessment Questionnaire (HAQ) for patient self-assessment of activity (50). Also a double blind placebo controlled trial in 86 OA patients failed to detect any benefit from taking either 10 ml cod liver oil (786 mg EPA) or olive oil per day for 24 weeks, next to the regular intake of NSAIDs (51). In contrast, one study reported beneficial effects of fish oil in OA patients. A randomised, double-blind multicentre trial investigated the effect of a low dose of 0,45 grams of fish oil (18% EPA and 12% DHA) per day versus a high dose of 4,5 grams on pain and function scores in 202 knee OA patients after 2 years (52). Both groups benefitted from the treatment, although unexpectedly, the group receiving low-dose fish oil benefitted the most, with significantly lower pain scores (WOMAC index) at 18 and 24 months and better function limitation scores after 24 months. No beneficial effects were observed on cartilage volume or bone marrow lesions. It should be mentioned however, that the low-dose fish oil preparation also contained Sunola oil which includes n-9 monounsaturated oleic acid. Therefore, it is difficult to conclude whether the observed effects were due to the n-3 fatty acids alone (53).

Despite the unclear effects of n-3 PUFA on pain in humans, they were shown to reduce radiographic damage in mouse models of OA, both in spontaneous (54) and surgically-induced models (55). Interestingly, their beneficial effect was correlated to a better wound-healing, while saturated fatty acids and n-6 fatty acids had opposite effects on OA severity and wound healing (55), suggesting the existence of common mechanisms involved in both processes. Moreover, it suggests a possible benefit of n-3 PUFA on structural damage, which still needs to be investigated in humans.

Effects on RA human tissues in vitro

Free fatty acids have been described in serum and SF of RA patients and they could affect joint tissues and immune cells involved in disease pathogenesis. Indeed, in vitro treatment of RA synovial fibroblast with free fatty acids induced pro-inflammatory cytokines IL-6, IL-8, MCP-1 and MMPs and this effect was independent of the length or saturation degree of the fatty acid, but variable between donors (56). For chondrocytes, lipids isolated from SF could inhibit chondrocyte proliferation (57), while saturated fatty acids induced IL-6 (56). Endothelial cells only responded to high concentrations of fatty acids such as palmitic acid and linoleic acid, but not oleic acid (56). Taken together, these data indicate that different cell types respond differently to individual fatty acids

and that combinations of fatty acids could have a different effect than the individual components. Moreover, free fatty acids and especially dietary fatty acids can potently influence bone metabolism through regulation of PGE_2 and leukotrienes, stimulating bone resorption or prostaglandin-mediated regulation of IGF-1, a growth factor that stimulated bone formation (58). The role of these processes in bone metabolism in RA remain to be investigated.

Besides their effect on joint tissues, fatty acids also have immune modulatory effects. Effects of n-3 PUFA on cells derived from RA patients have been investigated, while much less attention has been given to other fatty acids. N-3 PUFA had an anti-inflammatory effect on cytokine secretion by monocytes derived from RA patients (59), while inhibiting formation of reactive oxygen species (60) and the AA-mediated induction of TNFRI and –II on RA neutrophils (61). Fatty acids can also affect T cell function (62) and B cells function (63), although the effects of fatty acid type, saturation and length, as well as the specific effects on T and B cells from RA patients remain to be addressed.

Some of the phospholipids found in serum or SF have been shown to affect joint tissues and cells thereby potentially contributing to RA. Among these, LPC and LPA have received special attention, as they were described to have potent immune modulatory effects (reviewed in (64)). For example LPA has been shown to induce COX2 expression in RA synovial fibroblasts, either alone or in combination with pro-inflammatory cytokines such as IL-1α or b (22), to induce their proliferation and enhance production of IL-6, VEGF, CCL 2 and MMP3, as well as expression of VCAM and migration (65). Although the effects of LPC on human RA tissues were not yet investigated, LPC was shown to induce COX2 expression in vascular endothelial cells (66) and macrophages (67), which would suggest a possible pro-inflammatory role of this lipid in RA.

Another product of autotaxin found in RA SF, S1P, was also shown to have effects on RA-derived tissues, including to stimulate proliferation of RA synovial fibroblasts (68) and enhancing COX2 and PGE_2 production in synovial fibroblasts (68) and chondrocytes (69), or by decreasing aggrecan production by chondrocytes (69) and stimulating osteoblast differentiation (70).

Together, these data indicate a pro-inflammatory effect of the PC/LPC/LPA axis in RA.

Effects on OA human tissues in vitro

While intervention studies with fatty acid supplementation mainly focused on reducing pain in patients, the in vitro studies performed focused predominantly on the effects of fatty acids on OA tissues and the mechanisms underlying these effects. Chondrocytes

were most studied in this respect and they were usually stimulated with cytokines believed to have a prominent role in OA. Chondrocytes from knee cartilage explants of OA patients were shown to take up linoleic, oleic and palmitic acid, regardless of whether they were stimulated with TNF-α (71). Upon oleic acid exposure, TNF-α stimulated chondrocytes secrete less GAG and downregulate expression of MMP-1 and COX-2, indicative for an anti-inflammatory effect. Palmitic acid had similar effects on GAG release and MMP-1 expression but did not affect PGE_2 release or COX-2 expression. In contrast, AA precursor linoleic acid increased the release of PGE_2 by TNF-α stimulated chondrocytes. These results suggest that local fatty acid concentrations can contribute to cartilage damage. In another study, the effect of palmitate and oleic acid on chondrocytes of OA patients was investigated in comparison to post-mortem control chondrocytes (72). The data indicated that IL-1β together with palmitate synergistically increased IL-6 and COX-2 expression, whereas oleic acid did not in both donor types, indicating a rather pro-inflammatory effect of palmitic acid. In addition, increased apoptotic cell death was observed in chondrocytes stimulated with both IL-1β and palmitate in the post-mortem cartilage. The contrasting effects of palmitic acid on chondrocytes in these two studies could be due to differences in stimulus used to mimic OA-related inflammation in chondrocytes.

Oxylipins

Association with clinical characteristics and intervention studies in RA

It is generally accepted that PGE_2 contributes to the disease process in RA (14). This is primarily based on studies in mouse models of arthritis, as well as on intervention studies in humans. Deficiencies in enzymes involved in PGE_2 generation, such as $cPLA_2$ (73), COX-1 (74) and COX-2 (75) or mPGES-1 (76), as well as blockers of PGE_2 receptors (77) were associated with diminished disease in mouse models of arthritis. Moreover, intervention studies using pharmacological inhibitors used also in humans indicated that PGE_2 contributes to pain in antibody-induced models of arthritis, especially in the inflammatory phase of the disease (78,79). Taken together, these data indicate that PGE_2 plays a role in this disease. Intriguingly, however, it was also suggested that PGE_2 has a dual role in arthritis, being pro-inflammatory in the induction phase, while also contributing to disease resolution, by inducing the pre-resolving lipid mediator LXA_4 during the later phases (80). LXA_4 has been shown to have anti-inflammatory and pro-resolving properties also in other murine arthritis models, such as zymosan-induced arthritis (81).

In humans, most evidence for a deleterious contribution of PGE_2 to pain was obtained from studies investigating the effect of non-steroidal anti-inflammatory drugs (NSAIDS) in RA (reviewed in (82)). The involvement of PGE_2 in inflammation in RA is less clear,

although some studies indicate that local administration of corticosteroids in knee RA diminishes also swelling besides pain and this is paralleled by a reduction in mPGES-1, COX-1 and COX-2 expression, as well as less PGE_2 production (83). In line with this, randomized-control trials indicate that drugs aimed at blocking COX activity affect also the number of swollen joints (84). Because COX is involved in generation of several lipid species, including anti-inflammatory ones, such as PGJ_2 or the pro-resolving E series resolvins, it would be highly interesting to test the clinical efficacy of inhibitors of mPGES or antagonists of PGE_2 receptors, which would be expected to modulate more specifically PGE_2-mediated effects.

Besides prostaglandins, leukotrienes, especially LTB_4, could also play a role in RA. Serum levels of LTB_4 were associated with higher disease activity (85), while SF levels of LTB_4 were correlated with inflammatory markers (e.g. cellular infiltrate) in RA patients (86). However, one should be careful in interpreting these data, as 5S,12S-diHETE, a less active isomer of LTB_4, was also described in SF of RA patients (11). These two lipids are difficult to distinguish with ELISA or HPLC techniques (own unpublished data). Evidence for a role of LTB_4 in RA originates from murine studies, in which 5-LOX or LTA_4H deficiency prevented development of antibody-induced arthritis and this could be restored by transfer of neutrophils capable of secreting LTB_4, indicating a role for this lipid and neutrophils in this model (87). Similarly, absence of the LTB_4 receptor BLT1, especially on neutrophils, also resulted in less arthritis in the K/BxN and CIA models (88,89). Data in RA patients are less clear. Zileuton, a 5-LOX inhibitor, did not decrease joint tenderness and pain after a four week treatment in patients with active RA. However, there was a nonsignificant decline in the number of joints effected (90).

Association with clinical characteristics and intervention studies in OA

Studies investigating the role of oxylipins in OA patients are scarce. In a recent study, plasma levels of PGE_2 and 15-HETE, as well as TNF-α, IL-1β and COX-2 expression in peripheral blood leukocytes (PBL)were investigated in three separate cohorts of knee OA patients (28). Higher levels of plasma PGE_2 and 15-HETE were associated with presence of knee OA, while higher expression of TNFα, IL-1b and COX-2 in PBL at baseline predicted more rapid progression of joint space narrowing (JSN) 24 months later.

In a recent systematic review, the efficacy of pharmacological interventions for knee OA was investigated (91). A hundred and thirty-seven randomized controlled trials were summarized. Treatments included COX inhibitors such as diclofenac, ibuprofen, naproxen, celecoxib. All treatments were more efficient than placebo in controlling pain, while intra-articular treatments were superior to oral treatments. Regarding function and stiffness, all treatments with COX inhibitors were more effective than oral placebo,

indicating a possible beneficial effect of these pharmacological agents in OA. Another enzyme involved in generation of lipid mediators is 5-LOX. The safety of a natural 5-LOX inhibitor was evaluated in seventy-five OA patients (92). The inhibitor showed pain reduction in the treated group compared to placebo, however, effects on structural damage in patients remains to be defined.

A third approach is to inhibit both COX and 5-LOX enzymes, as several studies now showed that the dual COX/5-LOX inhibitor licofelone is equally effective in diminishing pain as the COX inhibitor naproxen, but has less gastrointestinal related adverse effects (93). Moreover, in one study with 355 OA patients, licofelone was more efficient than naproxen in reducing cartilage volume loss in the global joint and lateral compartment at 6,12 and 24 months (94). However, the effects of this compound on OA structural damage compared to placebo was not yet investigated in humans. Nevertheless, 2 studies in OA models in dogs indicated less cartilage lesions and decreased levels of PGE_2, LTB_4, collagenase 1 and IL-1β in the joint in the treated group (95), as well as reduced size of cartilage lesions and development of osteophytes (96). These findings are supported by in vitro studies, in which IL-1b-treated chondrocytes displayed decreased MMP-13 production upon treatment with Licofelone (97).

Effects on RA human tissues in vitro

Several studies investigated the effect of prostaglandins, especially PGE_2, on tissues derived from RA patients. In line with what was observed in vivo, PGE_2 can display both pro- and anti-inflammatory effects. PGE_2 has been shown to upregulate IL-6 and mPGES in RA synovial fibroblasts (98,99), IL-6 in chondrocytes (100), while inhibiting RA fibroblast growth (101) and MMP-1 expression (JI 2009; 15:1328) as well as osteoclast development (101). Also on immune cells, PGE_2 can have pro-inflammatory effects on dendritic cells, by inducing IL-23 production (102) and anti-inflammatory effects on monocytes and macrophages (103) by reducing the p40 subunit of IL-12 and IL-23. These data suggest that the inhibition of PGE_2 in RA should be carefully considered. For 15-deoxy-PGJ_2, predominantly anti-inflammatory effects were shown, such as downregulation of IL-6 (100) and induction of apoptosis in chondrocytes (104), as well as inhibition of TNFα-induced MMP-13 production in RA synovial fibroblasts (105).

In vitro studies indicate that leukotriene B4 has a pro-inflammatory effect on RA tissues. LTB_4 induced TNFα and IL-1b in RA synovial fibroblasts (106) and was capable of inducing osteoclast differentiation (107).

The effect of anti-inflammatory lipid mediators was much less studied. Intriguingly, one study indicated that 15-HETE, a derivative of 15-LOX, could upregulate MMP-2 in RA synovial fibroblasts (108), suggesting a possible pro-inflammatory role of this lipid in RA.

Effects on human tissues in vitro OA

Several studies investigated the effect of oxylipins on OA tissues in vitro. 15-LOX products as well as PGD (COX product) dose dependently decreased IL-1β induced MMP-1 and MMP-13 production by chondrocytes isolated from OA cartilage (109), suggesting a beneficial role for these lipids in OA. In contrast, the 5-LOX product LTB_4 was shown to increase IL-1b secretion by OA synovial membranes (110), while increasing osteocalcin secretion by OA osteoblasts (111), indicating that LTB_4 might contribute to inflammation and structural damage in OA patients.

PGE_2 is the most studied oxylipin in the context of OA and has been shown to induce IL-6, VEGF and M-SCF production by human OA synovial fibroblasts (112) and to have deleterious effects on OA chondrocytes. On articular cartilage of OA patients, PGE_2 inhibits proteoglycan release, stimulated MMP-13 production and collagen type II breakdown via engagement of EP receptors. Blocking the EP4 receptor could inverse these effects, indicating it as potential therapeutic target, more specific than COX inhibitors (113). However, it was also shown that PGE_2 at concentrations lower than found in SF, are important in maintaining normal chondrocyte phenotype (114). Taken together, these data indicate that future studies on PGE_2 and its signalling pathways are needed to fully understand its contribution to OA.

Practice points

- Lipid mediators, such as prostaglandins and leukotrienes are increased in synovial fluid of both RA and OA patients
- Anti-inflammatory lipid mediators were detected in RA synovial fluid, indicating activation of regulatory mechanisms
- PGE2 can have both deleterious and beneficial effects
- COX is involved in pain perception in RA and OA
- Targeting COX and LOX enzymes can affect both pro- and anti-inflammatory/pro-resolving lipid synthesis
- N-3 PUFA supplementation are suggested to have (small) beneficial effects, in RA patients

Research agenda

- The effects of n-3 PUFA supplementation in RA and OA should be further investigated in high quality randomized controlled trials
- Therapeutical interventions aimed at inhibiting lipid mediator receptors rather than the enzymes involved in their generation should be studied in humans
- The effects of PGE2 in inflammation in RA need further investigation
- The involvement of COX and LOX enzymes and their lipid products in development or severity of OA needs further pre-clinical investigation
- The overall effect of anti-inflammatory/pro-resolving lipids on OA and RA tissue needs to be addressed

Summary

Fatty acids, phospholipids and oxylipids can be detected in RA and OA patients. Most evidence points towards the activation of the COX/PGE_2 and autotaxin/LPA axes in RA, and the COX/PGE_2 and LOX pathways in OA. Inhibition of the COX pathway is beneficial for pain in both diseases, while the effects of inhibition of the LOX pathway are unclear. Whether and how lipids generated by these two pathways are involved in structural damage and inflammation in these diseases is still under investigation. Moreover, research is needed on the role of anti-inflammatory (oxy)lipids in RA and OA.

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References

1. Yusuf E, Kortekaas MC, Watt I, Huizinga TW, Kloppenburg M. Do knee abnormalities visualised on MRI explain knee pain in knee osteoarthritis? A systematic review. Ann Rheum Dis 2011;70: 60-67.
2. Berenbaum F. Osteoarthritis as an inflammatory disease (osteoarthritis is not osteoarthrosis!). Osteoarthritis Cartilage 2013;21: 16-21.
3. Boyer JF, Gourraud PA, Cantagrel A, Davignon JL, Constantin A. Traditional cardiovascular risk factors in rheumatoid arthritis: a meta-analysis. Joint Bone Spine 2011;78: 179-183.
4. de MW, van der Kraan PM, van den Berg WB, van Lent PL. High systemic levels of low-density lipoprotein cholesterol: fuel to the flames in inflammatory osteoarthritis? Rheumatology (Oxford) 2015;
5. Quehenberger O, Armando AM, Brown AH, Milne SB, Myers DS, Merrill AH, et al. Lipidomics reveals a remarkable diversity of lipids in human plasma. J Lipid Res 2010;51: 3299-3305.
6. Young SP, Kapoor SR, Viant MR, Byrne JJ, Filer A, Buckley CD, et al. The impact of inflammation on metabolomic profiles in patients with arthritis. Arthritis Rheum 2013;65: 2015-2023.
7. Ormseth MJ, Swift LL, Fazio S, Linton MF, Chung CP, Raggi P, et al. Free fatty acids are associated with insulin resistance but not coronary artery atherosclerosis in rheumatoid arthritis. Atherosclerosis 2011;219: 869-874.
8. Fuchs B, Bondzio A, Wagner U, Schiller J. Phospholipid compositions of sera and synovial fluids from dog, human and horse: a comparison by 31P-NMR and MALDI-TOF MS. J Anim Physiol Anim Nutr (Berl) 2009;93: 410-422.
9. Fuchs B, Schiller J, Wagner U, Hantzschel H, Arnold K. The phosphatidylcholine/lysophosphatidylcholine ratio in human plasma is an indicator of the severity of rheumatoid arthritis: investigations by 31P NMR and MALDI-TOF MS. Clin Biochem 2005;38: 925-933.
10. Moghaddami M, Ranieri E, James M, Fletcher J, Cleland LG. Prostaglandin D(2) in inflammatory arthritis and its relation with synovial fluid dendritic cells. Mediators Inflamm 2013;2013: 329494.
11. Giera M, Ioan-Facsinay A, Toes R, Gao F, Dalli J, Deelder AM, et al. Lipid and lipid mediator profiling of human synovial fluid in rheumatoid arthritis patients by means of LC-MS/MS. Biochim Biophys Acta 2012;1821: 1415-1424
12. Kosinska MK, Liebisch G, Lochnit G, Wilhelm J, Klein H, Kaesser U, et al. A lipidomic study of phospholipid classes and species in human synovial fluid. Arthritis Rheum 2013;65: 2323-2333.
13. Kosinska MK, Ludwig TE, Liebisch G, Zhang R, Siebert HC, Wilhelm J, et al. Articular Joint Lubricants during Osteoarthritis and Rheumatoid Arthritis Display Altered Levels and Molecular Species. PLoS One 2015;10: e0125192.
14. Korotkova M, Jakobsson PJ. Persisting eicosanoid pathways in rheumatic diseases. Nat Rev Rheumatol 2014;10: 229-241.
15. Hishinuma T, Nakamura H, Sawai T, Uzuki M, Itabash Y, Mizugaki M. Microdetermination of prostaglandin E2 in joint fluid in rheumatoid arthritis patients using gas chromatography/selected ion monitoring. Prostaglandins Other Lipid Mediat 1999;58: 179-186.
16. Pruzanski W, Vadas P, Kim J, Jacobs H, Stefanski E. Phospholipase A2 activity associated with synovial fluid cells. J Rheumatol 1988;15: 791-794.
17. Seilhamer JJ, Plant S, Pruzanski W, Schilling J, Stefanski E, Vadas P, et al. Multiple forms of phospholipase A2 in arthritic synovial fluid. J Biochem 1989;106: 38-42.

18. Westman M, Korotkova M, af KE, Stark A, Audoly LP, Klareskog L, et al. Expression of microsomal prostaglandin E synthase 1 in rheumatoid arthritis synovium. Arthritis Rheum 2004; 50:1774-1780.

19. Murakami M, Nakashima K, Kamei D, Masuda S, Ishikawa Y, Ishii T, et al. Cellular prostaglandin E2 production by membrane-bound prostaglandin E synthase-2 via both cyclooxygenases-1 and -2. J Biol Chem 2003;278: 37937-37947.

20. Shao XT, Feng L, Gu LJ, Wu LJ, Feng TT, Yang YM, et al. Expression of interleukin-18, IL-18BP, and IL-18R in serum, synovial fluid, and synovial tissue in patients with rheumatoid arthritis. Clin Exp Med 2009;9: 215-221.

21. Serhan CN, Chiang N, Dalli J. The resolution code of acute inflammation: Novel pro-resolving lipid mediators in resolution. Semin Immunol 2015;27: 200-215.

22. Nochi H, Tomura H, Tobo M, Tanaka N, Sato K, Shinozaki T, et al. Stimulatory role of lysophosphatidic acid in cyclooxygenase-2 induction by synovial fluid of patients with rheumatoid arthritis in fibroblast-like synovial cells. J Immunol 2008;181: 5111-5119.

23. Limaye V, Xia P, Hahn C, Smith M, Vadas MA, Pitson SM, et al. Chronic increases in sphingosine kinase-1 activity induce a pro-inflammatory, pro-angiogenic phenotype in endothelial cells. Cell Mol Biol Lett 2009;14: 424-441.

24. Song HY, Lee MJ, Kim MY, Kim KH, Lee IH, Shin SH, et al. Lysophosphatidic acid mediates migration of human mesenchymal stem cells stimulated by synovial fluid of patients with rheumatoid arthritis. Biochim Biophys Acta 2010;1801: 23-30.

25. Nikitopoulou I, Oikonomou N, Karouzakis E, Sevastou I, Nikolaidou-Katsaridou N, Zhao Z, et al. Autotaxin expression from synovial fibroblasts is essential for the pathogenesis of modeled arthritis. J Exp Med 2012;209: 925-933.

26. Castro-Perez JM, Kamphorst J, DeGroot J, Lafeber F, Goshawk J, Yu K, et al. Comprehensive LC-MS E lipidomic analysis using a shotgun approach and its application to biomarker detection and identification in osteoarthritis patients. J Proteome Res 2010;9: 2377-2389.

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

28. Attur M, Krasnokutsky S, Statnikov A, Samuels J, Li Z, Friese O, et al. Low-Grade Inflammation in Symptomatic Knee Osteoarthritis: Prognostic Value of Inflammatory Plasma Lipids and Peripheral Blood Leukocyte Biomarkers. Arthritis Rheumatol 2015;67: 2905-2915.

29. Rae SA, Davidson EM, Smith MJ. Leukotriene B4, an inflammatory mediator in gout. Lancet 1982; 1122-1124.

30. Lee TH, Hoover RL, Williams JD, Sperling RI, Ravalese J, III, Spur BW, et al. Effect of dietary enrichment with eicosapentaenoic and docosahexaenoic acids on in vitro neutrophil and monocyte leukotriene generation and neutrophil function. N Engl J Med 1985;312: 1217-1224.

31. Miles EA, Calder PC. Influence of marine n-3 polyunsaturated fatty acids on immune function and a systematic review of their effects on clinical outcomes in rheumatoid arthritis. Br J Nutr 2012;107 Suppl 2: S171-S184.

32. Vidgren HM, Agren JJ, Schwab U, Rissanen T, Hanninen O, Uusitupa MI. Incorporation of n-3 fatty acids into plasma lipid fractions, and erythrocyte membranes and platelets during dietary supplementation with fish, fish oil, and docosahexaenoic acid-rich oil among healthy young men. Lipids 1997;32: 697-705.

33. Skarke C, Alamuddin N, Lawson JA, Li X, Ferguson JF, Reilly MP, et al. Bioactive products formed in humans from fish oils. J Lipid Res 2015;56: 1808-1820.

34. Park Y, Lee A, Shim SC, Lee JH, Choe JY, Ahn H, et al. Effect of n-3 polyunsaturated fatty acid supplementation in patients with rheumatoid arthritis: a 16-week randomized, double-blind, placebo-controlled, parallel-design multicenter study in Korea. J Nutr Biochem 2013;24: 1367-1372.

35. Dawczynski C, Hackermeier U, Viehweger M, Stange R, Springer M, Jahreis G. Incorporation of n-3 PUFA and gamma-linolenic acid in blood lipids and red blood cell lipids together with their influence on disease activity in patients with chronic inflammatory arthritis--a randomized controlled human intervention trial. Lipids Health Dis 2011;10: 130.

36. Proudman SM, James MJ, Spargo LD, Metcalf RG, Sullivan TR, Rischmueller M, et al. Fish oil in recent onset rheumatoid arthritis: a randomised, double-blind controlled trial within algorithm-based drug use. Ann Rheum Dis 2015;74: 89-95.

37. Di Giuseppe D, Crippa A, Orsini N, Wolk A. Fish consumption and risk of rheumatoid arthritis: a dose-response meta-analysis. Arthritis Res Ther 2014;16: 446.

38. Di Giuseppe D, Wallin A, Bottai M, Askling J, Wolk A. Long-term intake of dietary long-chain n-3 polyunsaturated fatty acids and risk of rheumatoid arthritis: a prospective cohort study of women. Ann Rheum Dis 2014;73: 1949-1953.

39. Gan RW, Young KA, Zerbe GO, Demoruelle MK, Weisman MH, Buckner JH, et al. Lower omega-3 fatty acids are associated with the presence of anti-cyclic citrullinated peptide autoantibodies in a population at risk for future rheumatoid arthritis: a nested case-control study. Rheumatology (Oxford) 2015;

40. Leslie CA, Gonnerman WA, Ullman MD, Hayes KC, Franzblau C, Cathcart ES. Dietary fish oil modulates macrophage fatty acids and decreases arthritis susceptibility in mice. J Exp Med 1985;162: 1336-1349.

41. Ierna M, Kerr A, Scales H, Berge K, Griinari M. Supplementation of diet with krill oil protects against experimental rheumatoid arthritis. BMC Musculoskelet Disord 2010;11: 136.

42. Lee EY, Lee CK, Lee KU, Park JY, Cho KJ, Cho YS, et al. Alpha-lipoic acid suppresses the development of collagen-induced arthritis and protects against bone destruction in mice. Rheumatol Int 2007;27: 225-233.

43. Hah YS, Sung MJ, Lim HS, Jun JS, Jeong YG, Kim HO, et al. Dietary alpha lipoic acid supplementation prevents synovial inflammation and bone destruction in collagen-induced arthritic mice. Rheumatol Int 2011;31: 1583-1590.

44. Orosa B, Garcia S, Martinez P, Gonzalez A, Gomez-Reino JJ, Conde C. Lysophosphatidic acid receptor inhibition as a new multipronged treatment for rheumatoid arthritis. Ann Rheum Dis 2014;73: 298-305

45. Eros G, Ibrahim S, Siebert N, Boros M, Vollmar B. Oral phosphatidylcholine pretreatment alleviates the signs of experimental rheumatoid arthritis. Arthritis Res Ther 2009;11: R43.

46. Zhang W, Likhodii S, Zhang Y, Aref-Eshghi E, Harper PE, Randell E, et al. Classification of osteoarthritis phenotypes by metabolomics analysis. BMJ Open 2014;4: e006286.

47. Kosinska MK, Liebisch G, Lochnit G, Wilhelm J, Klein H, Kaesser U, et al. Sphingolipids in human synovial fluid--a lipidomic study. PLoS One 2014;9: e91769.

48. Baker KR, Matthan NR, Lichtenstein AH, Niu J, Guermazi A, Roemer F, et al. Association of plasma n-6 and n-3 polyunsaturated fatty acids with synovitis in the knee: the MOST study. Osteoarthritis Cartilage 2012;20: 382-387.

49. Lippiello L, Walsh T, Fienhold M. The association of lipid abnormalities with tissue pathology in human osteoarthritic articular cartilage. Metabolism 1991;40: 571-576.

50. Zawadzki M, Janosch C, Szechinski J. Perna canaliculus lipid complex PCSO-524 demonstrated pain relief for osteoarthritis patients benchmarked against fish oil, a randomized trial, without placebo control. Mar Drugs 2013;11: 1920-1935.

51. Stammers T, Sibbald B, Freeling P. Efficacy of cod liver oil as an adjunct to non-steroidal anti-inflammatory drug treatment in the management of osteoarthritis in general practice. Ann Rheum Dis 1992;51: 128-129.

52. Hill CL, March LM, Aitken D, Lester SE, Battersby R, Hynes K, et al. Fish oil in knee osteoarthritis: a randomised clinical trial of low dose versus high dose. Ann Rheum Dis 2015;

53. Felson DT, Bischoff-Ferrari HA. Dietary fatty acids for the treatment of OA, including fish oil. Ann Rheum Dis 2015;

54. Knott L, Avery NC, Hollander AP, Tarlton JF. Regulation of osteoarthritis by omega-3 (n-3) polyunsaturated fatty acids in a naturally occurring model of disease. Osteoarthritis Cartilage 2011;19: 1150-1157.

55. Wu CL, Jain D, McNeill JN, Little D, Anderson JA, Huebner JL, et al. Dietary fatty acid content regulates wound repair and the pathogenesis of osteoarthritis following joint injury. Ann Rheum Dis 2015;74: 2076-2083.

56. Frommer KW, Schaffler A, Rehart S, Lehr A, Muller-Ladner U, Neumann E. Free fatty acids: potential proinflammatory mediators in rheumatic diseases. Ann Rheum Dis 2015;74: 303-310.

57. Colantuoni G, Quintero M, Panasyuk A, Abderrahim L, Mitrovic DR. Do arachidonic acid and its metabolites, secreted by rheumatoid and osteoarthritic synovial tissue, account for the strong inhibition of DNA synthesis in cultured human articular chondrocytes? A novel approach to the mechanism of tissue damage. Joint Bone Spine 2005;72: 533-539.

58. Watkins BA, Lippman HE, Le BL, Li Y, Seifert MF. Bioactive fatty acids: role in bone biology and bone cell function. Prog Lipid Res 2001;40: 125-148.

59. Kremer JM, Lawrence DA, Jubiz W, DiGiacomo R, Rynes R, Bartholomew LE, et al. Dietary fish oil and olive oil supplementation in patients with rheumatoid arthritis. Clinical and immunologic effects. Arthritis Rheum 1990;33: 810-820.

60. Magaro M, Altomonte L, Zoli A, Mirone L, De SP, Di MG, et al. Influence of diet with different lipid composition on neutrophil chemiluminescence and disease activity in patients with rheumatoid arthritis. Ann Rheum Dis 1988;47: 793-796.

61. Moghaddami N, Irvine J, Gao X, Grover PK, Costabile M, Hii CS, et al. Novel action of n-3 polyunsaturated fatty acids: inhibition of arachidonic acid-induced increase in tumor necrosis factor receptor expression on neutrophils and a role for proteases. Arthritis Rheum 2007;56: 799-808.

62. de Jong AJ, Kloppenburg M, Toes RE, Ioan-Facsinay A. Fatty acids, lipid mediators, and T-cell function. Front Immunol 2014;5: 483.

63. Shaikh SR, Teague H. N-3 fatty acids and membrane microdomains: from model membranes to lymphocyte function. Prostaglandins Leukot Essent Fatty Acids 2012;87: 205-208.

64. Sevastou I, Kaffe E, Mouratis MA, Aidinis V. Lysoglycerophospholipids in chronic inflammatory disorders: the PLA(2)/LPC and ATX/LPA axes. Biochim Biophys Acta 2013;1831: 42-60.

65. Miyabe Y, Miyabe C, Iwai Y, Yokoyama W, Sekine C, Sugimoto K, et al. Activation of fibroblast-like synoviocytes derived from rheumatoid arthritis via lysophosphatidic acid-lysophosphatidic acid receptor 1 cascade. Arthritis Res Ther 2014;16: 461.

66. Rikitake Y, Hirata K, Kawashima S, Takeuchi S, Shimokawa Y, Kojima Y, et al. Signaling mechanism underlying COX-2 induction by lysophosphatidylcholine. Biochem Biophys Res Commun 2001;281: 1291-1297.

67. Ruiperez V, Casas J, Balboa MA, Balsinde J. Group V phospholipase A2-derived lysophosphatidylcholine mediates cyclooxygenase-2 induction in lipopolysaccharide-stimulated macrophages. J Immunol 2007;179: 631-638.

68. Kitano M, Hla T, Sekiguchi M, Kawahito Y, Yoshimura R, Miyazawa K, et al. Sphingosine 1-phosphate/sphingosine 1-phosphate receptor 1 signaling in rheumatoid synovium: regulation of synovial proliferation and inflammatory gene expression. Arthritis Rheum 2006;54: 742-753.

69. Masuko K, Murata M, Nakamura H, Yudoh K, Nishioka K, Kato T. Sphingosine-1-phosphate attenuates proteoglycan aggrecan expression via production of prostaglandin E2 from human articular chondrocytes. BMC Musculoskelet Disord 2007;8: 29.

70. Sato C, Iwasaki T, Kitano S, Tsunemi S, Sano H. Sphingosine 1-phosphate receptor activation enhances BMP-2-induced osteoblast differentiation. Biochem Biophys Res Commun 2012;423: 200-205.

71. Bastiaansen-Jenniskens YM, Siawash M, van de Lest CH, Verhaar JA, Kloppenburg M, Zuurmond AM, et al. Monounsaturated and Saturated, but Not n-6 Polyunsaturated Fatty Acids Decrease Cartilage Destruction under Inflammatory Conditions: A Preliminary Study. Cartilage 2013;4: 321-328.

72. Alvarez-Garcia O, Rogers NH, Smith RG, Lotz MK. Palmitate has proapoptotic and proinflammatory effects on articular cartilage and synergizes with interleukin-1. Arthritis Rheumatol 2014;66: 1779-1788.

73. Raichel L, Berger S, Hadad N, Kachko L, Karter M, Szaingurten-Solodkin I, et al. Reduction of cPLA2alpha overexpression: an efficient anti-inflammatory therapy for collagen-induced arthritis. Eur J Immunol 2008;38: 2905-2915.

74. Chen M, Boilard E, Nigrovic PA, Clark P, Xu D, FitzGerald GA, et al. Predominance of cyclooxygenase 1 over cyclooxygenase 2 in the generation of proinflammatory prostaglandins in autoantibody-driven K/BxN serum-transfer arthritis. Arthritis Rheum 2008;58: 1354-1365.

75. Myers LK, Kang AH, Postlethwaite AE, Rosloniec EF, Morham SG, Shlopov BV, et al. The genetic ablation of cyclooxygenase 2 prevents the development of autoimmune arthritis. Arthritis Rheum 2000;43: 2687-2693.

76. Trebino CE, Stock JL, Gibbons CP, Naiman BM, Wachtmann TS, Umland JP, et al. Impaired inflammatory and pain responses in mice lacking an inducible prostaglandin E synthase. Proc Natl Acad Sci U S A 2003;100: 9044-9049.

77. Clark P, Rowland SE, Denis D, Mathieu MC, Stocco R, Poirier H, et al. MF498 [N-{[4-(5,9-Diethoxy-6-oxo-6,8-dihydro-7H-pyrrolo[3,4-g]quinolin-7-yl)-3-methylbe nzyl]sulfonyl}-2-(2-methoxyphenyl)acetamide], a selective E prostanoid receptor 4 antagonist, relieves joint inflammation and pain in rodent models of rheumatoid and osteoarthritis. J Pharmacol Exp Ther 2008;325: 425-434.

78. Christianson CA, Corr M, Firestein GS, Mobargha A, Yaksh TL, Svensson CI. Characterization of the acute and persistent pain state present in K/BxN serum transfer arthritis. Pain 2010;151: 394-403.

79. Bas DB, Su J, Sandor K, Agalave NM, Lundberg J, Codeluppi S, et al. Collagen antibody-induced arthritis evokes persistent pain with spinal glial involvement and transient prostaglandin dependency. Arthritis Rheum 2012;64: 3886-3896.

80. Chan MM, Moore AR. Resolution of inflammation in murine autoimmune arthritis is disrupted by cyclooxygenase-2 inhibition and restored by prostaglandin E2-mediated lipoxin A4 production. J Immunol 2010;184: 6418-6426.

81. Conte FP, Menezes-de-Lima O, Jr., Verri WA, Jr., Cunha FQ, Penido C, Henriques MG. Lipoxin A(4) attenuates zymosan-induced arthritis by modulating endothelin-1 and its effects. Br J Pharmacol 2010;161: 911-924.

82. Martel-Pelletier J, Pelletier JP, Fahmi H. Cyclooxygenase-2 and prostaglandins in articular tissues. Semin Arthritis Rheum 2003;33: 155-167.

83. Korotkova M, Westman M, Gheorghe KR, af KE, Trollmo C, Ulfgren AK, et al. Effects of antirheumatic treatments on the prostaglandin E2 biosynthetic pathway. Arthritis Rheum 2005;52: 3439-3447.

84. Garner S, Fidan D, Frankish R, Judd M, Shea B, Towheed T, et al. Celecoxib for rheumatoid arthritis. Cochrane Database Syst Rev 2002;CD003831.

85. Gursel T, Firat S, Ercan ZS. Increased serum leukotriene B4 level in the active stage of rheumatoid arthritis in children. Prostaglandins Leukot Essent Fatty Acids 1997;56: 205-207.

86. Ahmadzadeh N, Shingu M, Nobunaga M, Tawara T. Relationship between leukotriene B4 and immunological parameters in rheumatoid synovial fluids. Inflammation 1991;15: 497-503.

87. Chen M, Lam BK, Kanaoka Y, Nigrovic PA, Audoly LP, Austen KF, et al. Neutrophil-derived leukotriene B4 is required for inflammatory arthritis. J Exp Med 2006;203: 837-842.

88. Chou RC, Kim ND, Sadik CD, Seung E, Lan Y, Byrne MH, et al. Lipid-cytokine-chemokine cascade drives neutrophil recruitment in a murine model of inflammatory arthritis. Immunity 2010;33: 266-278.

89. Shao WH, Del PA, Bock CB, Haribabu B. Targeted disruption of leukotriene B4 receptors BLT1 and BLT2: a critical role for BLT1 in collagen-induced arthritis in mice. J Immunol 2006;176: 6254-6261.

90. Weinblatt ME, Kremer JM, Coblyn JS, Helfgott S, Maier AL, Petrillo G, et al. Zileuton, a 5-lipoxygenase inhibitor in rheumatoid arthritis. J Rheumatol 1992;19: 1537-1541.

91. Bannuru RR, Schmid CH, Kent DM, Vaysbrot EE, Wong JB, McAlindon TE. Comparative effectiveness of pharmacologic interventions for knee osteoarthritis: a systematic review and network meta-analysis. Ann Intern Med 2015;162: 46-54.

92. Sengupta K, Alluri KV, Satish AR, Mishra S, Golakoti T, Sarma KV, et al. A double blind, randomized, placebo controlled study of the efficacy and safety of 5-Loxin for treatment of osteoarthritis of the knee. Arthritis Res Ther 2008;10: R85.

93. Cicero AF, Laghi L. Activity and potential role of licofelone in the management of osteoarthritis. Clin Interv Aging 2007;2: 73-79.

94. Raynauld JP, Martel-Pelletier J, Bias P, Laufer S, Haraoui B, Choquette D, et al. Protective effects of licofelone, a 5-lipoxygenase and cyclo-oxygenase inhibitor, versus naproxen on cartilage loss in knee osteoarthritis: a first multicentre clinical trial using quantitative MRI. Ann Rheum Dis 2009;68: 938-947.

95. Jovanovic DV, Fernandes JC, Martel-Pelletier J, Jolicoeur FC, Reboul P, Laufer S, et al. In vivo dual inhibition of cyclooxygenase and lipoxygenase by ML-3000 reduces the progression of experimental osteoarthritis: suppression of collagenase 1 and interleukin-1beta synthesis. Arthritis Rheum 2001;44: 2320-2330.

96. Moreau M, Boileau C, Martel-Pelletier J, Brunet J, Laufer S, Pelletier JP. Licofelone reduces progression of structural changes in a canine model of osteoarthritis under curative conditions: effect on protease expression and activity. J Rheumatol 2006;33: 1176-1183.

97. Boileau C, Pelletier JP, Tardif G, Fahmi H, Laufer S, Lavigne M, et al. The regulation of human MMP-13 by licofelone, an inhibitor of cyclo-oxygenases and 5-lipoxygenase, in human osteoarthritic chondrocytes is mediated by the inhibition of the p38 MAP kinase signalling pathway. Ann Rheum Dis 2005;64: 891-898.

98. Sommerfelt RM, Feuerherm AJ, Skuland T, Johansen B. Cytosolic phospholipase A2 modulates TLR2 signaling in synoviocytes. PLoS One 2015;10: e0119088.

99. Kojima F, Naraba H, Sasaki Y, Beppu M, Aoki H, Kawai S. Prostaglandin E2 is an enhancer of interleukin-1beta-induced expression of membrane-associated prostaglandin E synthase in rheumatoid synovial fibroblasts. Arthritis Rheum 2003;48: 2819-2828.

100. Wang P, Zhu F, Konstantopoulos K. Interleukin-6 synthesis in human chondrocytes is regulated via the antagonistic actions of prostaglandin (PG)E2 and 15-deoxy-Delta(12,14)-PGJ2. PLoS One 2011;6: e27630.

101. Shibata-Nozaki T, Ito H, Mitomi H, Akaogi J, Komagata T, Kanaji T, et al. Endogenous prostaglandin E2 inhibits aberrant overgrowth of rheumatoid synovial tissue and the development of osteoclast activity through EP4 receptor. Arthritis Rheum 2011;63: 2595-2605.

102. Sheibanie AF, Tadmori I, Jing H, Vassiliou E, Ganea D. Prostaglandin E2 induces IL-23 production in bone marrow-derived dendritic cells. FASEB J 2004;18: 1318-1320.

103. Klein-Wieringa IR, Andersen SN, Kwekkeboom JC, Giera M, de Lange-Brokaar BJ, Van Osch GJ, et al. Adipocytes modulate the phenotype of human macrophages through secreted lipids. J Immunol 2013;191: 1356-1363.

104. Shan ZZ, Masuko-Hongo K, Dai SM, Nakamura H, Kato T, Nishioka K. A potential role of 15-deoxy-delta(12,14)-prostaglandin J2 for induction of human articular chondrocyte apoptosis in arthritis. J Biol Chem 2004;279: 37939-37950.

105. Lin TH, Tang CH, Wu K, Fong YC, Yang RS, Fu WM. 15-deoxy-Delta(12,14) -prostaglandin-J2 and ciglitazone inhibit TNF-alpha-induced matrix metalloproteinase 13 production via the antagonism of NF-kappaB activation in human synovial fibroblasts. J Cell Physiol 2011;226: 3242-3250.

106. Xu S, Lu H, Lin J, Chen Z, Jiang D. Regulation of TNFalpha and IL1beta in rheumatoid arthritis synovial fibroblasts by leukotriene B4. Rheumatol Int 2010;30: 1183-1189.

107. Chen ZK, Lv HS, Jiang J. LTB4 can stimulate human osteoclast differentiation dependent of RANKL. Artif Cells Blood Substit Immobil Biotechnol 2010;38: 52-56.

108. Wu MY, Lin TH, Chiu YC, Liou HC, Yang RS, Fu WM. Involvement of 15-lipoxygenase in the inflammatory arthritis. J Cell Biochem 2012;113: 2279-2289.

109. Chabane N, Zayed N, Benderdour M, Martel-Pelletier J, Pelletier JP, Duval N, et al. Human articular chondrocytes express 15-lipoxygenase-1 and -2: potential role in osteoarthritis. Arthritis Res Ther 2009;11: R44.

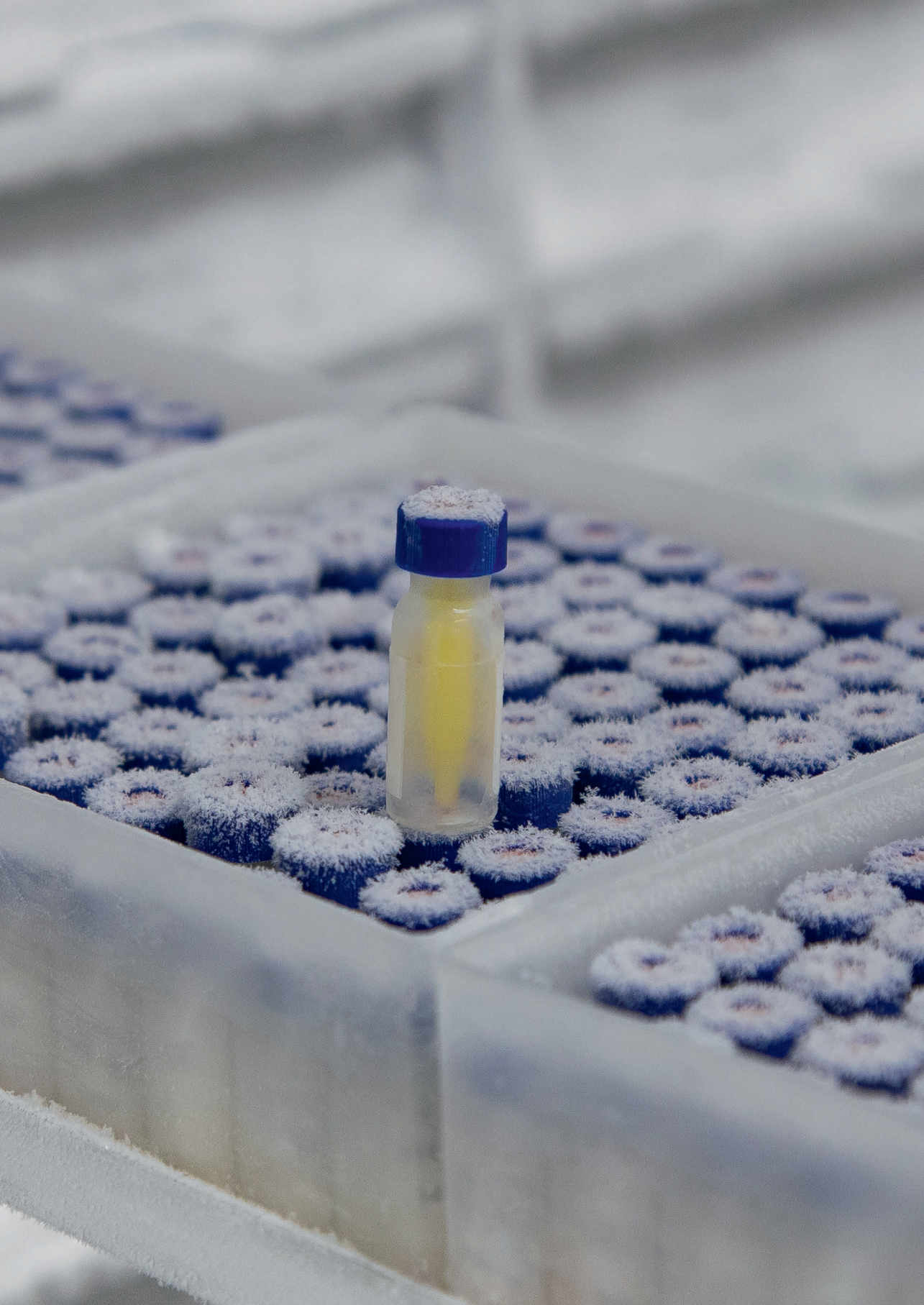
110. Marcouiller P, Pelletier JP, Guevremont M, Martel-Pelletier J, Ranger P, Laufer S, et al. Leukotriene and prostaglandin synthesis pathways in osteoarthritic synovial membranes: regulating factors for interleukin 1beta synthesis. J Rheumatol 2005;32: 704-712.

111. Paredes Y, Massicotte F, Pelletier JP, Martel-Pelletier J, Laufer S, Lajeunesse D. Study of the role of leukotriene B()4 in abnormal function of human subchondral osteoarthritis osteoblasts: effects of cyclooxygenase and/or 5-lipoxygenase inhibition. Arthritis Rheum 2002;46: 1804-1812.

112. Inoue H, Takamori M, Shimoyama Y, Ishibashi H, Yamamoto S, Koshihara Y. Regulation by PGE2 of the production of interleukin-6, macrophage colony stimulating factor, and vascular endothelial growth factor in human synovial fibroblasts. Br J Pharmacol 2002;136: 287-295.

113. Attur M, Al-Mussawir HE, Patel J, Kitay A, Dave M, Palmer G, et al. Prostaglandin E2 exerts catabolic effects in osteoarthritis cartilage: evidence for signaling via the EP4 receptor. J Immunol 2008;181: 5082-5088.

114. Tchetina EV, Di Battista JA, Zukor DJ, Antoniou J, Poole AR. Prostaglandin PGE2 at very low concentrations suppresses collagen cleavage in cultured human osteoarthritic articular cartilage: this involves a decrease in expression of proinflammatory genes, collagenases and COL10A1, a gene linked to chondrocyte hypertrophy. Arthritis Res Ther 2007;9: R75.



3

Targeted lipidomics reveals activation of resolution pathways in knee osteoarthritis in humans

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Summary

Objective: To investigate the presence of inflammation and resolution pathways in osteoarthritis (OA).

Design: Tissues were obtained from knee OA patients and control rheumatoid arthritis (RA) patients. Cells in synovial fluid (SF) were visualized by flow cytometry. Cytokines and chemokines were measured by multiplex assay. Lipid mediators (LMs) were determined by targeted lipidomics using liquid-chromatography mass spectrometry.

Results: SF of OA patients contained less cells, especially neutrophils, less cytokines and comparable levels of chemokines compared to RA controls.

Thirty-seven lipids were detected in the soluble fraction of SF, including polyunsaturated fatty acids (PUFAs) and their pro-inflammatory and pro-resolving lipoxygenase (LOX) and cyclooxygenase (COX) pathway markers in both OA and RA patients. Among these, pro-inflammatory LM such as prostaglandin E2 and thromboxane B2, as well as precursors and pathway markers of resolution such as 17-HDHA and 18-HEPE were detected. Interestingly, the pro-resolving lipid RvD2 could also be detected, but only in the insoluble fraction (cells and undigested matrix). Ratios of metabolites to their precursors indicated a lower activity of 5-LOX and 15-LOX in OA compared to RA, with no apparent differences in COX-derived products . Interestingly, synovial tissue and SF cells could produce 5-LOX and 15-LOX metabolites, indicating these cells as possible source of LM.

Conclusions: By using a state-of-the-art technique, we show for the first time that resolution pathways are present in OA patients. A better understanding of these pathways could guide us to more effective therapeutic approaches to inhibit inflammation and further structural damage in OA and RA.

Keywords: osteoarthritis, rheumatoid arthritis, lipid mediators, resolution, inflammation

Introduction

Osteoarthritis (OA) is the most common form of arthritis, with a prevalence of more than 70% in the elderly population. Characteristic radiographic features of OA are cartilage degradation and the presence of osteophytes (bone spurs). It has recently become evident that synovitis is an accompanying feature of OA in a significant number of patients and that inflammation is an important player in OA (reviewed in (1) and (2)) as it is associated with pain, as well as radiographic progression. The association with radiographic progression seems to be even stronger in patients with persistent inflammation (3). The reason for persistent inflammation in some patients is unclear, but one intriguing possibility is that the essential resolution pathways are incompletely/ not activated.

Inflammation is usually a self-resolving process initiated as a response to danger signals. This response is tightly regulated and involves the concerted and timely action of several molecular and cellular players. Extensive studies of acute inflammation in a model of self-resolving inflammation in mice indicated that the initial phases of inflammation are characterized by neutrophil recruitment, followed by macrophage accumulation during the resolution process (4, 5).

At the molecular level, cytokines and lipids are involved in regulating inflammation. Pro-inflammatory mediators, such as cytokines, chemokines and eicosanoids (e.g. prostaglandins and leukotrienes), a class of lipid mediators (LMs) derived from arachidonic acid (AA), are released during the initial phases of inflammation, driving recruitment and activation of immune cells (4). Resolution of inflammation has been shown to be an active process originating early in inflammation, being driven by anti-inflammatory and pro-resolving mediators (6-8). Among these, several families of specialized pro-resolving mediators (SPMs) have been identified (lipoxins, resolvins, protectins and maresins) that can induce resolution of inflammation in murine acute inflammatory models (9-11). Moreover, they appear to be regulated during the disease course in asthma, Alzheimer's disease (12-14), multiple sclerosis (15), cystic fibrosis (16), as well as ulcerative colitis patients (17), indicating a possible role for SPMs in regulating inflammation in human disease.

The SPMs known to date are synthesized enzymatically through lipoxygenase (LOX), cyclooxygenase (COX), or cytochrome P450 (CYP) pathways from polyunsaturated fatty acids (PUFAs) such as AA, docosahexaenoic acid (DHA), or eicosapentaenoic acid (EPA), mostly through transcellular processes involving different types of cells (18, 19). More recently, other mechanisms such as microparticle uptake, phagocytosis and

sequential stimulation with different stimuli have been implicated in the generation of SPMs (20, 21).

In contrast to cytokines and chemokines, the presence of bioactive (oxy)lipids has only scarcely been investigated in OA. The available studies indicated the presence of the 15-LOX product 15-HETE and the COX product prostaglandin E_2 (PGE_2) in plasma and these appeared to be higher in OA patients than healthy controls (22). Similarly, the 5-LOX product leukotriene B_4 (LTB_4) was described in synovial fluid (SF) of OA patients (23), as well as $PGF_{2\alpha,}$ the non-enzymatically made 8-iso-$PGF_{2\alpha}$, and the deactivation product 15-keto-13,14-dihydro-$PGF_{2\alpha}$ in both SF and plasma (24). Both LTB_4 and PGE_2 have been shown to be secreted by OA synovial explants (25). Interestingly, 5-LOX and 15-LOX have been shown to be present in the OA synovium, however most of their LM products were not yet studied in detail (26). Specifically, the presence of LMs associated with resolution of inflammation, SPMs or their precursors, has not yet been investigated in OA, despite the important role inflammation plays in the progression of structural damage.

The aim of this study was to investigate the activation of resolution in OA by studying the presence of bioactive lipids associated with resolution pathways in SF of OA patients. To this end, we employed a state-of-the-art targeted lipidomics approach to detect SPMs and their precursors in end-stage knee OA patients. Moreover, we extensively characterized inflammatory cells, cytokines and chemokines in SF and compared the results to rheumatoid arthritis (RA), as a control chronic inflammatory disease.

Materials and methods

Chemicals and materials

Listing of chemicals and other materials can be found in *Supplementary Materials and Methods*.

Patients and tissue sample collection

SF and synovial tissues from knee OA and RA patients were obtained as anonymized leftover material from patients undergoing knee arthroscopy at the department of Rheumatology or undergoing knee-replacement surgery at the departments of Orthopaedic surgery in the LUMC or Alrijne hospital in Leiden, performed for standard clinical care. Diagnosis in all patients was established by the treating physician. Age, gender, and BMI are reported in suppl. Table 1. This procedure was approved by the local ethical committee. SF samples were treated as described below and in Supplementary Fig. 1 and were stored at -80° until analysis. The average time to analysis was 7 months (range: 1 day – 19 months).

Isolation of soluble and insoluble fraction of SF

One mL synovial fluid was treated with hyaluronidase, followed by centrifugation at 931 ×g for 10 min. as described in Suppl. Fig.1. The supernatant (soluble fraction) was removed and the pellet (insoluble fraction) was resuspended in 1 mL water. Proteins were precipitated from both soluble and insoluble fractions with 3 mL methanol (MeOH) (3184 ×g for 15 min at 4 °C). The MeOH supernatant was removed, the protein pellet washed again with 1 mL MeOH and internal standard (IS) was added (LTB_4-d4, 15(*S*)-HETE-d8 and PGE_2-d4, 150 pg each and DHA-d5 1500 pg). Next, the sample was spun again before combining the MeOH supernatants. After drying down the MeOH, diluting it with water and acidifying, the samples were loaded on 3 mL 500 mg Bond Elut C-18 solid-phase extraction (SPE) columns (Agilent Technologies Santa Clara, CA, USA) as described in the legend of Suppl. Fig.1 and lipids were analyzed as described below.

Lipid analysis

Targeted lipidomics analysis of the SF was carried out after solid phase extraction (SPE) as previously described (27) with some modifications (Suppl. Fig. 1). Liquid chromatography combined with mass spectrometry (LC-MS/MS) analysis was carried out as previously published (28) with some modifications (Suppl. Materials and Methods).

Synovial tissue cells

Synovial tissue cells (synoviocytes) were isolated from fresh synovial tissue digested for 1.5 hours with 1 mg/mL collagenase type 2 (Worthington biochemical corporation, Lakewood, NJ, USA) in serum free IMDM medium (Lonza, Basel, Switzerland). Digested tissue was filtered over a 70 μm cell strainer (Falcon, Corning Incorporated, Life Sciences, Durham, NC, USA) to obtain the cells present in synovium. The cells were washed 3 times with serum free IMDM medium before use.

Stimulation of synovial fluid cells and synoviocytes

Both SF cells (SFC) and synoviocytes were first filtered over a 70 μm cell strainer (Falcon, Corning Incorporated, Life Sciences, Durham, NC, USA), then isolated cells suspended in PBS with calcium and magnesium (PBS (+/+), Sigma Aldrich, Steinheim, Germany) were stimulated with 4 μM calcium ionophore A23187 (Sigma, Saint Louis, MO, USA) or vehicle control for 10 min. For LPS stimulation experiments, SFC and synoviocytes were suspended in PBS (+/+) containing 0,1% fatty acid free BSA (Sigma, Saint Louis, MO, USA) and stimulated with 10 ng/mL LPS (Sigma, Saint Louis, MO, USA) for 72 hours. Next, proteins were precipitated by adding 3 volumes of MeOH and IS (0.75 ng/mL final concentration). Samples were stored under argon at -80 °C until analysis. Before LC/MS-MS analysis, samples were centrifuged at 16,100 ×g for 10 min at 4 °C and supernatants diluted 1:1 with water. Precipitated protein was quantified using a Micro BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). The amount of

protein per sample varied between 1 μg and 15 μg for SFC and between 5 μg and 34 μg for synoviocytes. LM Lipid analysis was performed as described in the Suppl. Materials and Methods.

Cytokine and FACS analysis

Cytokine and FACS analysis were performed as described in the Suppl. Materials and Methods.

Data analysis and statistics

LC-MS/MS peaks were integrated with manual supervision and area corrected to corresponding IS with MultiQuant™ 2.1 (Sciex, MA, USA). When possible, lipids were quantified based on a calibration line. Values were normalized to the amount of SF from which they originated (presented as ng lipid/mL SF) or to the amount of protein present in the samples as surrogate for cell numbers (presented as area ratio/mg protein). Paired samples were compared by a 2-tailed Spearman's correlation (SPSS Statistics for Windows, IBM Corp, Armonk, NY USA) and analytes with p-values < 0.05 were used in further analyses.

Differences between the two batches of SF, and differences between OA and RA groups for cell numbers, cytokine concentrations, and lipid concentrations were assessed using Mann-Whitney signed rank tests with Bonferroni corrections (GraphPad Prism 6, GraphPad Software, La Jolla California USA). All p-values indicated in the figure legends are Bonferroni corrected. Uncorrected p-values for lipids can be found in Suppl. Table 2.

Results

Inflammatory cells and cytokines in SF

To assess the inflammatory state of the patients, FACS analysis of SFC was performed on samples from 11 OA patients and 12 RA patients. The number of inflammatory cells was low in most OA samples (Fig.1A). Neutrophils, monocytes and T cells were present in comparable numbers, while the number of B cells was very low. Interestingly, a relatively large percentage of cells could not be attributed to these populations and remains to be determined (Fig.1B, top). The RA samples contained higher cell numbers and neutrophils were predominant, while monocytes, T cells and B cells were present in percentages comparable to those in the OA samples (Fig.1B, bottom).

Both pro- and anti-inflammatory cytokines were detectable in the 30 OA patients and 15 RA patients we analyzed, but had lower levels in OA samples (Fig.1C), compared to RA samples. Most cytokines correlated well with total cell numbers (data not shown). All measured chemokines were similar in OA and RA (Fig. 1D).

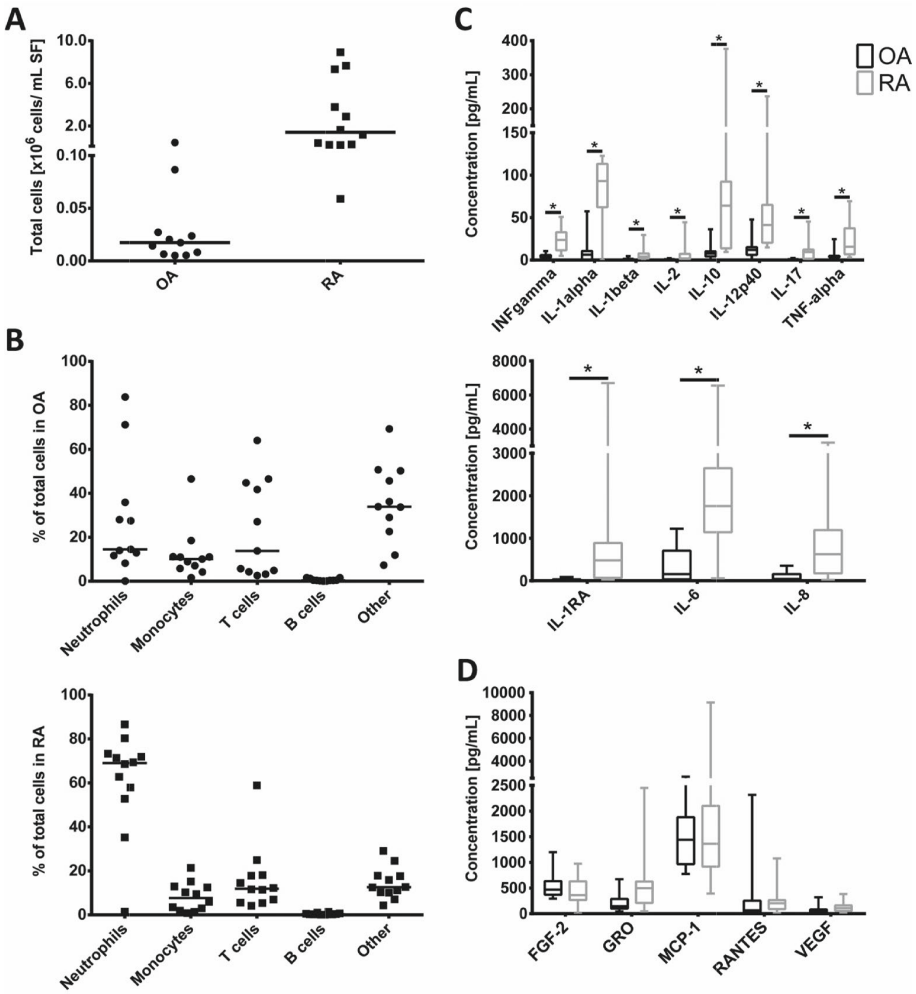

Figure 1. Inflammatory cells and cytokines in SF. **A,** Quantification of inflammatory cells in OA (n = 11, circles) and RA (n = 12, squares) samples. **B**, Percentages of different cell populations in SF of OA (top) and RA (bottom) are shown. Each dot represents one patient. Medians are depicted. Quantification of SF cytokines (**C**) and chemokines (**D**) in OA (n = 30) and RA (n = 15) samples. Medians with interquartile range are indicated. Whiskers indicate the minimum and maximum concentrations. Groups were compared using Mann-Whitney signed rank tests. *: $p < 0.002$

Targeted lipidomics analysis

With the LC-MS/MS platform used in this study, we can detect 60 analytes (Suppl. Table 2), including SPMs, such as for example resolvin E2 (RvE2) in whole blood supplemented with EPA (28) or RvD2 spiked into SF before hyaluronidase treatment (data not shown). Of these analytes, 37 were detected in at least one of the SPE worked-up samples (Suppl. Table 2). Concentrations in OA samples that were hyaluronidase-treated before storage were compared to samples treated after storage for several analytes (data

not shown) and as no systematic differences were found, the batches were combined and further analyzed as one. SPE precipitation after storage was compared to MeOH precipitation immediately upon collection in 20 randomly selected samples with rheumatic diseases (OA, RA and others) (29). For 28 of the 37 detected analytes, the concentrations determined by the two methods correlated well (Suppl. Table 2). Further analysis was restricted to these 28 analytes, which were determined upon SPE treatment in 24 OA and 10 RA samples.

Lipid mediators derived from w-6 and w-3 PUFA

Levels of PUFAs and oxylipids in both OA and RA samples are depicted in Fig. 2, Fig 3, and Suppl. Fig. 2. The analyte concentration in each patient group is depicted in Suppl. Table 2. Seven PUFAs were detected in SF of OA patients: the w-6 FAs, AA, adrenic acid (AdA) and linoleic acid (LA), and the w-3 FAs, EPA, DHA, docosapentaenoic acid (DPA_{n-3}) and alpha-linolenic acid (ALA)/gamma-linolenic acid (GLA). Moreover, oxidized products (both enzymatic and non-enzymatic) of these PUFAs were detected, including COX-1/2 and 12-LOX products of AA and 5- and 15-LOX products of multiple PUFAs. These included the precursors of SPMs: 15-HETE (precursor of lipoxin A_4), 17-HDHA (precursor of D series resolvins) and 18-HEPE (precursor of E series resolvins). LTB_4, 6-trans-LTB_4, and 20-OH-LTB_4 were low to undetectable in the OA samples. In general, the metabolites detected in OA were present at comparable levels in the RA samples, except the 5- and 15-LOX products of AA: 15-HETE, 6-trans-LTB_4, and 20-OH- LTB_4 (Fig. 2), and the 15-LOX metabolite of adrenic acid (AdA), 17-HDoTE (Fig.3), which were higher in RA than OA samples. None of the SPMs that can be measured with our platform (see Suppl. Table 2) could be detected in any of the samples.

Enzymatic pathways

The presence of a certain oxylipid is dependent on both the availability of its precursor and the activity of the enzyme involved in its generation. To assess the relative presence of certain enzymatic pathways in OA compared to RA patients, we established the ratios of oxylipids to their respective PUFA precursor. These ratios indicated that five metabolites of AA, one of DHA, and one of AdA, are less efficiently generated in OA than in RA (Fig. 4A and B and data not shown for 20-OH-LTB_4). Of these, four are generated via the 5-LOX pathway (Fig. 4A and 20-OH-LTB_4) and three via the 15-LOX pathway (Fig. 4B). These metabolites included the SPM precursors 15-HETE and 17-HDHA. No differences were found in metabolites generated via the COX-driven pathway (Fig. 4C), or the activity of leukotriene A4 hydrolase (LTA4H), assessed indirectly by using the concentration ratio of LTB_4 to 5-HETE (Fig. 4D). These data are interesting, as they indicate that the 5-LOX and 15-LOX pathways are less activated in OA than in RA, while other enzymatic pathways are similar.

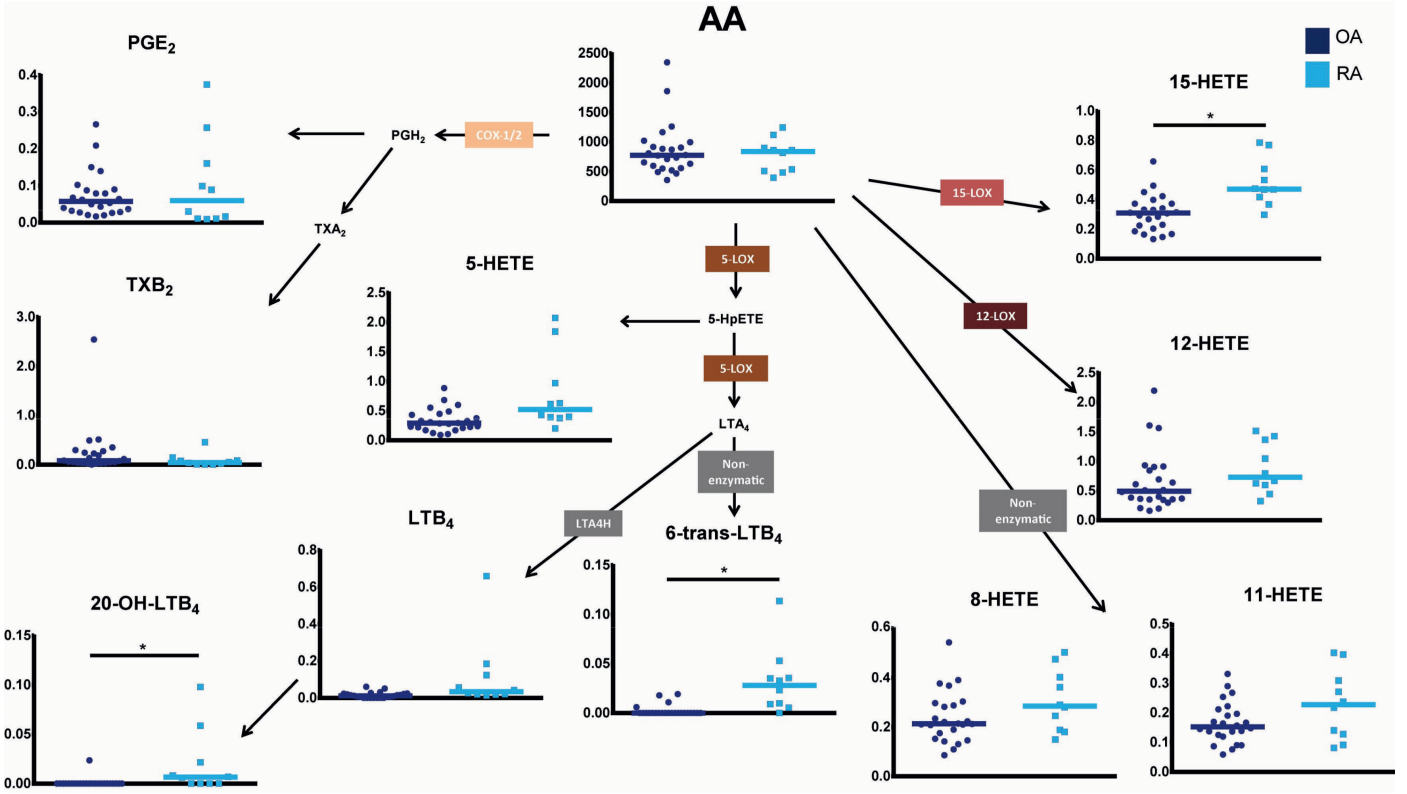

Figure 2. Concentrations (in ng per mL SF) of arachidonic acid (AA) and its metabolites measured in 24 OA and 10 RA samples. Each dot represents one patient. Medians are depicted. Groups were compared using Mann-Whitney signed ranked tests. *: $p < 0.01$. HETE: hydroxyeicosatetraenoic acid, LTA_4: leukotriene A_4, LTB_4: leukotriene B_4, PGH_2: prostaglandin H_2, PGE_2: prostaglandin E_2, TXA_2: thromboxane A_2, and TXB_2: thromboxane B_2. COX: cyclooxygenase, LOX: lipoxygenase, LTA4H: leukotriene A_4 hydrolase,.

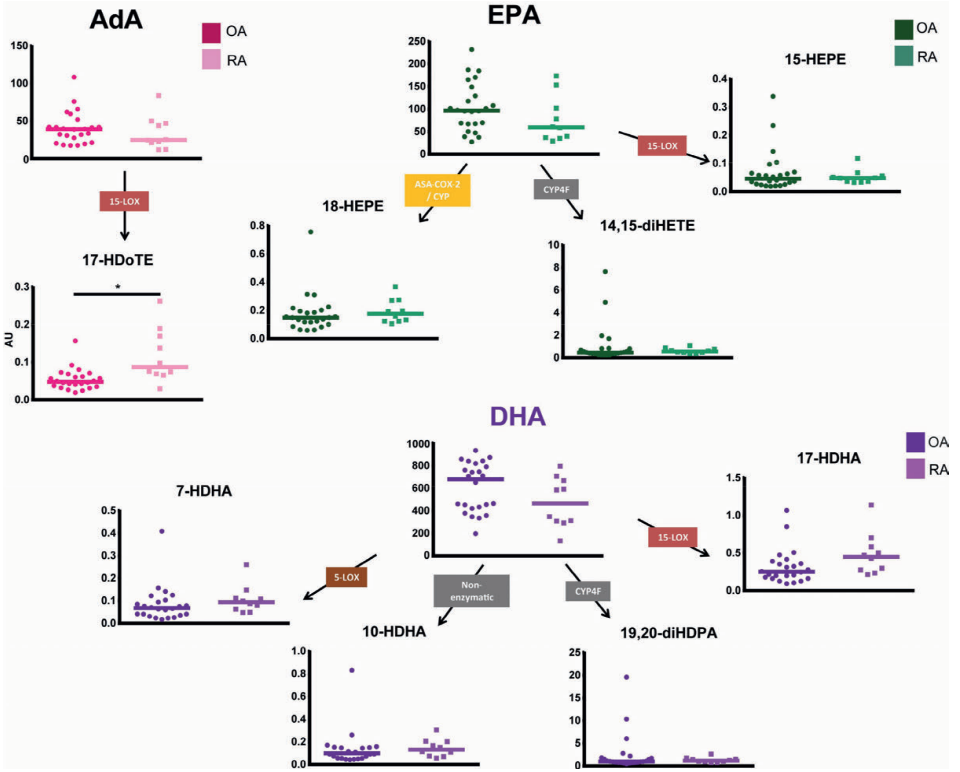

Figure 3. Concentrations (in ng per mL SF) of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), adrenic acid (AdA), and their metabolites measured in 24 OA and 10 RA samples. Levels of 17-HDoTE are in arbitrary units (AU). Each dot represents one patient. Medians are depicted. Groups were compared using Mann-Whitney signed ranked tests. *: $p < 0.05$. HEPE: hydroxyeicosapentaenoic acid, HDHA: hydroxydocosahexaenoic acid, diHETE: dihydroxyeicosatetraenoic acid, diHDPA: dihydroxydocosapentaenoic acid, HDoTE: hydroxydocosatetraenoic acid. ASA-COX: acetylated cyclooxygenase, LOX: lipoxygenase, CYP: Cytochrome P450, CYP4F: Cytochrome P450 4F.

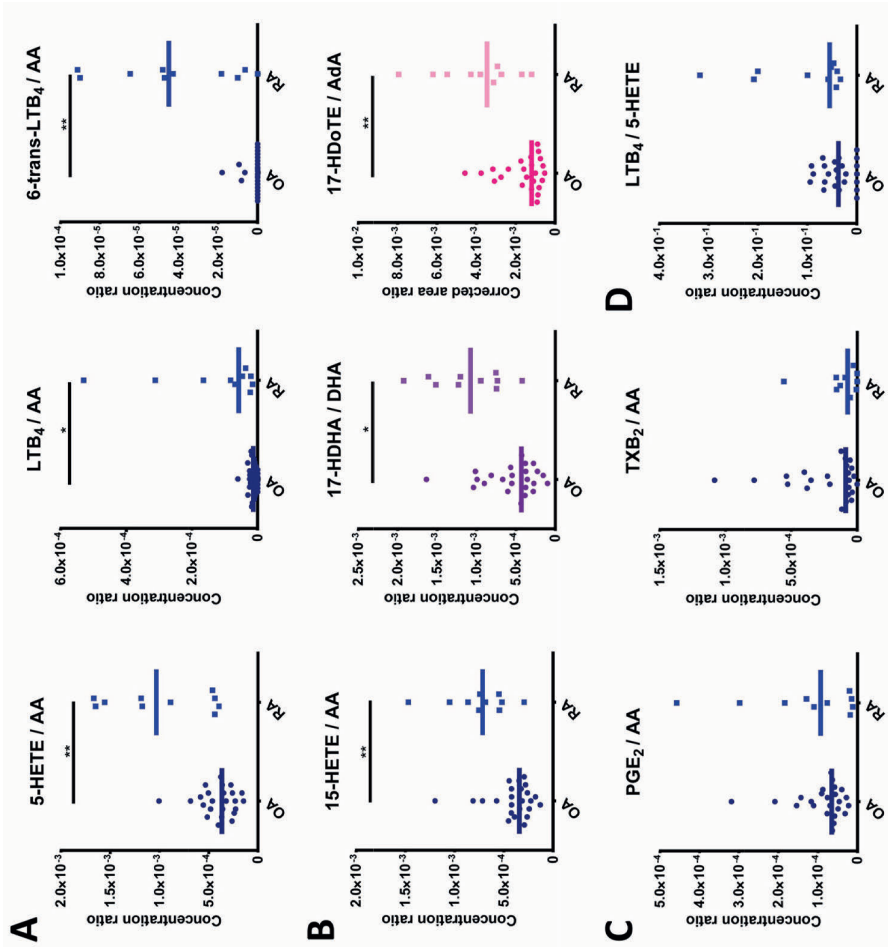
A
5-HETE / AA
LTB4 / AA
6-trans-LTB4 / AA
B
15-HETE / AA
17-HDHA / DHA
17-HDoTE / AdA
C
PGE2 / AA
TXB2 / AA
D
LTB4 / 5-HETE
Concentration ratio
Corrected area ratio
OA
RA

Figure 4.

< Figure 4. A, Concentrations ratios of 5-LOX products, 5-HETE, LTB_4, and 6-trans-LTB_4 to their precursor AA. **B**, Concentration ratios of 15-LOX products 15-HETE to AA, 17-HDHA to DHA and corrected area ratio of 17-HDoTE to AdA. **C**, Concentration ratios of COX products PGE_2 and TXB_2 to AA. **D**, Concentration ratio of 5-LOX products LTB_4 and 5-HETE. Each dot represents one patient (24 OA and 10 RA). Medians are depicted. Groups were compared using Mann-Whitney signed ranked tests. *: $p < 0.05$, **: $p < 0.01$. AA: arachidonic acid, DHA: docosahexaenoic acid, AdA: adrenic acid, HETE: hydroxyeicosatetraenoic acid, HDHA: hydroxydocosahexaenoic acid, HDoTE: hydroxydocosatetraenoic acid, LTB_4: leukotriene B_4, PGE_2: prostaglandin E_2, TXB_2: thromboxane B_2.

Bioactive lipid mediators and their precursors/pathway markers in OA joint cells

To assess which cells present in the knee joint could be responsible for the production of the oxylipids detected in OA SF, we isolated synoviocytes and SFC from OA patients. These were studied either unstimulated, directly *ex vivo* or after 3 days of culture, or after stimulation (Fig.5 and suppl. Fig. 3). Calcium ionophore stimulation was used as a potent activator of cPLA2 and subsequent bioactive lipid mediator synthesis (20, 30, 31), while LPS was used as a model TLR4 stimulus, as TLR4 is believed to mediate activation of synovial cells in OA through binding of extracellular matrix breakdown products (32). The unstimulated synoviocytes contained detectable levels of AA, EPA and DHA, as well as AA 5-LOX derivatives 5-HETE and LTB_4, and 15-LOX derivative 15-HETE indicating presence of activated 5-LOX and 15-LOX in these cells (Fig. 5A, "-"). In contrast, these lipids were only detectable in a part of the patients in SFC (Fig. 5B, "-"). Remarkably, LTB_4 could not be detected in SFC samples, while it was detectable in synoviocytes of all patients (Fig. 5A, B, "-"). After 3 days of culture, synoviocytes additionally contained detectable levels of the SPM precursors 17-HDHA and 18-HEPE, while these metabolites were undetectable in all SFC samples (Fig. 5C, D, "-"). Upon calcium ionophore stimulation, increased levels of AA, EPA, DHA, 5-HETE, 15-HETE, and LTB_4 (Fig. 5A, "+") were observed in synoviocytes of all patients. A similar trend was observed for SFC after calcium ionophore stimulation for AA, 5-HETE and LTB_4, although the data is likely underpowered to reach significance (Fig. 5B, "+"). LPS stimulation over 3 days had overall low effects and resulted in a significant, albeit small increase in EPA and 15-HETE in synoviocytes (Fig. 5C, "+"). Neither RvD2, nor other SPMs could be detected in either stimulated or unstimulated cells. These data indicate that both synoviocytes and SFC could contribute to the lipid mediator profile observed in SF of OA patients.

Lipid mediators in soluble and insoluble fraction of synovial fluid

Our data indicate the activation of resolution pathways in OA and RA. Because we did not detect the final pro-resolving lipids, we questioned whether this could be due to the isolation procedure. To investigate this possibility, we did a crude fractionation of five OA SF samples, in which we treated SF with hyaluronidase and then separated the supernatant (the soluble fraction) from the pellet (the insoluble fraction) (Fig. 6 and Suppl. Fig. 4).

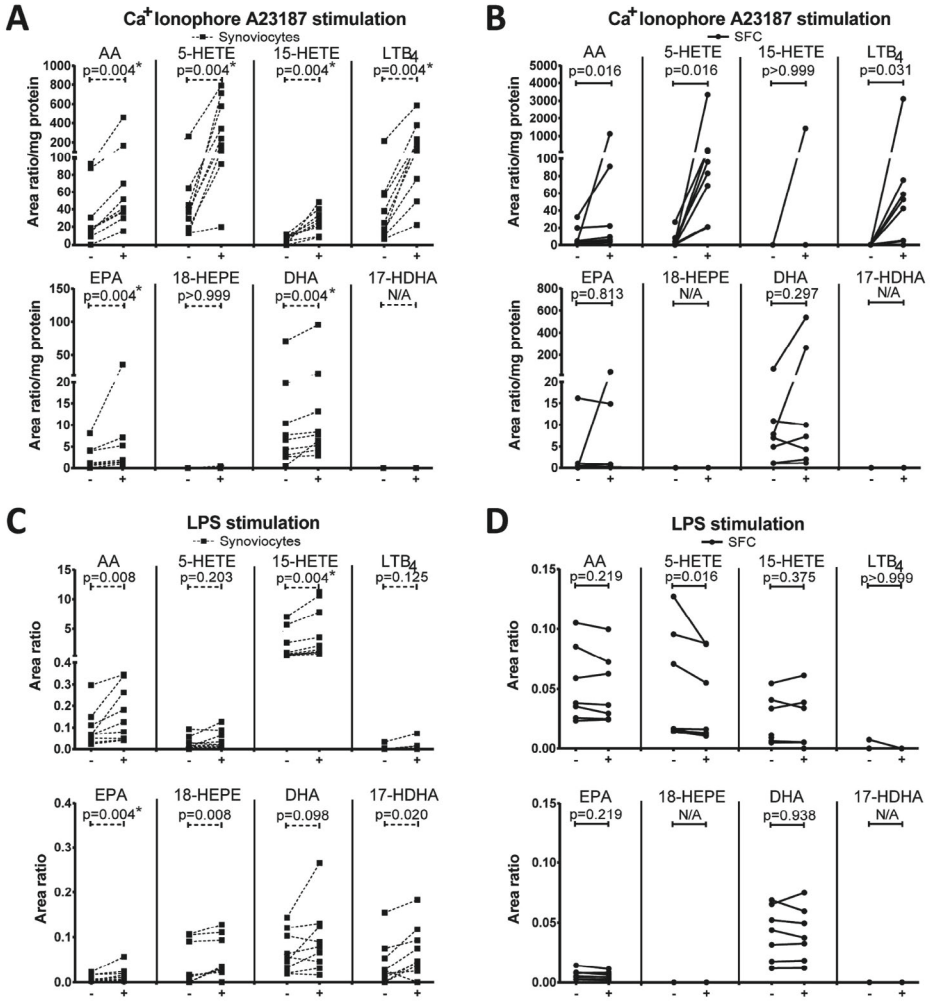

Figure 5. Protein corrected levels of PUFAs and oxylipids in calcium ionophore stimulated ("+") and unstimulated ("-") synoviocytes from nine patients (A) and synovial fluid cells (SFCs) from seven patients (**B**) . Areas corrected to IS of PUFAs and oxylipids in LPS stimulated ("+") and unstimulated ("-") synoviocytes from nine patients (**C**) and synovial fluid cells (SFCs) from seven patients (**D**)**.** Samples were compared using Wilcoxon matched-pairs signed rank test. *indicates significant differences after Bonferroni correction ($P < 0.0042$).

Consistent with the results in Fig. 2, Fig. 3, and Suppl. Fig. 2, we detected PUFAs, the monohydroxylated precursors of the SPMs like 15-HETE and 17-HDHA, as well as pro-inflammatory LMs such as PGE_2 and TXB_2 in the soluble fraction of all patients (Fig. 6 and data not shown). In the insoluble fraction, these analytes were also detectable in most patients (Fig. 6 and data not shown). Remarkably, although no SPMs could be detected in the soluble fraction, RvD2, a SPM derived from 17-HDHA, could be detected in the insoluble fraction in four out of five samples (Fig. 6 and Suppl. Fig. 4), indicating that the complete resolution pathway is activated in OA and is detectable in the joint.

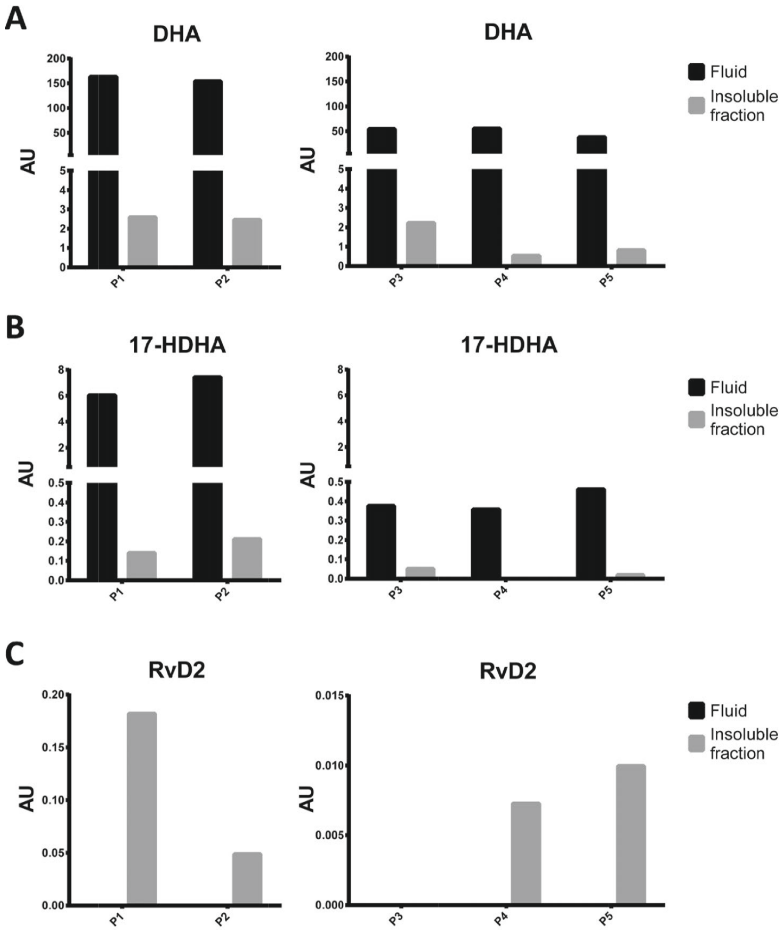

Figure 6. Both supernatant and insoluble fraction of OA SF samples were worked-up with SPE as described in *Materials and Method*s. Areas corrected to IS area (AU) are shown for DHA (**A**), its metabolites 17-HDHA (**B**) and RvD2 (**C**) in both the soluble (black) and insoluble (gray) fractions of the SF for five patients (P1-5). Samples were measured in 2 batches (presented left and right).

Discussion

In this study, we have characterized the inflammation present in the OA knee joint, by identifying inflammatory cells, cytokines, chemokines, PUFAs and oxylipids present in SF and comparing them to SF from RA patients (control). Our data indicate that inflammation is qualitatively and quantitatively different in OA compared to RA, being characterized by a lower inflammatory load (cells and cytokines), in agreement with previously published data (33). Moreover, by investigating the presence of SPMs and their precursors, as biomarkers of resolution, we found that resolution pathways are activated in OA, as well as RA. Remarkably, the SPM precursors are present in the fluid phase of synovial fluid,

while SPMs are detectable only in the insoluble fraction. Additionally, the enzymatic pathways involved in inflammation and its resolution seem to be less activated in OA than in RA. Finally, our data suggest that metabolites generated by these enzymatic pathways can be produced by OA synoviocytes and SFC.

Our data are in line with previous reports, indicating that SF of OA patients contains less inflammatory cells than of RA patients. Moreover, the composition of the cellular infiltrate was also different in these diseases, with OA SF containing relatively less infiltrating neutrophils than RA SF, but comparable percentages of monocytes, T cells and B cells. While GRO levels are similar in OA and RA, other neutrophil chemoattractants, such as IL-8 and LTB_4, which are lower in OA than RA, could account for the observed differences in the neutrophil population.

We have detected several pro- and anti-inflammatory cytokines, as well as pro- and anti-inflammatory LMs in SF of OA and RA. The inflammatory cell infiltrate correlated with the detected cytokine levels, suggesting that inflammatory cells in SF have a significant contribution to the production of these cytokines. In contrast, chemokine and most lipid levels were not different between OA and RA, despite differences in infiltrating cells numbers.

Interestingly, synovial cells (synoviocytes) and, to a lesser extent, SFC contained detectable levels of free fatty acids and derivatives even in the absence of extra stimulation, indicating them as a possible source of pro-inflammatory oxylipids, as well as SPM precursors in the joint. Moreover, synoviocytes and SFC were able to produce AA-derived oxylipids upon activation with calcium ionophore. Although the stimulus driving inflammation in OA is still unclear, there are indications that TLR4 could play a role in the disease by binding extracellular matrix breakdown products. Our data suggest a dual function for this receptor, both in the induction of inflammation and its resolution since TLR4 stimulation enhanced LXA_4 precursor 15-HETE production in synoviocytes. The temporal relationship between these functions remains to be elucidated. Moreover, in contrast to a previously published study (34), we could not detect LXA_4 upon stimulation of synoviocytes. The discrepancy could be explained by differences in experimental set-up, as the previous study has used synovial tissue explants, while we used isolated cells in order to minimize variations in cell number and composition inherent to explants. Moreover, the previous study has detected LXA_4 by ELISA, while we used a more specific targeted lipidomics approach based on LC-MS/MS.

As SPMs and their precursors are biomarkers of resolution pathways, our data indicate that the resolution pathways are activated in OA. Intriguingly, however, although the SPM

precursors were readily detectable in the soluble fraction of SF, only one SPM could be found, and only in the insoluble fraction containing cells and undigested tissue matrix. While the mechanisms underlying this dichotomy are still unclear, it is possible that other SPMs are either present only in low amounts in the SF volume that we tested, falling below our detection limit, or that they are short-lived. In either case, their association with the insoluble fraction could imply a short range of action around the cells that secrete them. Future studies are needed to address these possibilities. Likewise, the possible role for RvD2 in OA needs to be further studied. Previous data have indicated that RvD2 could attenuate pain in a fibromyalgia model (35) and in a model of inflammatory pain (36) in mice. Therefore, it is conceivable that the presence of RvD2 could be associated with less joint pain, which is a predominant feature of OA. However, due to lack of data regarding pain, we could not investigate this in our cohort. Likewise, 17-HDHA has been previously shown to reduce pain and tissue damage in a rat arthritis model (37), while 18-HEPE could reduce IL-6 production in cardiac fibroblasts (38). Whether these lipids also have an effect on cells/tissues involved in OA is yet unknown, but one could imagine that their described functions could be beneficial for OA.

However, despite the presence of RvD2, 17-HDHA and 18-HETE in SF, there is substantial inflammation still present in these patients, indicating that although resolution pathways are activated, they are probably incomplete or suboptimal in OA and RA. Moreover, they fail to counteract the pain and tissue destruction characteristics for these diseases. Likely explanations for this are that the LMs detected in SF might not be able to outcompete the inflammatory signals present in the joint of these patients or might not interfere with all pathways involved in the disease.

In contrast to our previous study (27) and a more recent study (39), we did not detect any SPMs in SF of RA patients. This could be due to differences in SF fluid volume and handling, as our previous samples were not treated with hyaluronidase and could therefore still have contained cells or other insoluble parts before storage and measurement. Additionally, different therapeutic treatments could influence the lipid profiles of the patients and could explain differences between cohorts.

A limited number of studies have previously shown the presence of 5- and 15-LOX in OA synovium, and have indicated in line with our findings, that the expression of these enzymes is lower in OA compared to RA synovium. However, our study now shows that lipids generated by these enzymes are present in SF of OA patients and detectable in synovial cells and to a lesser extent in SF cells, indicating that these enzymes are active in the OA synovium.

The (oxy)lipid profiles detected in RA SF were similar to the ones found in OA. Despite the higher inflammatory load present in the RA samples, the efficiency of the generation of (pro-) inflammatory LMs via the COX seemed similar in OA and RA. Together, these data suggest that SF inflammatory cells do not significantly add to the levels of these COX-derived LMs in SF in either disease.

In contrast, oxylipids that were different between OA and RA are generated via 5-LOX or 15-LOX pathways, suggesting a lower activity of these enzymes in OA compared to RA. This is also supported by differences in ratio between 5- or 15-LOX products and their PUFA precursors. Lower levels of LTB_4/AA in OA samples than RA could, in addition to decreased 5-LOX activity, also indicate a lower expression/activity of LTA4H. However, the ratio of LTB_4 to its pathway marker 5-HETE is not different between the two groups, indicating that the LTA4H activity is likely not different in the two patient groups. This is additionally supported by higher levels of the non-enzymatic LTA_4-derived 6-trans-LTB_4 in RA.

In conclusion, we have shown that knee SF of OA patients contains several inflammatory cells, as well as pro- and anti-inflammatory cytokines and oxylipids. In comparison to OA patients, the inflammatory load is higher in RA, with predominantly neutrophil infiltrate, which is accompanied by higher concentrations of cytokines and a higher activity of 5- and 15-LOX enzymes. By using a state-of-the-art technique, we now show for the first time that resolution pathways are present in OA patients. A better understanding of these pathways could guide us to a novel and effective therapeutic approach to inhibit inflammation and further structural damage in inflammatory joint disease as OA and RA.

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Author contributions

H. S. Jónasdóttir participated in acquiring the data, analysis and interpretation of the data, drafting of the article and critical revision of the article for important intellectual content, and final approval of the article.

H. Brouwers participated in acquiring the data, analysis and interpretation of the data, drafting of the article and critical revision of the article for important intellectual content, and final approval of the article.

J. C. Kwekkeboom participated in acquiring and analyzing the data, revising the article critically for important intellectual content, and final approval of the article.

E. M. J. van der Linden participated in acquisition of data, revising the article critically for important intellectual content, and final approval of the article.

T. Huizinga participated in interpretation of data, revising the article critically for important intellectual content, and final approval of the article.

M. Kloppenburg participated in interpretation of data, revising the article critically for important intellectual content, and final approval of the article.

R. E. M. Toes participated in interpretation of data, revising the article critically for important intellectual content, and final approval of the article.

M. Giera participated in the conception and design of the study, interpretation of data, revising the article critically for important intellectual content, and final approval of the article.

A. Ioan-Facsinay participated in the conception and design of the study, interpretation of data, drafting and critical revision of the article for important intellectual content, and final approval of the article.

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Conflict of interest

None

References

1. Yusuf E, Kortekaas MC, Watt I, Huizinga TWJ, Kloppenburg M. Do knee abnormalities visualised on MRI explain knee pain in knee osteoarthritis? A systematic review. Ann Rheum Dis. 2011; 70: 60-67.
2. Berenbaum F. Osteoarthritis as an inflammatory disease (osteoarthritis is not osteoarthrosis!). Osteoarthritis Cartilage. 2013; 21: 16-21.
3. Kortekaas MC, Kwok W-Y, Reijnierse M, Kloppenburg M. Inflammatory ultrasound features show independent associations with progression of structural damage after over 2 years of follow-up in patients with hand osteoarthritis. Ann Rheum Dis. 2015; 74: 1720-1724.
4. Lawrence T, Willoughby DA, Gilroy DW. Anti-inflammatory lipid mediators and insights into the resolution of inflammation. Nat Rev Immunol. 2002; 2: 787-795.
5. Newson J, Stables M, Karra E, Arce-Vargas F, Quezada S, Motwani M, et al. Resolution of acute inflammation bridges the gap between innate and adaptive immunity. Blood. 2014; 124: 1748-1764.
6. Levy BD. Resolvins and protectins: Natural pharmacophores for resolution biology. Prostaglandins Leukot Essent Fatty Acids. 2010; 82: 327-332.
7. Serhan CN, Savill J. Resolution of inflammation: the beginning programs the end. Nat Immunol. 2005; 6: 1191-1197.
8. Serhan CN, Clish CB, Brannon J, Colgan SP, Chiang N, Gronert K. Novel functional sets of lipid-derived mediators with antiinflammatory actions generated from omega-3 fatty acids via cyclooxygenase 2–nonsteroidal antiinflammatory drugs and transcellular processing. J Exp Med. 2000; 192: 1197-1204.
9. Bannenberg GL, Chiang N, Ariel A, Arita M, Tjonahen E, Gotlinger KH, et al. Molecular circuits of resolution: formation and actions of resolvins and protectins. J Immunol. 2005; 174: 4345-4355.
10. Fredman G, Li Y, Dalli J, Chiang N, Serhan CN. Self-limited versus delayed resolution of acute inflammation: Temporal regulation of pro-resolving mediators and microRNA. Sci Rep. 2012; 2: 639.
11. Serhan CN, Chiang N, Dalli J. The resolution code of acute inflammation: Novel pro-resolving lipid mediators in resolution. Seminars in Immunology. 2015; 27: 200-215.
12. Bhavsar P, Levy B, Hew M, Pfeffer M, Kazani S, Israel E, et al. Corticosteroid suppression of lipoxin A4 and leukotriene B4 from alveolar macrophages in severe asthma. Respir Res. 2010; 11: 71.
13. Zhu M, Wang X, Hjorth E, Colas R, Schroeder L, Granholm A-C, et al. Pro-resolving lipid mediators improve neuronal survival and increase Aβ42 phagocytosis. Mol Neurobiol, 9 December, 2015 ed: Springer US 2015.
14. Wang X, Zhu M, Hjorth E, Cortés-Toro V, Eyjolfsdottir H, Graff C, et al. Resolution of inflammation is altered in Alzheimer's disease. Alzheimers Dement. 2015; 11: 40-50.e42.
15. Prüss H, Rosche B, Sullivan AB, Brommer B, Wengert O, Gronert K, et al. Proresolution lipid mediators in multiple sclerosis — differential, disease severity-dependent synthesis — a clinical pilot trial. PLoS ONE. 2013; 8: e55859.
16. Rozsasi A, Heinemann A, Keck T. Cyclooxygenase 2 and lipoxin A4 in nasal polyps in cystic fibrosis. Am J Rhinol Allergy. 2011; 25: e251-e254.

17. Vong L, Ferraz JGP, Dufton N, Panaccione R, Beck PL, Sherman PM, et al. Up-regulation of annexin-A1 and lipoxin A4 in individuals with ulcerative colitis may promote mucosal homeostasis. PLoS ONE. 2012; 7: e39244.

18. Serhan CN, Petasis NA. Resolvins and protectins in inflammation resolution. Chem Rev. 2011; 111: 5922-5943.

19. Bannenberg G, Serhan CN. Specialized pro-resolving lipid mediators in the inflammatory response: An update. Biochim Biophys Acta. 2010; 1801: 1260-1273.

20. Dalli J, Serhan CN. Specific lipid mediator signatures of human phagocytes: microparticles stimulate macrophage efferocytosis and pro-resolving mediators. Blood. 2012; 120: e60.

21. Norris PC, Gosselin D, Reichart D, Glass CK, Dennis EA. Phospholipase A2 regulates eicosanoid class switching during inflammasome activation. Proceedings of the National Academy of Sciences. 2014; 111: 12746-12751.

22. Attur M, Krasnokutsky S, Statnikov A, Samuels J, Li Z, Friese O, et al. Low-grade inflammation in symptomatic knee osteoarthritis: prognostic value of inflammatory plasma lipids and peripheral blood leukocyte biomarkers. Arthritis Rheumatol. 2015; 67: 2905-2915.

23. Rae SA, Davidson EM, Smith MJH. Leukotriene B_4, an inflammatory mediator in gout. Lancet. 1982; 320: 1122-1124.

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

25. He W, Pelletier J-P, Martel-Pelletier J, Laufer S, Di Battista JA. Synthesis of interleukin 1beta, tumor necrosis factor-alpha, and interstitial collagenase (MMP-1) is eicosanoid dependent in human osteoarthritis synovial membrane explants: interactions with antiinflammatory cytokines. J Rheumatol. 2002; 29: 546-553.

26. Gheorghe KR, Korotkova M, Catrina AI, Backman L, af Klint E, Claesson H-E, et al. Expression of 5-lipoxygenase and 15-lipoxygenase in rheumatoid arthritis synovium and effects of intraarticular glucocorticoids. Arthritis Res Ther. 2009; 11: R83-R83.

27. Giera M, Ioan-Facsinay A, Toes R, Gao F, Dalli J, Deelder AM, et al. Lipid and lipid mediator profiling of human synovial fluid in rheumatoid arthritis patients by means of LC–MS/MS. Biochim Biophys Acta. 2012; 1821: 1415-1424.

28. Jónasdóttir H, Ioan-Facsinay A, Kwekkeboom J, Brouwers H, Zuurmond A-M, Toes R, et al. An advanced LC–MS/MS platform for the analysis of specialized pro-resolving lipid mediators. Chromatographia. 2015; 78: 391-401.

29. Barden AE, Mas E, Croft KD, Phillips M, Mori TA. Minimizing artifactual elevation of lipid peroxidation products (F2-isoprostanes) in plasma during collection and storage. Anal Biochem. 2014; 449: 129-131.

30. Song J, Liu X, Rao TS, Chang L, Meehan MJ, Blevitt JM, et al. Phenotyping drug polypharmacology via eicosanoid profiling of blood. Journal of Lipid Research. 2015; 56: 1492-1500.

31. Mukherjee PK, Marcheselli VL, Serhan CN, Bazan NG. Neuroprotectin D1: A docosahexaenoic acid-derived docosatriene protects human retinal pigment epithelial cells from oxidative stress. Proceedings of the National Academy of Sciences of the United States of America. 2004; 101: 8491-8496.

32. Robinson WH, Lepus CM, Wang Q, Raghu H, Mao R, Lindstrom TM, et al. Low-grade inflammation as a key mediator of the pathogenesis of osteoarthritis. Nat Rev Rheumatol. 2016; 12: 580-592.

33. de Lange-Brokaar BJE, Ioan-Facsinay A, van Osch GJVM, Zuurmond AM, Schoones J, Toes REM, et al. Synovial inflammation, immune cells and their cytokines in osteoarthritis: a review. Osteoarthritis Cartilage. 2012; 20: 1484-1499.

34. Marcouiller P, Pelletier J-P, Guévremont M, Martel-Pelletier J, Ranger P, Laufer S, et al. Leukotriene and prostaglandin synthesis pathways in osteoarthritic synovial membranes: regulating factors for interleukin 1beta synthesis. The Journal of Rheumatology. 2005; 32: 704-712.

35. Klein CP, Sperotto NDM, Maciel IS, Leite CE, Souza AH, Campos MM. Effects of D-series resolvins on behavioral and neurochemical changes in a fibromyalgia-like model in mice. Neuropharmacology. 2014; 86: 57-66.

36. Park C-K, Xu Z-Z, Liu T, Lü N, Serhan CN, Ji R-R. Resolvin D2 is a potent endogenous inhibitor for transient receptor potential subtype V1/A1, inflammatory pain, and spinal cord synaptic plasticity in mice: Distinct roles of resolvin D1, D2, and E1. The Journal of Neuroscience. 2011; 31: 18433.

37. Lima-Garcia JF, Dutra RC, da Silva K, Motta EM, Campos MM, Calixto JB. The precursor of resolvin D series and aspirin-triggered resolvin D1 display anti-hyperalgesic properties in adjuvant-induced arthritis in rats. British Journal of Pharmacology. 2011; 164: 278-293.

38. Endo J, Sano M, Isobe Y, Fukuda K, Kang JX, Arai H, et al. 18-HEPE, an n-3 fatty acid metabolite released by macrophages, prevents pressure overload–induced maladaptive cardiac remodeling. The Journal of Experimental Medicine. 2014; 211: 1673-1687.

39. Barden AE, Moghaddami M, Mas E, Phillips M, Cleland LG, Mori TA. Specialised pro-resolving mediators of inflammation in inflammatory arthritis. Prostaglandins Leukot Essent Fatty Acids. 2016; 107: 24-29.

Supplementary materials and methods

Chemicals and Materials

Hyaluronidase from bovine testes was from Sigma Aldrich (Schnelldorf, Germany) Phosphate buffered saline without calcium and magnesium 10× (PBS (-/-)) was from Life Technologies (Carlsbad, CA, USA). For LM analysis LC-MS grade methanol (MeOH), glacial acetic acid pro analysi (p.a.), LC-MS grade water, hydrochloric acid, and methyl formate were from Sigma Aldrich (Schnelldorf, Germany). Ethanol p.a. was from Merck (Darmstadt, Germany). All substances used as standards were from Cayman Chemicals (Ann Arbor, MI, USA), except RvE1, RvE2 18S-RvE3 and 18R-RvE3 (kind gifts from Dr. Makoto Arita, Tokyo, Japan), and 17-hydroxydocosatetraenoic acid (17-HDoTE) which was made in-house (see Production of 17-HDoTE below). Sample tubes (1.5mL) were from Eppendorf (Hamburg, Germany) and glass vials were from Corning Inc. (Corning, NY, USA) and Fisher Scientific (Hampton, NH, USA). C18 solid phase extraction cartridges were either from Waters (Boston, MA, USA), Sep-Pak C18, 200 mg, 3 mL; or Agilent Technologies (Santa Clara, CA, USA), Bond Elut C18, 500 mg, 3mL. Autosampler vials, caps and inserts were from Agilent Technologies (Waldbronn, Germany). The internal standard (IS) solution used for targeted lipid analysis consisted of LTB_4-d4, 15(*S*)-HETE-d8, PGE_2-d4 and DHA-d5, in MeOH.

Cytokine analysis

After hyaluronidase treatment, 70 μL SF was centrifuged over a 0.5 mL Costar spin-X column (Corning inc., Corning, NY, USA) at 13,400 ×g for 5 min. Sixteen cytokines/chemokines were determined using the Milliplex MAP Human Cytokine/chemokine kit (EMD Millipore Merck, Darmstadt, Germany), according to the manufacturer's instructions. Samples were measured on the Bio-Plex 200 system (Bio-Rad) and analysis was done using Bio-plex Manager 6.0 Software (Bio-Rad).

FACS analysis

SFC were stained with a mixture of CD3-AF700 (clone UCHT1), CD19-APC-Cy7 (clone SJ25C1), CD14-FITC (clone M5E2), CD15-APC (clone HI98) and CD16-PE (clone B73.1) (BD Pharmingen,San Diego, CA, USA) for 30 min. at 4 °C. Dead cells were excluded using DAPI (Molecular Probes, Eugene, OR, USA). The number of SFC was calculated using Flow-Count fluorospheres (Beckman Coulter, Brea, CA, USA). Samples were measured on a LSRFortessa (BD Biosciences, San Jose, CA, USA) and analyzed with FACSDiva software (BD Biosciences).

Targeted lipid analysis

Briefly: Lipid analysis was achieved using a QTrap 6500 mass spectrometer in negative ESI mode (Sciex, Nieuwerkerk aan den Ijssel, The Netherlands), coupled to a LC system employing two LC-30AD pumps, a SIL-30AC autosampler, and a CTO-20AC column oven. (Shimadzu, 's-Hertogenbosch, The Netherlands). The employed column was a Kinetex C_{18} 50 × 2.1 mm, 1.7 μm, protected with a C8 precolumn (Phenomenex, Utrecht, The Netherlands), kept at 50 °C. The following binary gradient of water (A) and MeOH (B) with 0.01% acetic acid was used: 0 min 30% B, held for 1 min, then ramped to 45% at 1.1 min, to 53.5% at 2 min, to 55.5% at 4 min, to 90% at 7 min, and to 100% B at 7.1 min, held for 1.9 min. The injection volume was 40 μL and the flow rate 400 μL/min. The MS was operated under the same conditions as in (1). In addition to the mass transition used for each analyte (See Supplementary Table 2), relative retention times (RRT) were used for identification. For quantification calibration lines, made with standard material for each analyte (see Suppl. Table 2 for range), were used and only peaks with a signal to noise (S/N) > 10 were quantified. For analytes where no calibration line was used, area ratios were used and S/N > 3 was used as a detection limit. The LC-MS/MS method used does not discriminate between alpha-linolenic acid (ALA) and gamma-linolenic acid (GLA), and therefore the detected fatty acid(s) is listed as ALA/GLA.

Production of 17-HDoTE

To 5 glass tubes containing 1 mg AdA were added 5 mL 0.15 M Tris buffer (pH 9.0). The samples were sonicated for one minute and subsequently bubbled for one minute with 99.90% oxygen. 150 μL of 157,000 units/mL soybean 15-LOX (Cayman Chemicals, Ann Arbor, MI, USA) was added to each sample and the reaction was quenched with 5 mL methanol after 10 min incubation. Hydroperoxides were reduced by addition of 30 μL $SnCl_2$ (160 mg/ml). After 5 min incubation, samples were centrifuged at 3184 ×g for 10 min. The supernatant was decanted and acidified with 200 μL formic acid. All samples were transferred into a separation funnel. 3 mL chloroform was added and mixed, and the lower organic phase was collected. This step was repeated twice. Anhydrous sodium sulfate was added to the combined organic extracts. The dried organic layer was decanted and the tube washed with a small amount of chloroform which was decanted likewise. After drying the sample under a stream of N_2, the residue was taken up in 1 mL methanol and stored at -20 °C before fractionation using LC-UV analysis.

Fractionation using LC-UV

To isolate the main monohydroxylated product of AdA by 15-LOX incubation, liquid chromatography-ultra violet spectroscopy (LC-UV) was used. The LC-UV fractionation was carried out on a Dionex Ultimate 3000 HPLC system interfaced with a Dionex RS Diode Array Detector. HPLC was performed using an eclipse plus 1.8 μm C18 column

(Agilent, 50 × 4.6 mm). The column oven was set to 50 °C and the injection-volume was 20 μL. The mobile phase consisted of solvent A, water containing 0.01% acetic acid, and solvent B, methanol containing 0.01% acetic acid. The flow rate was constant at 0.50 mL/min. For the isolation of the 15-LOX reaction products the system was operated under isocratic conditions of 90% B for 3.5 min. The eluent between 2.00 and 2.37 min was collected and dried with N_2 and the residue was dissolved in MeOH and stored at -20 °C.

Product analysis using LC-MS/MS

Reaction product analysis was executed on a Dionex Ultimate 3000 HPLC instrument interfaced with a Sciex QTrap 6500. HPLC was performed using a kinetex 1.7 μm C18 column (Phenomenx, 50 × 2.1 mm) and the column oven was set at 50 °C. The injection volume was 20 μL. The mobile phase consisted of solvent A and solvent B as described for LC-UV. The system was operated using gradient elution. Solvent B increased linearly from 40% to 85% in 10 min and from 10.1 until 13 min to 100% B and kept constant for 0.5 min. The flow rate was 250 μL/min. The tandem mass spectrometry analysis (MS/MS) was performed using electrospray ionization in the negative ion mode. Precursor ions (MS_1) were selected between 300 and 400 Da. Enhanced product ions (EPI) included *m/z* 379.1*,* 363.2 and 347.0 for generation of product ion spectra.

NMR analysis

890 μg 17-HDoTE was dissolved in $CDCl_3$ for nuclear magnetic resonance (NMR) data acquisition. The NMR spectra were recorded on a Bruker Avance II spectrometer (Bruker BioSpin, Karlsruhe, Germany) operating at frequencies of 600.13 MHz (^{1}H) and 150.92 MHz (^{13}C). The ^{1}H NMR chemical shifts were referenced to the signal of $CHCl_3$ (7.26 ppm), while the ^{13}C NMR chemical shifts were referenced to the signal of $CDCl_3$ (77.36 ppm).

1. Jónasdóttir H, Ioan-Facsinay A, Kwekkeboom J, Brouwers H, Zuurmond A-M, Toes R, et al. An advanced LC–MS/MS platform for the analysis of specialized pro-resolving lipid mediators. Chromatographia. 2015; 78: 391-401.

Supplementary data

Patient characteristics	FACS	Cytokines	SPE
Patients with OA	n = 11	n = 30	n = 24
Age [years], mean(SD), n	70(10), 10	68(7), 28	68(9), 23
BMI [kg/m^2], mean(SD), n	31(3), 10	29(4), 28	31(4), 22
Gender, number of females(%), n	7 (70), 10	16 (57), 28	12 (52), 23
Patients with RA	n = 12	n =15	n = 10
Age [years], mean(SD), n	58(17), 8	62(7), 4	55(16), 9
BMI [kg/m^2], mean(SD), n	25(4), 7	27(5), 3	25(3), 8
Gender, number of females(%), n	4 (50), 8	2 (50), 4	5 (56), 9

Supplementary Table 1. Available clinical data: Age, BMI and gender for OA and RA patients. Not all patient samples were used for all experiments.

Supplementary Table 2.

Lipid maps ID	Analyte	MRM transition in ESI (-) [*m/z*]	Calibration line injected concentration range [ng/mL]	OA Detected in at least one sample using SPE work-up	RA Detected in at least one sample using SPE work-up	OA Median (Concentration range) [ng/mL SF] n=24	RA Median (Concentration range) [ng/mL SF] n=10	SPE *vs.* MeOH Spearman's correlation [ρ]	SPE *vs.* MeOH Spearman's correlation [p-value]	OA *vs.* RA Concentration [p-value]	OA *vs.* RA Concentration ratio to PUFA [p-value]
LMFA04000027	10-HDHA	343.1→153	0.025 - 10	X	X	0.10 (0.04-0.83)	0.13 (0.06-0.31)	0.74	<0.001	0.1587	0.0040
LMFA03060085	11-HETE	319.1→167	0.025 - 10	X	X	0.15 (0.06-0.33)	0.23 (0.08-0.40)	0.74	<0.001	0.1826	0.0838
LMFA03060088	12-HETE	319.1→179	0.025 - 10	X	X	0.49 (0.16-2.20)	0.73 (0.33-1.51)	0.82	<0.001	0.1272	0.0204
LMFA02000228	13-HODE	295.0→195	N/A	X	X	N/A	N/A	0.73	<0.001	0.0163	0.0310
LMFA02000051	13-HOTrE	293.1→195	N/A	X	X	N/A	N/A	0.44	0.05	N/A	N/A
LMFA03060077	14,15-diHETE	335.1→207	0.01 - 2	X	X	0.47 (0.25-7.66)	0.56 (0.37-1.08)	0.58	0.007	0.3598	N/A
LMFA03070032	15-HEPE	317.1→219	0.025 - 10	X	X	0.05 (0.02-0.34)	0.05 (0.03-0.12)	0.72	<0.001	0.9820	0.1178
LMFA03060087	15-HETE	319.1→219	0.025 - 10	X	X	0.31 (0.13-0.66)	0.47 (0.30-0.79)	0.80	<0.001	0.0005*	0.0009*
LMFA04000032	17-HDHA	343.1→245	0.025 - 10	X	X	0.25 (0.09-1.07)	0.45 (0.22-1.14)	0.84	<0.001	0.0310	0.0015*
N/A	17-HDoTE	347.1→247	N/A	X	X	N/A	N/A	0.60	0.006	0.0017*	0.0006*
LMFA03070033	18-HEPE	317.1→259	0.025 - 10	X	X	0.15 (0.06-0.76)	0.18 (0.11-0.37)	0.89	<0.001	0.3035	0.0090
LMFA04000043	19,20-diHDPA	361.1→273	0.01 - 2	X	X	0.99 (0.47-19.58)	1.16 (0.74-2.60)	0.46	0.04	0.7300	N/A
LMFA03020018	20-OH-LTB$_4$	351.1→195	0.01 - 2	X	X	0 (0-0.02)	0.01 (0-0.10)	0.76	<0.001	0.0007*	0.0007*
LMFA03060084	5-HETE	319.1→115	0.025 - 10	X	X	0.29 (0.09-0.88)	0.52 (0.20-2.08)	0.49	0.03	0.0080	0.0013*
LMFA03020014	6-trans-12-epi-LTB4	335.1→195	0.01 - 2	X	X	0 (0-0.02)	0.02 (0-0.13)	0.28	0.23	N/A	N/A

Supplementary Table 2. *[Continued]*

Lipid maps ID	Analyte	MRM transition in ESI (-) [*m/z*]	Calibration line injected concentration range [ng/mL]	OA Detected in at least one sample using SPE work-up	RA Detected in at least one sample using SPE work-up	OA Median (Concentration range) [ng/mL SF] n=24	RA Median (Concentration range) [ng/mL SF] n=10	SPE *vs.* MeOH Spearman's correlation [ρ]	SPE *vs.* MeOH Spearman's correlation [p-value]	OA *vs.* RA Concentration [p-value]	OA *vs.* RA Concentration ratio to PUFA [p-value]
LMFA03020013	6-trans-LTB_4	335.1→195	0.01 - 2	X	X	0 (0-0.02)	0.03 (0-0.11)	0.73	<0.001	< 0.0001*	<0.0001*
LMFA04000025	7-HDHA	343.1→141	0.025 - 10	X	X	0.07 (0.02-0.41)	0.09 (0.05-0.26)	0.56	0.01	0.1178	0.0035
N/A	7S,17S-diHDPA	335.1→208	0.025 - 2	X	n.d	0 (0-0.13)	N/A	N/A	N/A	N/A	N/A
LMFA03060086	8-HETE	319.1→155	0.025 - 10	X	X	0.21 (0.09-0.54)	0.29 (0.15-0.50)	0.66	0.001	0.1587	0.0352
LMFA03110003	8-iso-PGE_2	351.1→271	0.01 - 2	X	X	0 (0-0.02)	0 (0-0.02)	0.21	0.38	N/A	N/A
LMFA03110001	8-iso-$PGF_{2\alpha}$	353.1→193	0.01 - 2	X	X	0.067 (0.02-0.27)	0.02 (0-0.12)	N/A	N/A	N/A	N/A
LMFA02000151	9-HODE	295.0→171	N/A	X	X	N/A	N/A	0.52	0.019	0.0222	0.7628
LMFA02000024	9-HOTrE	293.0→171	N/A	X	X	N/A	N/A	0.56	0.01	0.0426	0.6264
LMFA01030001	AA	303.0→205	5 - 500	X	X	$7.7x10^2$ ($3.5x10^2$-$2.3x10^3$)	$8.4x10^2$ ($3.9x10^2$-$1.2x10^3$)	0.83	<0.001	0.6798	N/A
LMFA01030178	AdA	331.1→233	1 - 200	X	X	$3.9x10^1$ ($1.8x10^1$-$1.1x10^2$)	$2.5x10^1$ ($1.2x10^1$-$8.4x10^1$)	0.62	0.003	0.4780	N/A
LMFA01030152	ALA/GLA	277.0→233	N/A	X	X	N/A	N/A	0.87	<0.001	0.0069	N/A

Supplementary Table 2. *[Continued]*

Lipid maps ID	Analyte	MRM transition in ESI (-) [*m/z*]	Calibration line injected concentration range [ng/mL]	OA Detected in at least one sample using SPE work-up	RA Detected in at least one sample using SPE work-up	OA Median (Concentration range) [ng/mL SF] n=24	RA Median (Concentration range) [ng/mL SF] n=10	SPE *vs.* MeOH Spearman's correlation [ρ]	SPE *vs.* MeOH Spearman's correlation [p-value]	OA *vs.* RA Concentration [p-value]	OA *vs.* RA Concentration ratio to PUFA [p-value]
LMFA01030185	DHA	327.1→229	5 - 500	X	X	$6.8x10^2$ ($2.0x10^2$-$9.4x10^2$)	$4.7x10^2$ ($1.3x10^2$-$8.0x10^2$)	0.92	<0.001	0.0645	N/A
LMFA04000044	DPA_{n-3}	329.1→231	5 - 500	X	X	$9.2x10^1$ ($4.1x10^1$-$2.6x10^2$)	$7.3x10^1$ ($3.6x10^1$-$3.2x10^2$)	0.89	<0.001	0.6264	N/A
LMFA01030759	EPA	301.0→203	1 - 200	X	X	$9.7x10^1$ ($2.7x10^1$-$2.3x10^2$)	$6.0x10^1$ ($2.9x10^1$-$1.7x10^2$)	0.88	<0.001	0.1335	N/A
LMFA01030120	LA	279.0→261	5 - 500	X	X	$4.1x10^3$ ($2.5x10^3$-$1.0x10^4$)	$3.7x10^3$ ($8.7x10^2$-$5.7x10^3$)	0.64	0.002	0.0590	N/A
LMFA03020001	LTB_4	335.1→195	0.01 - 2	X	X	0.01 (0-0.06)	0.03 (0.01-0.66)	0.91	<0.001	0.0019	0.0002*
LMFA03020006	LTD_4	495.1→177	0.01 - 2	n.d	X	N/A	0 (0-0.73)	0.29	0.21	N/A	N/A
LMFA03020002	LTE_4	438.1→333	0.01 - 2	X	X	0 (0-0.07)	0 (0-0.01)	0.27	0.26	N/A	N/A
LMFA03010004	PGD_2	351.1→233	0.01 - 2	X	X	0.01 (0-1.07)	0 (0-0.01)	N/A	N/A	N/A	N/A
LMFA03010003	PGE_2	351.2→271	0.01 - 2	X	X	0.06 (0.02-0.27)	0.06 (0.01-0.37)	0.96	<0.001	0.7628	0.9491

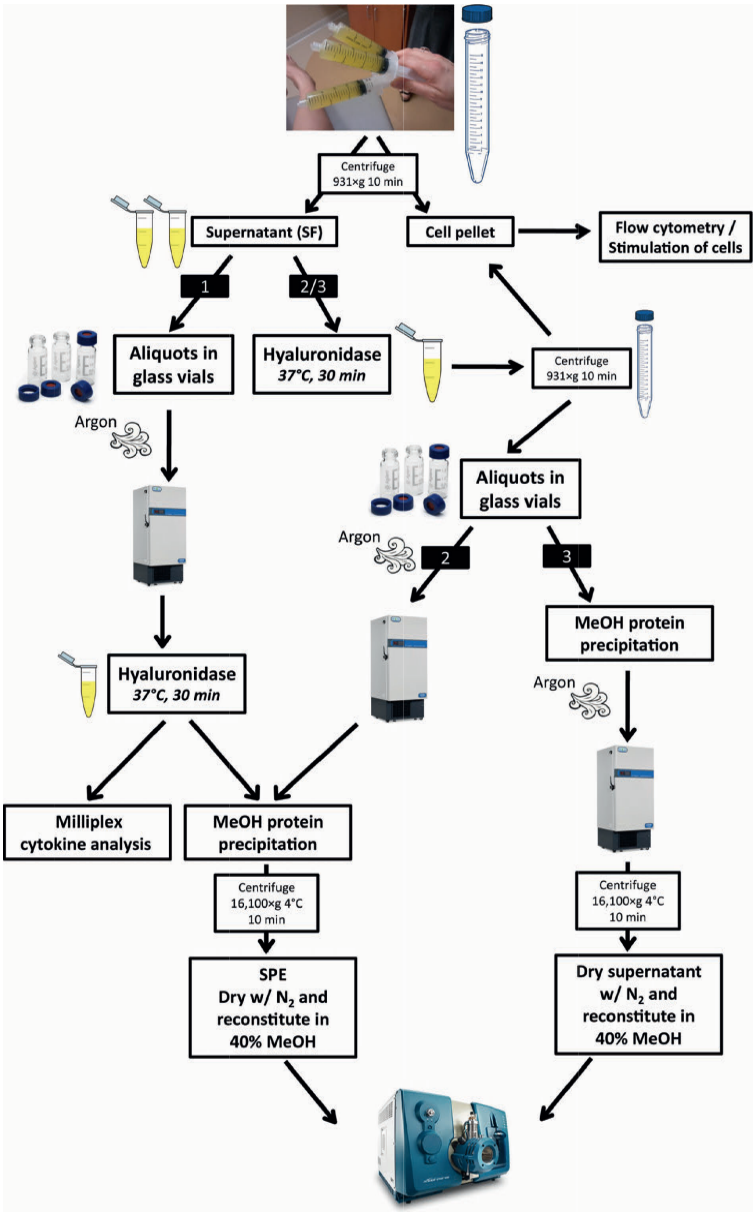
Supplementary Table 2. *[Continued]*

Lipid maps ID	Analyte	MRM transition in ESI (-) [*m/z*]	Calibration line injected concentration range [ng/mL]	OA Detected in at least one sample using SPE work-up	RA Detected in at least one sample using SPE work-up	OA Median (Concentration range) [ng/mL SF] n=24	RA Median (Concentration range) [ng/mL SF] n=10	SPE *vs.* MeOH Spearman's correlation [ρ]	SPE *vs.* MeOH Spearman's correlation [p-value]	OA *vs.* RA Concentration [p-value]	OA *vs.* RA Concentration ratio to PUFA [p-value]
LMFA03010002	$PGF_{2\alpha}$	353.1→193	0.025 - 2	X	X	0.03 (0.01-0.12)	0.02 (0-0.07)	0.41	0.070	N/A	N/A
LMFA03030002	TXB_2	369.1→169	0.01 - 2	X	X	0.08 (0-2.55)	0.04 (0-0.46)	0.99	<0.001	0.1587	0.2535
LMFA04000047	10S,17S-diHDHA	359.1→153	0.01 - 2	n.d	n.d						
LMFA03080004	11(12)EET	318.9→167	N/A	n.d	n.d						
LMFA03010031	13,14-dihydro-15-keto-PGE_2	351.1→235	N/A	n.d	n.d						
LMFA03010027	13,14-dihydro-15-keto-$PGF_{2\alpha}$	353.1→195	N/A	n.d	n.d						
LMFA03080005	14(15)EET	319.0→219	N/A	n.d	n.d						
LMFA03010021	15-deoxy-PGJ_2	315.0→203	N/A	n.d	n.d						
LMFA03040010	15-epi-LXA_4	351.1→115	0.01 - 2	n.d	n.d						
LMFA03010030	15-keto-PGE_2	349.0→235	0.01 - 2	n.d	n.d						
LMFA04000074	17-epi-RvD1	375.0→215	N/A	n.d	n.d						
LMFA03070049	18R-RvE3	333.1→245	N/A	n.d	n.d						

Supplementary Table 2. *[Continued]*

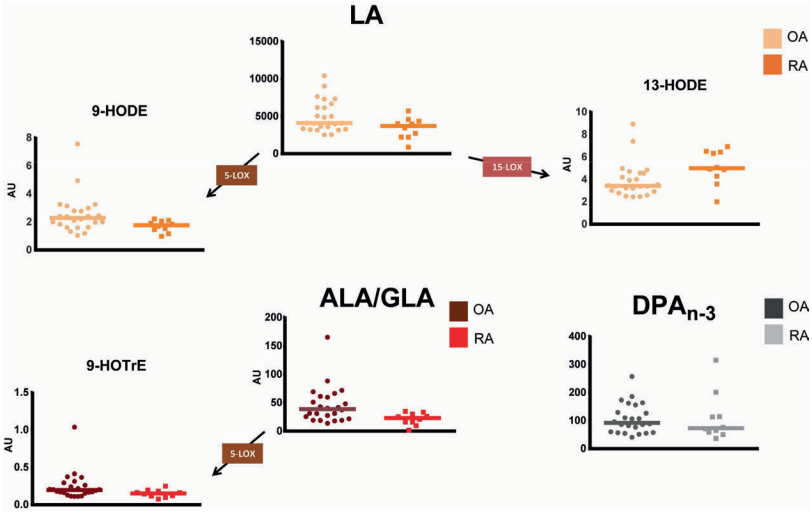
Lipid maps ID	Analyte	MRM transition in ESI (-) [*m/z*]	Calibration line injected concentration range [ng/mL]	OA Detected in at least one sample using SPE work-up	RA Detected in at least one sample using SPE work-up	OA Median (Concentration range) [ng/mL SF] n=24	RA Median (Concentration range) [ng/mL SF] n=10	SPE *vs.* MeOH Spearman's correlation [ρ]	SPE *vs.* MeOH Spearman's correlation [p-value]	OA *vs.* RA Concentration [p-value]	OA *vs.* RA Concentration ratio to PUFA [p-value]
LMFA03070048	18S-RvE3	333.1→245	N/A	n.d	n.d						
LMFA03060010	5S,15S-diHETE	335.0→173	N/A	n.d	n.d						
	7-epi-MaR1	359.1→250	N/A	n.d	n.d						
LMFA03080003	8(9) EET	319.0→155	N/A	n.d	n.d						
LMFA03060050	8S,15S-diHETE	335.1→208	0.01 - 2	n.d	n.d						
LMFA03040001	LXA_4	351.1→115	0.01 - 2	n.d	n.d						
LMFA03040002	LXB_4	351.1→221	0.01 - 2	n.d	n.d						
LMFA04000048	MaR1	359.2→250	N/A	n.d	n.d						
LMFA03010019	PGJ_2	333.0→271	N/A	n.d	n.d						
LMFA04000006	RvD1	375.1→215	N/A	n.d	n.d						
LMFA04000007	RvD2	375.1→277	N/A	n.d	n.d						
LMFA03070019	RvE1	349.1→195	N/A	n.d	n.d						
LMFA03070020	RvE2	333.1→115	N/A	n.d	n.d						

Supplementary Table 2. Analytes measured in SF with the Lipid Mediator platform and their corresponding Lipid maps ID (if available) are summarized. Mann-Whitney signed ranks test was used for comparing concentrations of each analyte and the concentration ratio of each analyte to its corresponding polyunsaturated fatty acid (PUFA) precursor between OA and RA. * indicates significant p-values after Bonferroni correction. SPE: Solid phase extraction, N/A: Not applicable, n.d: Not detected.

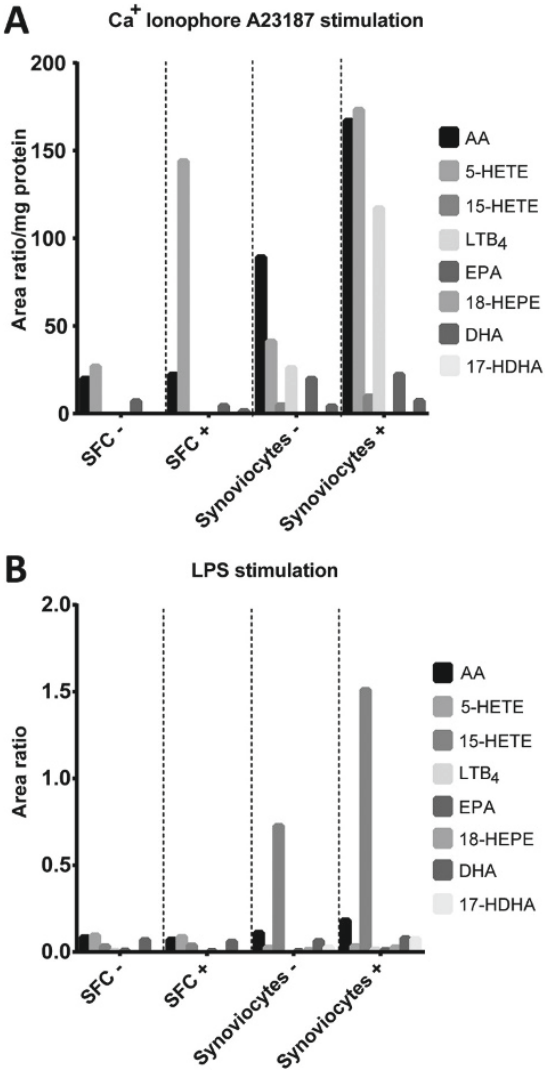


Supplementary Figure 1. After collection SF samples were centrifuged at 931 ×g for 10 min and the supernatant collected. The pelleted cells were filtered over a 70 *μ*m cell strainer (Falcon, Corning Incorporated, Life Sciences, Durham, NC, USA) to remove all insoluble parts and used for FACS analysis or for stimulation, as described in *Materials and Methods*. For part of the samples, the SF was stored and treated with hyaluronidase just before LM analyses (**path 1**). Most SF samples were directly treated with 1 mg/mL hyaluronidase PBS(-/-), vortexed for 5 min, incubated for 30 min at 37 °C, then centrifuged at 931 ×g for 10 min (**paths 2** and **3**). The pellet was combined with the first cell pellet and the supernatant aliquotted into glass vials, flushed with argon, and the aliquots frozen at -80 °C until LM analysis (**paths 1** and **2**)

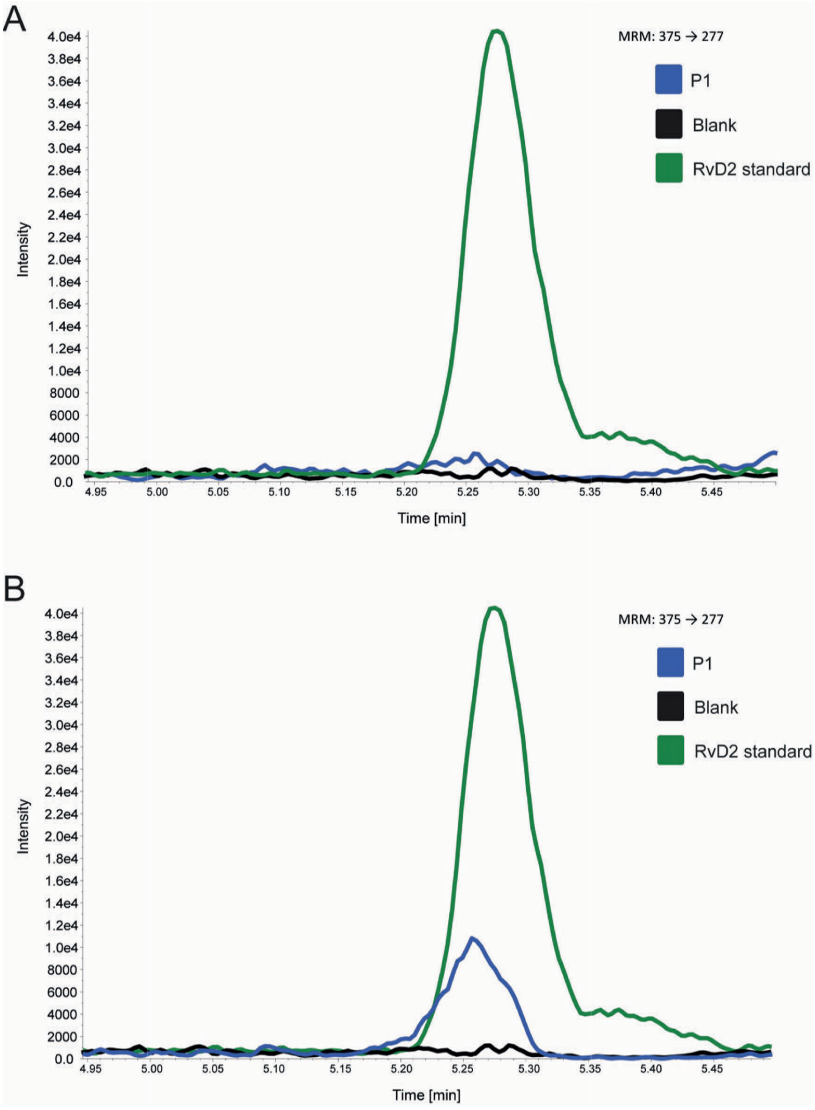
Additionally, in a subset of the hyaluronidase-treated samples, proteins from 100 μL SF were precipitated with 3 volumes of MeOH and internal standard (IS, LTB_4-d4, 15(*S*)-HETE-d8, PGE_2-d4 and DHA-d5) for lipid analysis added (final concentration 0.75 ng/mL each) prior to freezing. After vortexing, samples were stored under argon at -80 °C (**path 3**). After storage, for lipid analysis IS and MeOH (3:1 v:v) were added to 250 μL SF (final concentration 0.15 ng/mL each) for immediate protein precipitation (**path 2**). For samples aliquotted and stored without prior hyaluronidase treatment (**path 1)**, 1 mg/mL hyaluronidase in PBS (-/-) was added to 227 μL SF (1:10 (v:v)), and incubated for 30 min at 37 °C before the addition of IS and MeOH as specified above for path 2. The samples were then vortexed and kept at -20 °C for 30 min before centrifuging at 16,100 ×g for 10 min at 4 °C. The supernatant was transferred to a glass vial, diluted tenfold with water and acidified with 30 μL 6M HCl. After loading the samples on pre-conditioned SPE cartridges, the cartridges were subsequently washed with 3 mL H_2O before elution of analytes with 3 mL methyl formate. The organic extract was concentrated to dryness under a gentle stream of N_2 at 40 °C and samples reconstituted in 150 μL 40% MeOH, N_2 blown over and stored at -80 °C until analysis. Samples stored at -80 °C after protein precipitation with MeOH (**path 3**), were spun down at 16,100 ×g for 10 min at 4 °C before transferring supernatants to glass vials. The supernatant was concentrated to dryness under a gentle stream of N_2, reconstituted in 40% MeOH and stored at -80 °C until analysis.



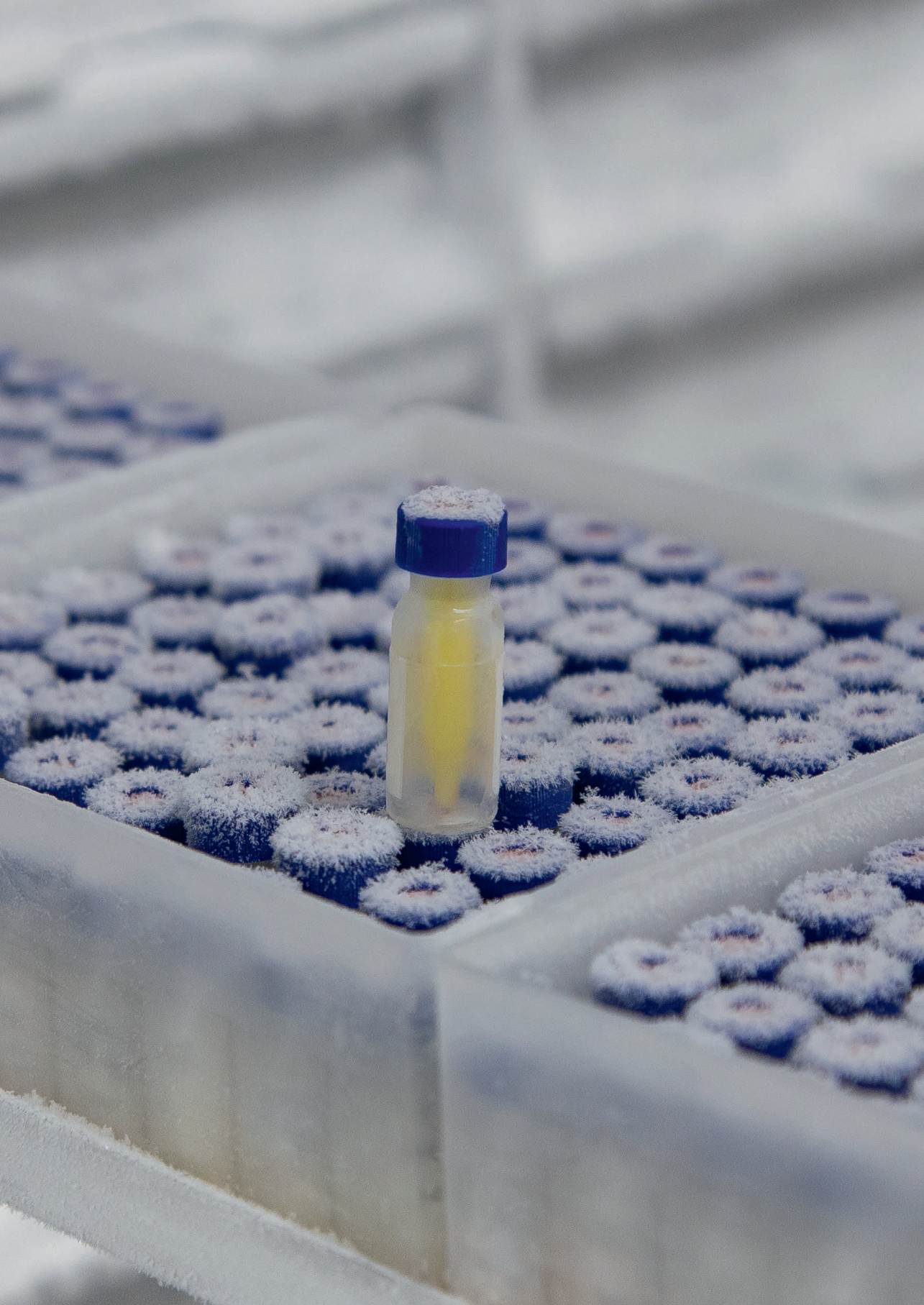
Supplementary Figure 2. Concentrations (in ng per mL SF) of ω-6 linoleic acid (LA) and ω-3 docosapentaenoic acid (DPA_{n-3}), levels of alpha-linolenic acid/gamma-linolenic acid (ALA/GLA) in arbitrary units (AU), and of their metabolites (in arbitrary units (AU)) measured in 24 OA and 10 RA samples. Each dot represents one patient. Medians are depicted. Groups were compared using Mann-Whitney signed ranked tests. HODE: hydroxyoctadecadienoic acid, HOTrE: hydroxyoctadecatrieonic acid.



Supplementary Figure 3 Protein corrected levels of PUFAs and oxylipids in calcium ionophore stimulated ("+") and unstimulated ("-") synovial fluid cells (SFCs) and synoviocytes from one OA patient (**A**). Areas corrected to IS of PUFAs and oxylipids in LPS stimulated ("+") and unstimulated ("-") synovial fluid cells (SFCs) and synoviocytes from one OA patient (**B**).



Supplementary Figure 4 Detection of RvD2 in the SF of an OA patient. MRM trace with transition *m/z* 375 to 277 was used. **A**, soluble fraction of the SF from patient 1 (P1) in blue, a blank in black, and the RvD2 standard material (0.5 ng/mL on column) in green. **B,** insoluble fraction of the SF from P1 in blue, a blank in black and the RvD2 standard material (0.5 ng/mL on column) in green.



4

Pro-resolving mediator treatment of high fat diet induced experimental osteoarthritis

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Manuscript submitted

Abstract

Objective: Specialized pro-resolving mediators (SPM) are bioactive lipids derived from omega-3 and omega-6 fatty acids which have been ascribed pro-resolving capacity. Since inflammation is a well-established feature of osteoarthritis (OA), we investigated the correlation between inflammation and structural damage in experimental OA. Furthermore, we evaluated the effect of SPM treatment on structural damage. In addition, we investigated SPM receptor expression on human OA synovial cells and chondrocytes and the effect of various SPM on IL-6 production by human synovial cells.

Design: Male C57BL/6 mice were fed a high fat diet (HFD) and were either treated with 100 nM Lipoxin A4, Lipoxin B4 and Maresin-1, or the vehicle control. Inflammation was evaluated by in vivo imaging and at the end of the experiment, inflammation and structural damage were evaluated by histology. Synovial and cartilage tissue of 42 OA patients was used to evaluate SPM receptor expression by flow cytometry and IL-6 production by synovial fibroblasts and synovial macrophages upon incubation with SPMs.

Results: The HFD induced OA, indicated by cartilage damage and osteophyte formation, correlated with synovial inflammation, but SPM treatment in HFD induced experimental OA did not result in decreased joint pathology. Both human chondrocytes and synovial cells expressed a subset of SPM receptors and SPM decreased IL-6 production under certain experimental conditions, *in vitro*.

Conclusions: There was no effect of Lipoxin A4, Lipoxin B4 and Maresin-1 treatment on joint pathology in HFD induced OA. Further research is warranted to determine whether SPM have therapeutic potential in OA.

Introduction

Osteoarthritis (OA) affects the weightbearing joints such as knees, hips and spine but also smaller joints such as those in the thumb, fingers and toes. To date, there are not effective treatments available and patients are prescribed NSAIDs to control their pain symptoms. Inside the joints, OA is characterized by breakdown of cartilage, changes to the underlying bone structures and low-grade synovial inflammation. OA is a progressive disease of which the end stage is characterized by a complete disruption of the joint and at this stage the patients should undergo total joint replacement surgery.

There is emerging evidence that the chronic low grade inflammation in OA plays are role in its pathophysiology(1). Clinical studies show correlations between the presence of synovial inflammation and disease progression in knee and hand OA(2–4). In addition, synovial macrophages can induce cartilage destruction and bone remodeling in an experimental model of OA(5,6).

Next to local inflammation, systemic inflammation is also implicated in the development of OA as obesity strongly predisposes to OA(7).

Several local and systemic soluble inflammatory mediators are implicated to play a role in disease pathogenesis such matrix metalloproteinases, cytokines, adipokines and prostaglandins(8–11). However, results of clinical trials have been disappointing as neither of the trials improved structural damage outcome. It is proposed that new treatment options for OA should focus on treating the early phases of OA, to prevent structural damage. We hypothesize that these early phases of OA are characterized by chronic low-grade inflammation which should be targeted by pro-resolving mediators rather than strong anti-inflammatory mediators. Specialized pro-resolving mediators (SPM) are small bioactive lipid molecules derived from poly unsaturated fatty acids (PUFAs) and they are produced by a wide range of immune and tissue cells by the actions of LOX and COX enzymes. Many SPM are now identified and include lipoxins, maresins, resolvins and protectins. SPM are shown to accumulate during periods of acute inflammation to limit progression to persistent chronic inflammation in many experimental models including infection, asthma and inflammatory arthritis models(12–15). In particular, recently, SPM resolvin D1 (RvD1) was shown to ameliorate OA in a surgically induced model of OA(16).

In this study, we investigated synovial inflammation in vivo over time, as well as development of osteophyte formation and cartilage loss in the HFD induced experimental model of OA. In addition, we evaluated SPM receptor expression in human joint cells and

investigated the effect of SPM on IL-6 production in synovial cells. Finally, we treated the HFD animals with SPM to investigate whether they can limit structural damage.

Materials and Methods

Preparation of SPM

LXA4, LXB4 and Mar1 were purchased from Cayman Chemicals. RvE2 was a kind gift from dr. Makoto Arita (RIKEN, Yokohama, Japan). SPM were diluted in HPLC grade EtOH (Sigma-Aldrich) using a Hamilton syringe and glass vials (Agilent Technologies). For in vitro experiments, EtOH was evaporated using a gentle stream of nitrogen, appropriate medium was added and SPM were homogenized by 2 rounds of vortex (10 seconds) and sonication (10 seconds). For in vivo experiments, SPM were diluted in HPLC grade EtOH and added directly to sterile 0,9% NaCl and homogenized by 2 rounds of vortex (10 seconds) and sonication (10 seconds). These procedures were also performed with plain HPLC grade EtOH which was used as vehicle control.

Experimental OA

Male C56BL/6 mice were purchased from Charles River and were random assigned to the chow diet control group or high fat diet (HFD) diet groups. The HFD groups were fed with Rodent Diet with 60% kcal% fat (Research Diet) after an acclimatization period of three weeks and the treated and untreated mice were cages together. Both diets were provided ad libitum. The mice were treated every 7 days with a pool of SPM consisting of 100 nM LxA4, LxB4 and Mar1. The mice were injected i.v. with 100 μL per 30 grams of bodyweight. Every month, the mice were injected with IRDye®800CW 2-DG Optical Probe (Li-Cor), 10 nmol per 20 grams of bodyweight and the next day the mice were subjected to imaging using the IVIS Spectrum In Vivo Imaging System (PerkinElmer, Ohio, USA). After 6 months of HFD and SPM treatment, the mice were sacrificed and the knees were prepared for histology. The study was approved by the Institutional Animal Care and Use Committee (IACUC) in the USA and the Ethical Committee for Experimental Animal Experimentation of the LUMC.

Histology of mouse knees

After sacrifice, the knees were cut from the hind leg by cutting through the femur and tibia and fibula halfway of the bones. The skin, the fat and some muscle tissue was removed and the knees were fixed in 4% formaldehyde for 24 hours. The knees were then rinsed thoroughly for 10 minutes using distilled water and incubated with Kristensens Solution for 2 weeks to decalcify. Residual Kristensens solution was removed by washing the knees for 3 hours under running tap water and the knees were processed for histology. Tissue slides of 7 μM were stained with fast green (Klinipath) and safranine O (Sigma)

and of each knee, three sections were scored which were at least 140 μM apart. The cartilage was scored at 4 positions (femur lateral, femur medial, tibia lateral and tibia medial) and was given a score of grade 0 (no damage), grade 0.5 (faded Safranine O staining), grade 1 (superficial layer has frays), grade 2 (superficial layer has frays and clefts), grade 3 (<25% of the width of the tidemark is damaged), grade 4 (25-50% of the width of the tidemark is damaged), grade 5 (50-75% of the width of the tidemark is damaged) or grade 6 (>75% of the width of the tidemark is damaged). The total score of the knee is the sum of the 4 places with a maximum score of 24. At the same 4 locations, the osteophytes were counted and given a score (0-3) depending on the size of the osteophyte. Osteophyte scoring resulted in a score of the total number of osteophytes on the 4 locations combined with a maximum score of 4, as well as a total osteophyte score with a maximum of 12. Synovial inflammation was scored by grading the synovium thickness from 0-3 at 4 locations, with grade 0 (flat almost invisible synovium layer with flattened cells), grade 1 (more rounded cells), grade 2 (2-3 cell layers thick), grade 3 (dark purple staining and more than 3 cell layers thick). The total score of the knee is the sum of the 4 places with a maximum score of 12. Scoring was performed blinded by two observers which were blinded for the experimental groups and their score was averaged.

Human synoviocyte and chondrocyte isolation and culture

Synovium and cartilage were obtained as anonymized left-over material from total knee replacement surgery procedures and stored at 4 °C before processing the same day. Synovium was cut in small pieces and incubated in serum free IMDM medium (Lonza, Basel, Switzerland) supplemented with 1 mg/mL collagenase type II (Worthington biochemical corporation, Lakewood, NJ, USA) for 1.5 hours. Cartilage was cut into small pieces and incubated with 2 mg/mL Pronase (Roche) for 1.5 hours. Next, the cartilage was resuspended in DMEM/F12 medium with 0.75 mg/mL collagenase type II overnight. Digested tissue was separately filtered over a 70 μm cell strainer (Falcon, Corning Incorporated, Life Sciences, Durham, NC, USA) to obtain the synoviocytes and chondrocytes. The cells were washed 3 times before use. All digestion steps were performed at 37°C on a lab roller.

SPM receptor analysis

Isolated cells were stained with a different set of antibodies depending on the SPM receptor detection. For the detection of ERV1/ChemR23 (also known as ChemR23 or CMKLR1), isolated cells were stained using anti-CD31-AF647 (BD), anti-CD90-PE (Biolegend), anti-CD45-AF700 (Biolegend), anti-CD14-PECy (BD), anti-CD55-Biotin (BD), streptavidin-eFluor450 (eBioscience), anti-CMKLR1-FITC (AbD Serotec), anti-CD117-APC (BD), anti-CD3-PE (BD) and anti-CD19-APC-Cy7 (BD). For the detection of ALX/FPR2, isolated cells were stained using anti-CD31-FITC(BD), anti-CD90-APC

(Biolegend), anti-CD45-AF700 (Biolegend) or anti-CD45-FITC (BD), anti-CD14-PECy (BD), anti-CD55-Biotin (BD), streptavidin-eFluor450 (eBioscience), anti-CD117-APC (BD), anti-CD3-AF700 (BD) and anti-CD19-APC-Cy7 (BD). The anti-FPR2 antibody (Genovac) and the isotype for anti-FPR2 were labelled with PE using LYNX rapid R-Phycoerythrin (RPE) antibody conjugation kit (AbD Serotec) according to the manufacturer's protocol. CD31+ cells, CD55+ cells and CD90+ cells were gated from the CD45+ population. CD14+ cells, CD19+ cells and CD117+ cells were gated from the CD45+ population. For the detection of BLT1R, isolated cells were directly stained with anti-LTB4R-PE (BD). For the detection of DRV1/GPR32, isolated cells were directly stained with anti-GPR32 (Genetex) and subsequently with anti-rabbit IgG-FITC (Santa-Cruz). Isotypes for all antibodies were used to determine positivity. Dead cells were excluded using the Fixation and Dead Cell Discrimination Kit (Miltenyi) according to the manufacturer's protocol. Remaining red blood cells were lysed using lysisbuffer (BD) after staining, according to the manufacturer's protocol.

IL-6 production by synoviocytes

For effect on IL-6 production, freshly isolated synoviocytes were resuspended in IMDM supplemented with 10 % FCS, P/S and glutamax (2 x 10^6/0.5 mL) and incubated overnight at 37°C in a pre-warmed 24- wells plate. The next day, cells were washed with DMEM/F12 medium and incubated with SPM and 10ng/mL LPS overnight at 37°C using DMEM/F12 medium with 2% fatty acid free (FFA) BSA and penicillin/streptomycin (P/S). The next day, supernatant was collected and IL-6 production was quantified using a human IL-6 ELISA kit (R&D Systems) according to the manufacturer's protocol. For intracellular staining, 10 μg/mL Brefeldin A was added for 4 hours after O/N incubation with SPM. Alternatively, SPM were added after O/N incubation with DMEM/F12 medium with 2% FFA BSA, P/S for one hour. After 1 hour of incubation, Brefeldin was added for 4 hours. Subsequently, the cells were harvested using Accutase (Sigma), washed with PBS and stained with anti-CD45-AF700, anti-CD14-PECy7 and anti-IL-6-PE (eBioscience) for flowcytometry. Flowcytometry analysis was performed using FACSDiva™ Software (BD).

Statistical analysis

Wilcoxon signed rank tests, Mann-Whitney tests were performed sing Graphpad Prim 9.1.1 to evaluate significance.

Results

We used the HFD induced OA model to investigate the disease development in mice. Metabolic stress induced inflammation was shown to play a role in OA development in this model(17,18). We have fed male C57BL/6 mice a HFD that contained 35% lard

and we followed the mice for 6 months. These mice gained 3 times more weight than the control mice (Figure 1A). To investigate the course of inflammation in these mice, we visualized the inflammation *in vivo* using fluorescent 2-deoxy-glucose (2-DG). We could detect inflammation, as indicated by the strength of the 2-DG signal in the fore paws of the mice. The HFD mice showed significantly more inflammation in their fore paws after 4 months compared to their chow controls (Figure 1B). We could not detect any inflammation in the mice knees. To determine whether the HFD developed OA, we evaluated cartilage loss, osteophyte formation and synovial synovitis in the knees of these mice. We could not detect cartilage degradation in either of the groups, however there was a trend towards more inflammation in the HFD mice (Figure 1C). In contrast, all of the HFD mice developed osteophytes during these 6 months (Figure 1C). We next evaluated whether the osteophyte formation correlated with synovial inflammation in these mice. Indeed, there was a strong correlation between both osteophyte severity and osteophyte number but not with cartilage loss (Figure 1D).

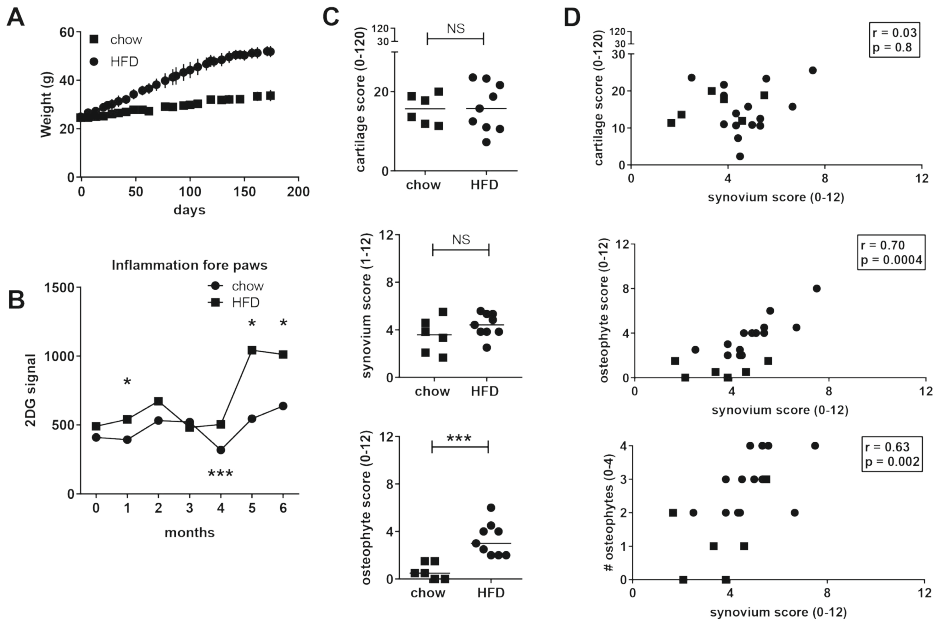


Figure 1. HFD induces inflammation which correlates with structural damage. A) Weight gain of the lean chow fed mice and HFD mice. Mean and SD are shown of n=6 chow and n=9 HFD mice. B) Mean quantified fluorescent 2-deoxyglucose signal in the fore paws of mice in vivo. C) Cartilage score, osteophyte score and synovium scores obtained by histology of the right knee. Each dot represents a mouse. n=6 chow and n=9 HFD mice. D) Correlations between synovium score and cartilage score, number of osteophytes an osteophyte score respectively. Scores are obtained by histology of the right knee. n=20 mice. Closed squares are chow mice and closed circles HFD mice. Statistical significance in this figure was obtained using Mann-Whitney *p<0.05, **p<0.01, ***p<0.001.

Expression of SPM receptors in human articular tissue

To determine candidate SPMs for treatment of experimental OA, we investigated SPM receptor expression on tissues of the human joint. We were able to evaluate the Lipoxin receptor (ALX)/N-formyl peptide receptor (FPR)-2 (ALX/FPR2), the Resolvin E1 receptor/ Chemerin receptor 23 (ERV1/ChemR23) also known as chemokine-like receptor 1 (CMKLR1), The leukotriene B4 receptor (BLT1) and the resolving D 1 receptor/G protein-coupled receptor 32 (DRV1/GPR32) expression on isolated chondrocytes and ALX/ FPR2 and ERV1/ChemR23 expression on cells isolated from synovium (synoviocytes), containing synovial immune cells and fibroblasts. We could detect the expression of the resolvin E1 and (partial) resolvin E2 receptor ERV1/ChemR23 on human chondrocytes (Figure 2A)(19). No expression of leukotriene B4 receptor BLT1 or resolvin D receptor DRV1/GPR32 was detected on chondrocytes. Synovium of 16 OA patients was tested for SPM receptor positivity. Only a small percentage of the CD31+ endothelial cells and fibroblasts showed expression of the ERV1/ChemR23. In contrast, expression of the Lipoxin A4/Resolvin D1 receptor ALX/FPR2 was only detected in CD45+ cells(20). Of the CD45+ cells, on average more than half of the CD14+ cells shower positivity for ALX/ FPR2. In addition, a small percentage of the mast cells, characterized by CD117 positivity showed expression of ALX/FPR2.

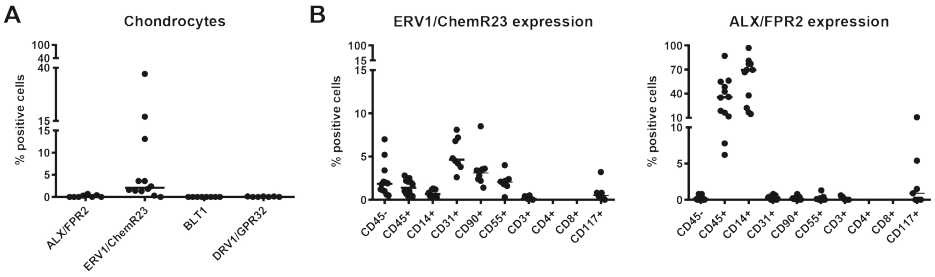


Figure 2. SPM receptor expression in chondrocytes and synovial cells. SPM receptor expression was determined using flowcytometry. A) Isolated chondrocytes, n=8 donors for ALX/FPR2, BLT1 and DRV1/GPR32 and n=12 donors for ERV1/ChemR23. B) Isolated synoviocytes, n=16 donors.

Effects of SPM on synoviocytes and macrophages

Since inflammation correlates with structural damage in the HFD mice model, and we detect expression of some of the SPM receptors, we investigated effects of a set of SPM on cells in the human synovium. We evaluated the effect of SPM on IL-6 production by synoviocytes including synovial fibroblasts and immune cells. Since synovium expresses ERV1/ and ALX/FPR2 we chose to incubate the cells with either LXA4, LXB4, Mar-1 or RvE2. Only with the lowest concentration (1 nM) we could detect an inhibition of IL-6 production by LXB4 and RvE2 in all the donors tested, although this was not significant ($p=0.06$, Figure 3A). We also investigated the effect of the pooled SPM, however

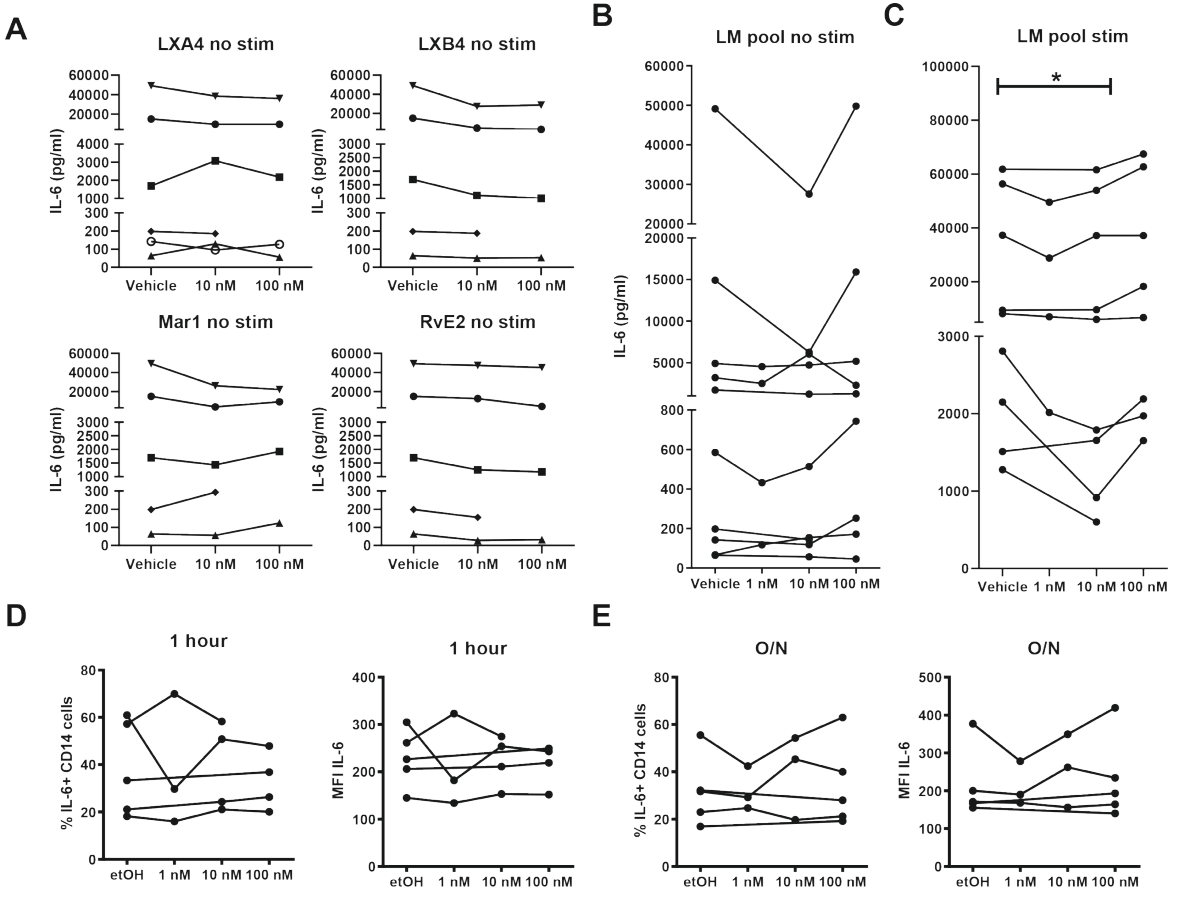
there was no difference in IL-6 production between the groups (Figure 3B). However when we stimulated the synoviocytes with LPS after the SPM stimulation to mimic the inflammatory environment, there was a significant inhibition of IL-6 production in the 10 nM concentration compared to the control group (Figure 3C). Next, we evaluated the effect of LXA4 on inflammatory cytokine IL-6 production As the CD14+ monocyte/ macrophage population expresses ALX/FPR2.We digested the synovium to obtain individual cells and incubated the cells either for 1 hour (figure 3D) or overnight (Figure 3E) before we added brefeldin A for 4 hours. Neither of the experiments showed an effect on intracellular IL-6 levels evaluated using numbers of IL-6 MFI (Figure 3) or number of IL-6 positive cells (data not shown) in the CD14+ population.

Treatment of HFD mice with pro-resolving mediators

Based on the receptor expression profile on human joint tissue cells and the previous reports that show macrophages are involved in osteophyte formation, we decided to treat the mice with agonists of the ALX/FPR2 receptor, Lipoxin A4 and Maresin-1[5]. In addition, Lipoxin A4 and Maresin-1 were detected in synovial fluid of arthritis patients and Lipoxin A4 was shown to attenuate adipose inflammation and produced by synovial tissue(21–23). Lipoxin B was added to complete the pool of SPM based on its yet unidentified role in inflammation and the most promising effect on synoviocytes (p=0.06 using 10 nM LXB4). Mice were treated weekly with 100 nM of Lipoxin A4, Lipoxin B4 and Maresin-1. After 6 months of treatment, there was no effect of these SPM on structural damage to the knee joints (Figure 4B-D).

> **Figure 3.** Effect of SPM on IL-6 production by synoviocytes. A-C) Isolated synoviocytes were incubated with SPM O/N and IL-6 production was determined in the supernatant by ELISA. Synoviocytes were either stimulated with individual SPM (A), a pool of SPM containing LXA4, LXB4, Mar1 and RvE2, or a pool of SPM containing LXA4, LXB4, Mar1 and RvE2 and LPS. Each line represents a donor, n=4-10 donors. D) Isolated synoviocytes were either incubated for 1 hour (A) or overnight (O/N) (E) with different concentrations of LXA4. IL-6 positivity and IL-6 mean fluorescence intensity (MFI) on CD14+ cells was determined by flowcytometry. Each line represents a donor, n=5 donors. Statistical significance in this figure was obtained using Wilcoxon signed rank test, *p<0.05, **p<0.01, ***p<0.001.

Figure 3.


A
LXA4 no stim
LXB4 no stim
Mar1 no stim
RvE2 no stim
IL-6 (pg/ml)
Vehicle
10 nM
100 nM
B
LM pool no stim
IL-6 (pg/ml)
Vehicle 1 nM 10 nM 100 nM
C
LM pool stim
*
Vehicle 1 nM 10 nM 100 nM
D
1 hour
% IL-6+ CD14 cells
MFI IL-6
etOH 1 nM 10 nM 100 nM
E
O/N
% IL-6+ CD14 cells
MFI IL-6
etOH 1 nM 10 nM 100 nM

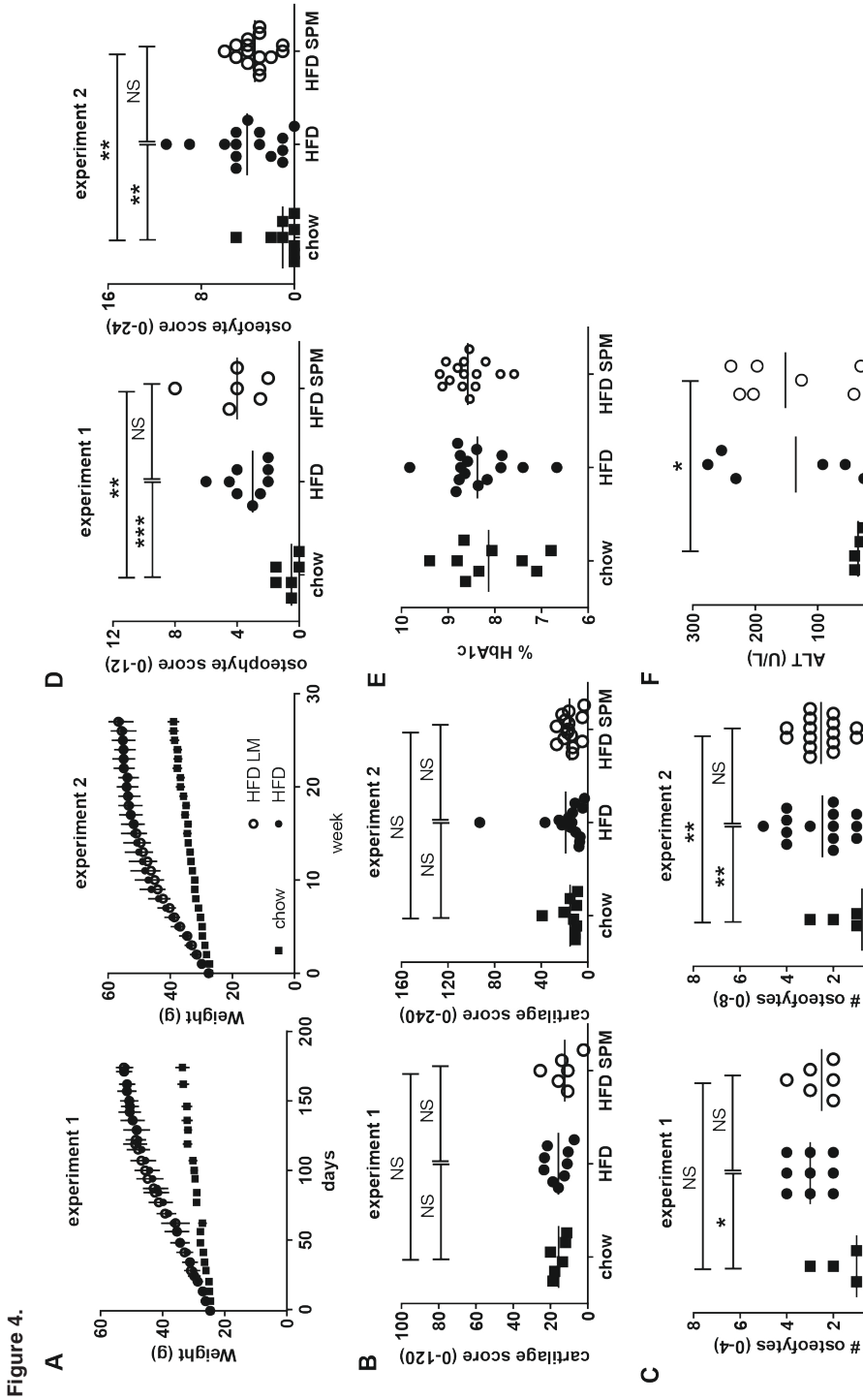

A
experiment 1
Weight (g)
days
experiment 2
HFD LM
HFD
chow
week
B
experiment 1
cartilage score (0-120)
NS
chow
HFD
HFD SPM
C
experiment 1
osteofytes (0-4)
*
D
experiment 1
osteophyte score (0-12)
**
***
experiment 2
osteofyte score (0-24)
E
% HbA1c
F
ALT (U/L)
*

Figure 4.

< Figure 4. Pro-resolving mediator treatment of experimental OA. A) Weight gain of the chow control and HFD mice of two independent experiments. Mean and SD are shown of n=6 chow control, n=9 HFD untreated (vehicle control) and 6-9 HFD treated (SPM, 100 nM Lipoxin A4, Lipoxin B4 and Maresin-1) mice for experiment 1. In experiment 2, means are shown for n=9 chow control and 15 HFD mice of both the untreated and treated group. B) Cartilage score, C) osteophyte number and D) osteophyte score of two experiments. For experiment 1, scores of the right knee are shown. For experiment 2, mean scores of the left and right knee combined are shown. E) HbA1c and F) ALT levels were measured in serum of mice from experiment 2. Statistical significance for B-D was established using Mann-Whitney *p<0.05, **p<0.01, ***p<0.001.

No effects of treatment metabolic inflammatory factors

Although we could not detect any effect on structural damage in the knees of the mice, we wanted to explore whether the SPM attenuated the overall inflammatory status of the mice. We investigated the systemic blood levels of HbA1c as a marker of glycemic control in mice but there was no difference between the groups (Figure 4E)(24). Blood alanine aminotransferase (ALT) levels are a marker for liver damage and shown to be increased in HFD mice and lowered by the administration of Lipoxin A4(23). Indeed we detected an increase in ALT levels in the HFD treated mice, however the SPM treatment did not significantly alter ALT levels (Figure 4F).

Discussion

There is a stressing need for novel therapeutics to treat OA patients which limit structural damage progression. SPM were shown to limit inflammatory processes and promote resolution of inflammation and tissue repair(25).We show that human chondrocytes, synovial macrophages and synovial fibroblasts express SPM receptors ERV1/ChemR23 and ALX/FPR2. In addition, our results show a slight decrease of IL-6 production by synovial cells after treatment with a pool of SPM targeting ERV1/ChemR23 and ALX/FPR2 receptors, however only in the specific condition were the cells we additionally triggered with LPS. The individual SPM could not elicit the same response although LXB4 and RvE2 showed a similar trend as the pool of SPM. Treatment the mice with HFD induced experimental OA however did not prevent the development of structural damage.

We show for the first time that synovial inflammation is correlated with structural damage in HFD induced experimental OA(26). This correlation is also demonstrated for human OA, although whereas some reports show a good correlation, other show a moderate or no correlation, depending on the joints or patient population studied, emphasizing the heterogeneous disease we call OA(27–30). We were unable to show that the inflammation preceded the development of structural damage in the knees as the in vivo imaging technique was likely not optimized to detect (low grade) inflammation in the knees compared to the mouse paws which do not have much tissue or hair surrounding the joints.

We show that a small percentage of human chondrocytes are positive for the chemoattractant chemerin, and SPM Resolvin E1 and E2 receptor ERV1/ChemR23. It was shown before that all chondrocytes stained positive for ERV1/ChemR23, however this was after weeks of culture in which they might have upregulated the receptor(31). Fibroblast-like synoviocytes (FLS) from RA patients were shown to produce IL-6 upon ERV1/ChemR23 signaling via chemerin stimulation (32). It is proposed that the response to either inflammatory chemerin and pro-resolving RvE1 via ERV1/ChemR23 is tightly regulated and RvE1 might play a role in the resolution of inflammation in RA(33,34). We show that in OA patients there is a trend, but no significant effect of RvE2 on IL-6 production, which acts through the same receptor. It remains to be shown whether RvE1 might have a more potent effect.

In contrast to the chondrocytes and FLS, the CD14+ macrophages express the lipoxin A4 receptor ALX/FPR2. Macrophages are shown to be important mediators in OA disease development in human and experimental OA(35). However, we could not detect an effect of lipoxin A4 or resolvin D1 on macrophage IL-6 production in contrast to previous results. Sun at al showed decreased IL-6 production in OA synovial macrophages after RvD1 incubation. However, the contradictive results might easily be explained by the differences in experimental set up as we show that slight changes to the experimental protocol can change the outcome. Indeed, some studies reported the need for a specific experimental set up before an effect of LXA4 or aspirin triggered LXA4 could be seen on phagocytosis by THP-1 cells or neutrophils respectively(36,37). Our in vitro findings emphasize that the effect of SPM is not robust and provokes the thought how these SPM can cause a robust effect during inflammation.

Weekly treatment of mice with a pool of LXA4, LXB4 and Mar-1 did not limit the development of structural damage. A possible explanation could be that the cells in the joint to not respond to the SPM, as we also observe for the human cells. However, it could also be that the admission route should be altered. Recently it was shown that intra-articular injection RvD1 reduced the severity of mechanically induced experimental OA(16). In addition, the administration route might also explain why we could not reproduce the LXA4 induced lowered ALT levels, since LXA4 was previously shown to lower ALT levels after intra peritoneal injection.

In conclusion, we show for the first time that inflammation is correlated with structural damage in the HFD induced experimental OA model. We show expression of SPM receptors ERV1/ChemR23 and ALX/FPR2 joint tissues of OA patients, however only in one of a large variety of conditions and cell types tested, we could detect an effect of SPM on inflammatory IL-6 production by synoviocytes. Furthermore, weekly treatment

of mice with a pool of lipoxins and Maresin-1 did not prevent structural damage in HFD induced experimental OA.

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Author contributions

Hilde Brouwers: Conception and design, Collection and assembly of data, Analysis and interpretation of the data, Drafting of the article, Final approval of the article

Joanneke Kwekkeboom: Administrative, technical, or logistic support, Collection and assembly of data

Kosaku Murakami: Collection and assembly of data

Simone Perniola: Collection and assembly of data

Margreet Kloppenburg: Provision of study materials or patients, Critical revision of the article for important intellectual content

Rene Toes: Critical revision of the article for important intellectual content

Andreea Ioan-Facsinay: Obtaining of funding, Conception and design, Critical revision of the article for important intellectual content, Final approval of the article

Role of the funding source

The study sponsors had no role in the study design, in the collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication.

Competing interests

None of the authors have competing interests

References

1. Robinson WH, Lepus CM, Wang Q, et al. Low-grade inflammation as a key mediator of the pathogenesis of osteoarthritis. *Nat Rev Rheumatol*. 2016;12(10):580-592. doi:10.1038/nrrheum.2016.136
2. Ayral X, Pickering EH, Woodworth TG, Mackillop N, Dougados M. Synovitis: a potential predictive factor of structural progression of medial tibiofemoral knee osteoarthritis -- results of a 1 year longitudinal arthroscopic study in 422 patients. *Osteoarthr Cartil*. 2005;13(5):361-367. doi:10.1016/j.joca.2005.01.005
3. Roemer FW, Guermazi A, Felson DT, et al. Presence of MRI-detected joint effusion and synovitis increases the risk of cartilage loss in knees without osteoarthritis at 30-month follow-up: the MOST study. *Ann Rheum Dis*. 2011;70(10):1804-1809. doi:10.1136/ard.2011.150243
4. Kortekaas MC, Kwok W-Y, Reijnierse M, Kloppenburg M. Inflammatory ultrasound features show independent associations with progression of structural damage after over 2 years of follow-up in patients with hand osteoarthritis. *Ann Rheum Dis*. 2015;74(9):1720-1724. doi:10.1136/annrheumdis-2013-205003
5. Blom AB, van Lent PLEM, Holthuysen AEM, et al. Synovial lining macrophages mediate osteophyte formation during experimental osteoarthritis. *Osteoarthr Cartil*. 2004;12(8):627-635. doi:10.1016/j.joca.2004.03.003
6. Blom AB, van Lent PL, Libregts S, et al. Crucial role of macrophages in matrix metalloproteinase-mediated cartilage destruction during experimental osteoarthritis: involvement of matrix metalloproteinase 3. *Arthritis Rheum*. 2007;56(1):147-157. doi:10.1002/art.22337
7. Visser AW, de Mutsert R, le Cessie S, et al. The relative contribution of mechanical stress and systemic processes in different types of osteoarthritis: the NEO study. *Ann Rheum Dis*. 2015;74(10):1842-1847. doi:10.1136/annrheumdis-2013-205012
8. Burrage PS, Mix KS, Brinckerhoff CE. Matrix metalloproteinases: Role in arthritis. *Front Biosci*. 2006;11(1 P.447-888):529-543. doi:10.2741/1817
9. Chevalier X, Eymard F, Richette P. Biologic agents in osteoarthritis: Hopes and disappointments. *Nat Rev Rheumatol*. 2013;9(7):400-410. doi:10.1038/nrrheum.2013.44
10. Kroon FPB, Veenbrink AI, de Mutsert R, et al. The role of leptin and adiponectin as mediators in the relationship between adiposity and hand and knee osteoarthritis. *Osteoarthr Cartil*. August 2019. doi:10.1016/j.joca.2019.08.003
11. Martel-Pelletier J, Pelletier J-P, Fahmi H. Cyclooxygenase-2 and prostaglandins in articular tissues. *Semin Arthritis Rheum*. 2003;33(3):155-167. http://www.ncbi.nlm.nih.gov/pubmed/14671726. Accessed October 23, 2019.
12. Buckley CD, Gilroy DW, Serhan CN. Proresolving lipid mediators and mechanisms in the resolution of acute inflammation. *Immunity*. 2014;40(3):315-327. doi:10.1016/j.immuni.2014.02.009
13. Serhan CN, Chiang N. Endogenous pro-resolving and anti-inflammatory lipid mediators: A new pharmacologic genus. In: *British Journal of Pharmacology*. Vol 153. ; 2008. doi:10.1038/sj.bjp.0707489
14. Levy BD, Vachier I, Serhan CN. Resolution of Inflammation in Asthma. *Clin Chest Med*. 2012;33(3):559-570. doi:10.1016/j.ccm.2012.06.006
15. Arnardottir HH, Dalli J, Norling L V, Colas RA, Perretti M, Serhan CN. Resolvin D3 Is Dysregulated in Arthritis and Reduces Arthritic Inflammation. *J Immunol*. 2016;197(6):2362-2368. doi:10.4049/jimmunol.1502268

16. Sun AR, Wu X, Liu B, et al. Pro-resolving lipid mediator ameliorates obesity induced osteoarthritis by regulating synovial macrophage polarisation. *Sci Rep*. 2019;9(1). doi:10.1038/s41598-018-36909-9

17. Griffin TM, Huebner JL, Kraus VB, Yan Z, Guilak F. Induction of osteoarthritis and metabolic inflammation by a very high-fat diet in mice: Effects of short-term exercise. *Arthritis Rheum*. 2012;64(2):443-453. doi:10.1002/art.33332

18. Gierman LM, van der Ham F, Koudijs A, et al. Metabolic stress-induced inflammation plays a major role in the development of osteoarthritis in mice. *Arthritis Rheum*. 2012;64(4):1172-1181. doi:10.1002/art.33443

19. Oh SF, Dona M, Fredman G, Krishnamoorthy S, Irimia D, Serhan CN. Resolvin E2 formation and impact in inflammation-resolution. *J Immunol*. 2012;188(9):4527. doi:10.4049/JIMMUNOL.1103652

20. Krishnamoorthy S, Recchiuti A, Chiang N, et al. Resolvin D1 binds human phagocytes with evidence for proresolving receptors. *Proc Natl Acad Sci U S A*. 2010;107(4):1660. doi:10.1073/PNAS.0907342107

21. Giera M, Ioan-Facsinay A, Toes R, et al. Lipid and lipid mediator profiling of human synovial fluid in rheumatoid arthritis patients by means of LC–MS/MS. *Biochim Biophys Acta - Mol Cell Biol Lipids*. 2012;1821(11):1415-1424. doi:10.1016/J.BBALIP.2012.07.011

22. Börgeson E, McGillicuddy FC, Harford KA, et al. Lipoxin A4 attenuates adipose inflammation. *FASEB J*. 2012;26(10):4287-4294. doi:10.1096/fj.12-208249

23. Börgeson E, Johnson AMF, Lee YS, et al. Lipoxin A4 Attenuates Obesity-Induced Adipose Inflammation and Associated Liver and Kidney Disease. *Cell Metab*. 2015;22(1):125-137. doi:10.1016/j.cmet.2015.05.003

24. Asare-Bediako B, Noothi SK, Li Calzi S, et al. Characterizing the Retinal Phenotype in the High-Fat Diet and Western Diet Mouse Models of Prediabetes. *Cells*. 2020;9(2). doi:10.3390/cells9020464

25. Bannenberg G, Serhan CN. Specialized pro-resolving lipid mediators in the inflammatory response: An update. *Biochim Biophys Acta*. 2010;1801(12):1260-1273. doi:10.1016/J.BBALIP.2010.08.002

26. V S, RC A, P DL, et al. Does a high-fat diet affect the development and progression of osteoarthritis in mice?: A systematic review. *Bone Joint Res*. 2020;8(12):582-592. doi:10.1302/2046-3758.812.BJR-2019-0038.R1

27. Mathiessen A, Conaghan PG. Synovitis in osteoarthritis: current understanding with therapeutic implications. *Arthritis Res Ther*. 2017;19(1). doi:10.1186/S13075-017-1229-9

28. E Y, RG N, A I-F, et al. Association between weight or body mass index and hand osteoarthritis: a systematic review. *Ann Rheum Dis*. 2010;69(4):761-765. doi:10.1136/ARD.2008.106930

29. Kroon FPB, van Beest S, Ermurat S, et al. In thumb base osteoarthritis structural damage is more strongly associated with pain than synovitis. *Osteoarthr Cartil*. 2018;26(9):1196-1202. doi:10.1016/J.JOCA.2018.04.009

30. Kraus VB, Blanco FJ, Englund M, Karsdal MA, Lohmander LS. Call for Standardized Definitions of Osteoarthritis and Risk Stratification for Clinical Trials and Clinical Use. *Osteoarthritis Cartilage*. 2015;23(8):1233. doi:10.1016/J.JOCA.2015.03.036

31. Berg V, Sveinbjörnsson B, Bendiksen S, Brox J, Meknas K, Figenschau Y. Human articular chondrocytes express ChemR23 and chemerin; ChemR23 promotes inflammatory signalling upon binding the ligand chemerin21-157. *Arthritis Res Ther 2010 126*. 2010;12(6):1-12. doi:10.1186/AR3215

32. Kaneko K, Miyabe Y, Takayasu A, et al. Chemerin activates fibroblast-like synoviocytes in patients with rheumatoid arthritis. *Arthritis Res Ther*. 2011;13(5). doi:10.1186/AR3475

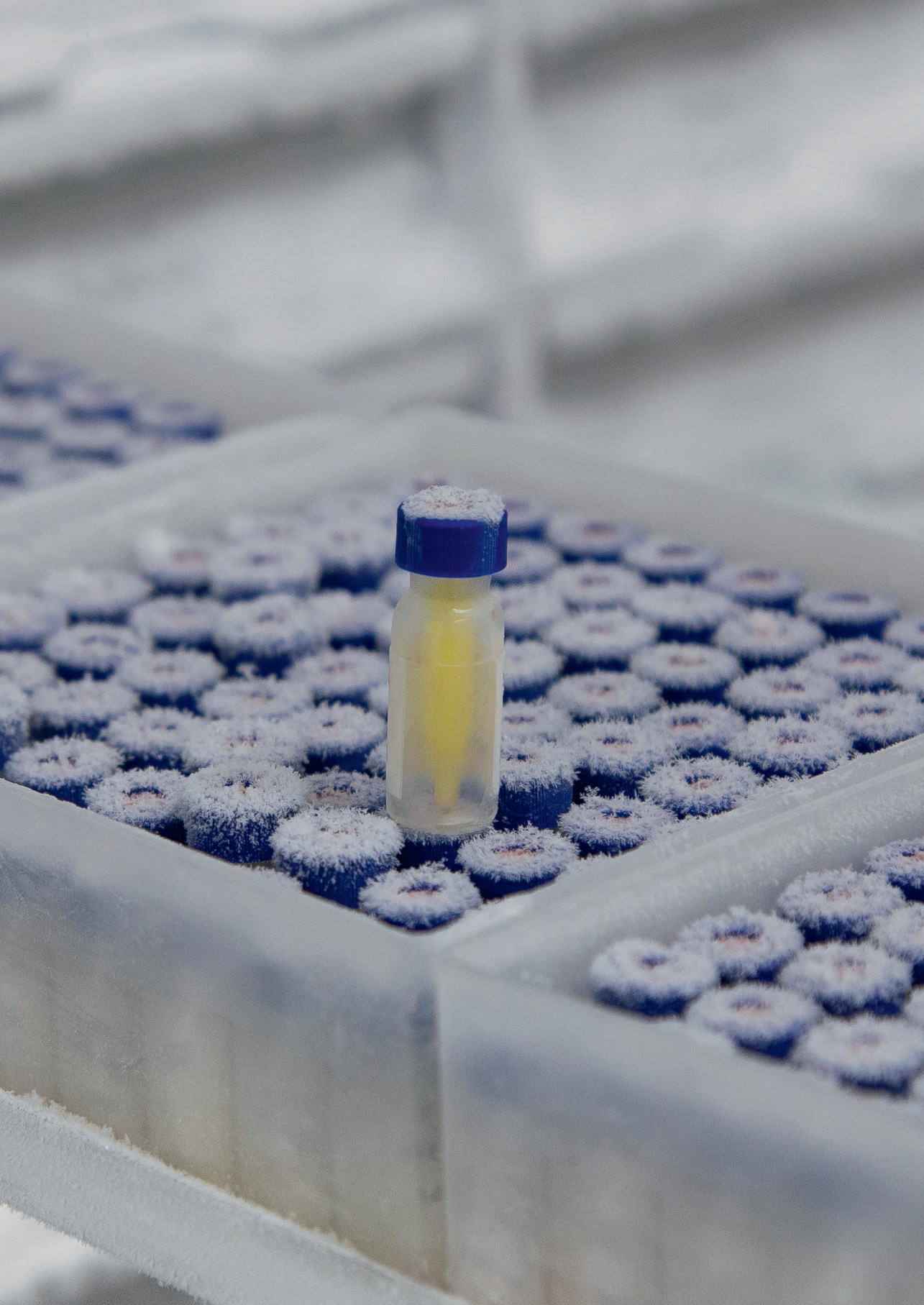
33. M H, M S, C G, M H. ChemR23, the receptor for chemerin and resolvin E1, is expressed and functional on M1 but not on M2 macrophages. *J Immunol*. 2015;194(5):2330-2337. doi:10.4049/JIMMUNOL.1402166

34. Diaz-Rubio GI, Corona-Meraz F-I, Madrigal-Ruiz P-M, et al. CCR2/CCL2 and CMKLR1/RvE1 chemokines system levels are associated with insulin resistance in rheumatoid arthritis. *PLoS One*. 2021;16(1). doi:10.1371/JOURNAL.PONE.0246054

35. Griffin TM, Scanzello CR. Innate inflammation and synovial macrophages in osteoarthritis pathophysiology. *Clin Exp Rheumatol*. 2019;37(5):57-63. /pmc/articles/PMC6842324/?report=abstract. Accessed November 21, 2020.

36. Prescott D, Mckay DM. Aspirin-triggered lipoxin enhances macrophage phagocytosis of bacteria while inhibiting inflammatory cytokine production. *Am J Physiol Gastrointest Liver Physiol*. 2011;301:487-497. doi:10.1152/ajpgi.00042.2011.-The

37. Wu B, Walker J, Spur B, Rodriguez A, Yin K. Effects of Lipoxin A4 on antimicrobial actions of neutrophils in sepsis. *Prostaglandins, Leukot Essent Fat Acids*. 2015;94:55-64. doi:10.1016/J.PLEFA.2014.11.005



5

Anti-inflammatory and pro-resolving effects of the omega-6 polyunsaturated fatty acid adrenic acid

PRO-RESOLVING EFFECTS OF ADRENIC ACID

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Abstract

Polyunsaturated fatty acids (PUFAs) and their metabolites are potent regulators of inflammation. Generally, omega (n) -3 PUFAs are considered pro-resolving whereas n-6 PUFAs are classified as pro-inflammatory. Here we characterized the inflammatory response in murine peritonitis and unexpectedly found the accumulation of adrenic acid (AdA), a poorly studied n-6 PUFA. Functional studies revealed that AdA potently inhibited the formation of the chemoattractant leukotriene B4 (LTB_4) specifically in human neutrophils, and this correlated with a reduction of its precursor arachidonic acid (AA) in free form. AdA exposure in human monocyte-derived macrophages enhanced efferocytosis of apoptotic human neutrophils. *In vivo,* AdA treatment significantly alleviated arthritis in an LTB_4-dependent murine arthritis model. Our findings are the first to indicate that the n-6 fatty acid AdA effectively blocks production of LTB_4 by neutrophils and could play a role in resolution of inflammation in vivo.

Key points

Adrenic acid is the first omega-6 fatty acid with pro-resolving functions.
Adrenic acid inhibits leukotriene B4 production specifically in neutrophils.
Adrenic acid treatment alleviates leukotriene B4 mediated experimental arthritis.

Introduction

Inflammation is an important first line response to barrier breach and tissue damage. Usually, inflammation and its resolution are tightly controlled processes that involve the recruitment of immune cells and soluble mediators. Lack of proper resolution can lead to chronic inflammation, which is associated with several diseases such as atherosclerosis, autoimmune diseases, asthma, and cancer. During the past 20 years, it has been established that polyunsaturated fatty acids (PUFAs) and PUFA-derived lipid mediators are potent regulators of inflammation and its resolution(1).

Historically, n-6 PUFAs such as arachidonic acid (AA) and linoleic acid (LA) are considered pro-inflammatory, whereas n-3 PUFAs are considered to be anti-inflammatory and/or pro-resolving(2). This is primarily due to the fact that the potent pro-inflammatory leukotrienes and prostaglandins are derived from AA. In contrast, n-3 PUFAs such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA) are metabolized into so-called specialized pro-resolving lipid mediators (SPMs) like resolvins, protectins and maresins(1, 3–5). In addition to SPMs derived from EPA, DHA and DPA, more recently, additional n3-PUFA derived metabolites have been associated with anti-inflammatory effects, such as the 15-lipoxygenase (LOX) products from α-linoleic acid (ALA)(6). Furthermore, biological functions have been assigned to the free n-3 PUFAs themselves (7). For instance, EPA, DHA and DPA can compete with AA as substrates for enzyme conversion, leading to a diminished production of pro-inflammatory oxylipins(8). Moreover, free n-3 PUFAs can mediate anti-inflammatory effects by activating their specific G-protein coupled receptor GPR120(9).

However, this pro- versus anti-inflammatory classification of n-6 and n-3 PUFAs might be too strict. On the molecular level, anti-inflammatory effects of n-6 PUFAs and their derivatives have been shown both *in vitro* and *in vivo*. For instance, AA can be metabolized into pro-resolving lipoxins as well as anti-inflammatory cytochrome P450 (Cyp450) derivatives(10, 11). Furthermore, AA-derived prostaglandin E_2 (PGE_2) was shown to have both pro-inflammatory and pro-resolution properties(12). In addition, earlier studies proposed that the n-6 PUFAs dihomo-γ-linolenic acid (DGLA) and adrenic acid (AdA) might compete with AA for pro-inflammatory oxylipin production(13–18).

Clinical trials using n-3 PUFA supplementation in several inflammatory diseases have shown both beneficial but also negative results. Systematic reviews in asthma and rheumatoid arthritis report inconsistent or modest beneficial effects and also in cardiovascular disease, n-3 PUFA supplementation trials did not show conclusive beneficial effects(19–23). Furthermore, some studies report beneficial effects of dietary

n-6 PUFA intake, in particular with regard to LA(24, 25). In summary, several reports highlight that a simple dichotomous classification of n-6 and n-3 PUFA as exclusively pro- and anti-inflammatory, respectively, does not sufficiently describe the underlying biology and that the pro- and anti-inflammatory roles of PUFAs are not completely understood.

To better understand the roles of the n-3 and n-6 PUFA in the context of inflammation, we determined their kinetics in an experimental model of self-resolving peritonitis. We performed a comprehensive analysis of lipids, using LC-MS/MS and we discovered the accumulation of the n-6 PUFA AdA during the resolution phase of inflammation. This finding triggered us to further study the biological function of AdA *in vitro* and *in vivo.*

Materials and Methods

Cell isolation and culture

Human primary neutrophils were isolated from EDTA blood of healthy donors. Most of the erythrocytes were removed by dextran sedimentation using 3% dextran T-500 (Pharmacosmos) in phosphate buffered saline without calcium and magnesium (PBS (-/-), Lonza). Next, neutrophils were separated from peripheral blood mononuclear cells (PBMCs) by Ficoll density gradient centrifugation and remaining erythrocytes were removed by hypotonic lysis. Human PBMCs were isolated from buffy coats of healthy donors (Sanquin, The Netherlands) by Ficoll density gradient centrifugation. Monocytes were isolated from PBMCs by magnetic cell sorting using CD14+ microbeads (Miltenyi Biotec) and were differentiated in RPMI 1640 (Gibco, Life Technologies) supplemented with 8% FCS using 5 ng/mL GM-CSF (R&D Systems) for 7 days. Written informed consent was obtained from all donors and the study was approved by the medical ethical committee of the Leiden University Medical Center (LUMC).

Cell culture incubations for lipid analysis

Neutrophils (1×10^6 cells/mL) were incubated with 5 μg/mL AdA (Nu-Check Prep) in PBS with calcium and magnesium (+/+, B. Braun) for 10 minutes. Macrophages were incubated for either 10 minutes or 24 hours, using the same cell density and AdA concentration as for neutrophils, but in RPMI 1640 without phenol red (Gibco, Life Technologies) containing 0.1% fatty acid free BSA (Sigma Aldrich). AdA was pre-diluted in ethanol (EtOH, absolute HPLC grade, Thermo Scientific) and suspended in the appropriate medium using a ultrasonic bath. After stimulation with AdA, both neutrophils and macrophages were stimulated with 4 μM calcium ionophore A23187 (Sigma-Aldrich) for 10 minutes. For lipid mediator analysis, methanol (MeOH) containing internal standards (IS) was added to the cells and their supernatant in a 3:1 volume ratio. The IS consisted of LTB_4-d4, PGE_2-d4, 15-HETE-d8 (all 50 ng/mL) and 500 ng/mL of DHA-d5. Samples were stored under argon

at -80 °C until analysis. For quantitative lipidomic analysis using the Lipidyzer™, the cells were washed twice with PBS (+/+) containing 0.1% fatty acid free BSA after stimulation. The pelleted cells were stored at -80 °C until analysis.

Calcium flux assay

Leukocytes were obtained from heparinized blood of healthy donors. To this end, HetaSep (Stemcell) was added to the blood at a ratio of 1:6 (HetaSep:blood) and incubated for 30 minutes at 37 °C to sediment the erythrocytes. Leukocytes were harvested and remaining erythrocytes were lysed using NH4Cl/KHCO3 (5 minutes, room temperature). Leukocytes (1×107/mL) in RPMI 1640 containing 2% FCS and 0.02% pluronic acid (Molecular Probes) were labelled with 2 μM Indo-1 AM (Molecular Probes) for 35 minutes at 37°C. Cells were washed and resuspended in RPMI 1640 containing 2% FCS and 1 mM CaCl2. Cells were pre-incubated (10 minutes, 37°C) with 5 μg/mL AdA before acquisition. After 2 minutes of acquisition, calcium ionophore A23187 was added and samples were acquired another 5 minutes. Cells were analyzed by flow cytometry using a LSRII (BD Biosciences) and analyzed using FlowJo software.

Phagocytosis assay

THP-1 cells were differentiated in the presence of phorbol 12-myristate 13-acetate (PMA, 50 ng/mL) for 48 hours followed by medium exchange to RPMI 1640 supplemented with 10% FCS for 24 hours. Differentiated THP-1 cells (96-well plate, 35000 cells/well) were pre-treated with AdA or vehicle (15 minutes, 37 °C), followed by the addition of opsonized FITC-labeled zymosan A (Molecular Probes) at a ratio of 10:1 (particles:cell) for 1 hour at 37 °C. FITC-labeled zymosan A was opsonized using opsonizing reagent containing purified rabbit polyclonal IgG antibodies (Molecular Probes, Eugene, Oregon). Subsequently, trypan blue solution (0.04%, Sigma-Aldrich) was used to quench surface-bound FITC-zymosan A particles for 2 minutes at room temperature after which the solution was washed away and substituted with PBS (+/+). Fluorescence intensity was measured in a FluoStar Optima microplate reader (BMG Labtech) using an excitation of 485 nm and an emission of 520 nm.

Efferocytosis assay

PBMCs were isolated by density centrifugation of leukocyte cones from the NHS Blood and Transplant bank. PBMCs were plated 30 x 10^6 cells per 10 cm^2 in PBS(+/+). After 1 hour, the cells were washed twice after which monocytes remained adhered to the plate. The monocytes were differentiated in RPMI 1640 (Sigma) supplemented with 20% FBS in the presence of 50 ng/mL hM-CSF (Peprotech) for 7 days. Macrophages (M0 macrophages) were harvested and plated (96-well plate, 2.5 ×10^4 cells/well) and polarized to become M1 macrophages under the influence of IFNγ (20ng/ml, Peprotech) and LPS

(10ng/ml, Sigma) for 24 hours. Apoptotic HL-60 cells were prepared by incubating the cells in serum free PBS for 24 hours (4 x 10^6 cells/mL) after which approximately 60% is apoptotic. Apoptotic HL-60 cells were labelled with CFSE (2μM, 37°C, 10 minutes). M0 and M1 macrophages were pre-treated with AdA (Cayman Chemicals) for 30 minutes at 37 °C. Apoptotic HL-60 cells were added in a 1:5 ratio (macrophages/HL-60 cells) and left to incubate for 1 hour at 37°C. Subsequently, macrophages were washed twice and surface-bound HL60 were quenched with trypan blue solution (0.04%, Sigma-Aldrich) for 2 minutes at room temperature. CFSE positive efferocytic macrophages were detected using an LSR Fortessa II and data were analysed using FlowJo software.

Migration assay

Isolated human neutrophils (1×10^6 cells/mL) were incubated with 5 μg/mL AdA in PBS (+/+) or vehicle (1% EtOH) for 10 minutes and subsequently stimulated with 4 μM calcium ionophore A23187 for 10 minutes. Next, the cells were centrifuged (16,100 *g*, 10 minutes) and supernatant was stored at -80°C until further use. For the migration assay, 29 μL of supernatant was added to the bottom well of a ChemoTx plate (101-3, Neuro Probe) and 25 μL containing 2×10^5 freshly isolated neutrophils was added to the upper well. Migrated cells were collected from the lower well after 90 minutes at 37°C. The cells were quantified by flow cytometry using a LSRFortessa (BD Biosciences), Flow-Count fluorospheres (Beckman Coulter) and FACSDiva software (BD Biosciences).

Enzyme activity assays

Activity assays were performed according to the manufacturers' protocol. The Lipase Activity Assay Kit (Sigma-Aldrich) and the $cPLA_2$ Assay Kit (Cayman chemicals) were used.

***In vivo* peritonitis model**

This experiment was both carried out at the University of Iceland and at the LUMC. Male C57BL/6 mice of 8 weeks old were purchased from Charles River. Animals were housed in the local animal facility in individually ventilated cages. Peritonitis was induced by injecting 1 mg of zymosan A (Saccharomyces cerevisiae, Sigma-Aldrich) in 100 μL saline intraperitoneally (*i.p.*). Control mice were injected with saline only. AdA treated mice were injected with 10 μg AdA in saline either at timepoint T=0 or T=2 hours. In the experiments where mice were treated with AdA at T=0, AdA was mixed with the zymosan A to limit the number of injections. AdA was pre-diluted in absolute HPLC grade EtOH and EtOH was evaporated using a SpeedVac (Zirbus). Next, AdA was suspended in saline using an ultrasonic bath. Saline for control mice was treated identically, without the addition of AdA. Control and AdA treated mice were randomized over the cages and were housed together in a cage. At the indicated time-points after induction of peritonitis, mice were sacrificed

by exposure to carbon dioxide and peritoneal lavage was collected using 5 mL ice cold PBS. From this lavage, cells were collected by centrifugation (10 minutes, 225 *g*). Part of the recruited cells were analyzed by flow cytometry using anti-F4/80 (BM8, Biolegend) and anti-GR1 (1A8, BD Biosciences), or anti-CD11b (M1/70, BD Bioscience) and anti-CXCR2 (TG11, Biolegend). Cells were counted by light microscopy and were analyzed using Navios Flow Cytometer and Kaluza software (Beckman Coulter). For lipid mediator analysis, the remaining peritoneal cells were centrifuged at 16,100 *g* for 5 minutes and snap frozen. The pelleted cell were stored at -80°C until use. On the day of analysis, 250 μL EtOH was added to the cells, samples were shaken for 5 minutes, ultra-sonicated for 10 seconds and centrifuged at 16,100 *g* for 5 minutes. The remaining pellet was used to measure protein content using Pierce BCA Protein Assay Kit (Thermo Scientific). Peritoneal lavage samples were worked up using SPE as published previously(26) without the *n*-hexane washing step, as free fatty acids were to be evaluated, and resuspended in a final volume of 200 μL of 40% MeOH. The study was approved by the Experimental Animal Committee, Ministry for the Environment in Iceland and the Ethical Committee for Animal Experimentation of the LUMC.

K/BxN Serum-transferred arthritis and anti-GPI ELISA

These experiments were both carried out at the University of Minnesota Medical School and the LUMC. Male C57BL/6 mice of 8 weeks old were purchased from Charles River or The Jackson Laboratory. Animals were housed in the local animal facility in individually ventilated cages. Mice on different treatments were kept in separate cages. Arthritis was induced by *i.p* injection of 100 μL arthritic serum, obtained from T cell receptor transgenic K/BxN mice; injections were done on day 0 and day 2. Mice were treated daily with 2.5 mg AdA from day -3 till the end of the experiment. AdA was dissolved fresh each day in Cremophor EL (Calbiochem) in a 1:1 ratio before dissolving in saline, resulting in a 2.5% final concentration Cremophor. Control mice were treated with 2.5% Cremophor in saline. Clinical scores were obtained in a blinded fashion as previously described(27). In short, a clinical score of 0-3 was given to each paw daily. In addition, ankle thickness of both left and right ankle was measured daily using a precision caliper. Ankle difference was calculated by combining the score of the left and right ankle daily and subtracting the combined baseline thickness, divided by two. Anti-glucose-6-phosphate isomerase (GPI) antibodies were measured in plasma taken at day 10 and day 14 via a tail vein bleed, or in serum obtained at the end of the experiment via heart puncture. The study was approved by the Institutional Animal Care and Use Committee (IACUC) in the USA and the Ethical Committee for Experimental Animal Experimentation of the LUMC, depending on where the experiments were carried out. Anti-GPI antibodies were analyzed as described before(28). AdA was diluted in serum of either arthritic or non-arthritic mice

or PBS, resulting in different AdA concentrations to test interference with the detection of anti-GPI antibodies by ELISA.

Chemicals

5-HETE, 5*S*-HETE, 8-HETE, 11-HETE, 12-HETE, 15-HETE, 15-HEPE, 18-HEPE, 17R-HDHA, 14,15-diHETE, 7S,17S-diHDPA, LXA_4, AT-LXA_4, LXB_4, LTB_4, 6-*trans*,12-*epi*-LTB_4, 6-*trans*-LTB_4, 20-OH-LTB_4, PGD_2, PGE_2, $PGF_{2\alpha}$, TXB_2, RvD1, RvD2, 10*S*,17*S*-diHDHA, MaR1, LTD_4, LTE_4, 19,20-diHDPA, 8*S*,15*S*-diHETE, DPAn-3, DHA and EPA were from Cayman Chemicals and used to construct calibration lines. 15*S*-HETE-d8, LTB_4-d4, PGE_2-d4 and DHA-d5 were used as IS (Cayman Chemicals). RvE1, RvE2, synthetic 18*S*-RvE3 and 18*R*-RvE3 were kind gifts from Dr. Makoto Arita (RIKEN, Yokohama, Japan). AdA and the GLC-85 standard mix were from Nu-Check Prep Inc. All other chemicals including Fluka LC-MS CHROMASOLV grade MeOH were from Sigma-Aldrich.

Lipidomic analyses

LC-MS/MS analysis was carried out as previously described (26) on a QTrap 6500 mass spectrometer (Sciex), coupled to a Shimadzu Nexera LC30-system including auto-sampler and column oven (Shimadzu). The employed column was a Kinetex C18 50 × 2.1 mm, 1.7 μm, protected with a C8 pre-column (Phenomenex). Water (A) and MeOH (B) both with 0.01% acetic acid were used. The gradient program started at 40% eluent B and was kept constant for 1 minute, then linearly increased to 45% B at 1.1 minutes, then to 53.5% B at 4 minutes, to 55% B at 6.5 minutes, then to 90% B at 12 minutes and finally to 100% B at 12.1 minutes, kept constant for 3 minutes. The flow rate was set to 400 µL/minute. The MS was operated under the following conditions: the collision gas flow was set to medium, the drying temperature was 400 °C, the needle voltage -4500 V, the curtain gas was 30 psi, ion source gas 1 was 40 psi and the ion source gas 2 was 30 psi (air was used as drying gas and nitrogen as curtain gas). LC-MS/MS peaks were integrated with manual supervision and area corrected to corresponding IS using MultiQuant™ 2.1 (Sciex). For quantification, the multiple reaction monitoring (MRM) transitions and collision energies (CE) were used, combined with calibration lines..

For GC/MS analysis, to 1×10^6 neutrophils or macrophages were added 250 μL acetone (HPLC grade, Sigma-Aldrich) in 20 μL PBS(-/-) and 10 μL EtOH (Merck). Next, lipids were hydrolyzed using 10 μL 10 mM sodium hydroxide for 30 minutes at 60 °C. After hydrolysis, 1 μL/mL IS was added and sample workup and PUFA quantification was performed as previously described(29). GC/MS was performed using a Scion 436 gas chromatograph coupled to a Scion triple quadrupole mass spectrometer (both from Bruker) including a G6501-CTC PAL auto sampler (CTC analytics).

For Lipidyzer™ analysis, 250 μL 2-propanol was added to 5×10^6 neutrophils and centrifuged for 10 minutes at 16000 g at 20 °C. Supernatant was collected and the pellet was subjected to additional extraction using 200 μL 2-propanol. 100 μL IS in MeOH:dichloromethane was added and the sample was dried under a gentle stream of nitrogen. The sample was suspended in 250 μL 10 mM ammonium acetate in (50:50) MeOH:dichloromethane. Acquisition and quantification was performed as previously described using a Qtrap 5500 mass spectrometer (Sciex) with differential mobility separation (DMS) coupled to a Shimadzu Nexera X2 LC system for flow injection and Lipidomics Workflow Manager Software (30, 31).

Statistical analysis

Significance was calculated by means of two-tailed unpaired Student's t tests, one-way or two-way ANOVA or a two-tailed Wilcoxon signed-rank test for paired samples using GraphPad Prism version 7.04 for Windows. To justify using two-way ANOVA in figures 5, 6 and Supplementary figure 3, the Shapiro-Wilk test was used. To determine statistical significance by linear mixed model analysis or repeated measures ANOVA as a mixed model, IBM SPSS statistics 25 for Windows was used. The statistical test used to determine significance is indicated for each experiment.

Results

Cellular accumulation of adrenic acid during resolution

First, we carried out a detailed molecular mapping of the self-resolving zymosan A-induced murine peritonitis model. Using a validated LC-MS/MS platform we analyzed the cellular PUFA and oxylipin content in a longitudinal fashion (Fig. 1A). It was previously shown in this model that PUFAs, as well mono-hydroxylated fatty acids, leukotrienes and prostaglandins accumulate in the cell free lavage fluid during the peak of inflammation and their levels return to baseline during resolution(29, 30). We confirmed this accumulation of PUFAs in the cell free lavage in our first experiment (Supplemental Fig. 1A). Moreover, we show that inside the cells, the kinetics are different: the mono-hydroxylated products accumulate only during the resolution phase, which was characterized by the decrease of neutrophil numbers and the increase of F4/80+ expression on macrophages (Supplemental Fig. 1B,C)(31). Leukotriene B_4 (LTB_4) levels peak at 12 hours after which levels again decrease, in contrast to the prostaglandins, which show a biphasic pattern. The analysis revealed a particular accumulation of free PUFAs in the cellular fraction during the resolution phase of inflammation (Fig. 1A). The PUFA accumulation varied largely between different PUFAs: levels of free LA and ALA changed only marginally (between 3- to 5-fold) compared to baseline, while DHA levels increased the most (133-fold). Furthermore, we detected a strong increase of 80-fold

for the n-6 fatty acid AdA within the cellular fraction (Fig. 1B), suggesting a previously unappreciated pro-resolving role for this n-6 PUFA. This increase was observed in three independent experiments of which two were performed at the University of Iceland and one at the LUMC in the Netherlands (Fig. 1C). To test the pro-resolving capacity of AdA, we administered AdA either simultaneously with zymosan or 2 hours after peritonitis induction. In both experiments we detected an enhanced neutrophil clearance from the peritoneal cavity (Fig. 1D,E). These results prompted us to further investigate the role of AdA in inflammatory responses.

Adrenic acid inhibits leukotriene B_4 production in neutrophils

We initially investigated the effect of AdA on the oxylipin and PUFA profile of human primary neutrophils and macrophages, as these are key players in acute inflammation in humans as well as the main cell populations present in the peritoneal cavity during the first 72 hours of inflammation(29, 32). Our analysis revealed that AdA diminished LTB_4 biosynthesis upon calcium ionophore A23187 stimulation in neutrophils, whereas there was an inconsistent effect on macrophages due to inter-donor variations (Fig. 2A). In unstimulated cells the LTB_4 production pathway was not active (Supplemental Fig. 2A) Macrophages were incubated longer with AdA to reach similar AdA uptake but experiments performed with 10 minute incubation show similar results (data not shown). A similar effect was observed for the LTB_4 pathway marker 5-HETE (Fig. 2B). Besides LTB_4 and 5-HETE production we could not detect any other eicosanoid formation such as prostaglandins, nor could we detect any SPM formation within the experiment time frame. Since LTB_4 is produced upon AA release from phospholipids and triglycerides(33, 34) (Fig. 2C), we also measured the levels of released AA in AdA-treated cells (Fig. 2D). Interestingly, AA levels dramatically decreased upon AdA exposure in neutrophils, indicating that the entire LTB_4 production pathway is attenuated. In contrast, AdA led to an increase in AA in macrophages. Using three different cell viability assays we confirmed that the observed effect of AdA on neutrophil leukotriene synthesis was not caused by cell death (Supplemental Fig. 2B). In addition, we carried out intracellular calcium flux analysis showing that AdA-exposed neutrophils have an undisturbed intracellular calcium flux in response to calcium ionophore A23187 (Fig. 2E).

> Figure 1. Time course of PUFA and their oxidative products during zymosan A-induced peritonitis. **(A)** Heat map of investigated lipids in the cellular fraction of peritoneal fluid (ng/mg protein). n = 3 mice/time point, one representative experiment out of three is shown. (**B)** Fold change of detected PUFAs relative to the lowest detected concentration (timepoint zero) of the experiment as shown in Fig. 1A. **(C)** Time course of free AdA accumulation in the cellular fraction of peritoneal fluid (ng/μg protein) in three independent experiments depicted individually. Average with SEM of 3-4 mice/group is shown. **(D,E)** Mice were administered AdA at T=0 hours ***(D)*** or T=2 hours ***(E)*** and number of neutrophils in the peritoneal lavage was determined by flow cytometry. Mean and SEM are shown, *n*=5-6 mice per group of two independent experiments.

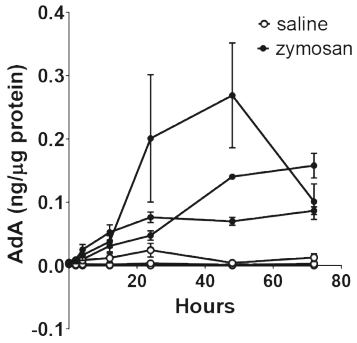
A

Analyte	Time (hours) 0	2	4	12	24	48	72
5-HETE	0,1	0,7	3,2	3,9	3,2	2,5	10,6
8-HETE	0,3	0,5	0,4	0,3	0,2	0,6	2,6
11-HETE	1,1	0,4	2,2	1,5	1,3	0,8	5,7
12-HETE	28,1	12,7	10,7	9,2	4,8	17,5	64,5
15-HETE	1,0	1,7	3,3	2,1	1,5	2,5	12,4
15-HEPE	0,1	0,5	0,8	0,5	0,4	1,9	2,5
18-HEPE	0,0	0,0	0,1	0,0	0,0	0,0	0,1
17-HDHA	0,7	2,6	6,0	2,3	1,5	12,6	32,5
LTB_4	0,0	0,1	2,1	2,4	1,6	0,0	0,0
6-trans-LTB_4	3,0	0,2	0,2	0,3	0,2	0,1	0,3
trans-12-epi-LTB_4	4,0	0,2	0,2	0,2	0,2	0,1	0,5
20-OH-LTB_4	0,0	0,0	0,0	0,0	0,0	0,0	0,0
LTD_4	0,0	0,2	0,1	0,0	0,2	0,0	0,4
LTE_4	0,1	0,4	0,2	0,0	0,4	0,4	2,4
PGD_2	0,1	0,1	0,5	0,8	0,4	0,3	0,7
PGE_2	0,8	0,1	6,4	7,3	4,1	3,9	31,8
$PGF_{2\alpha}$	0,5	0,3	5,1	3,0	1,7	0,5	2,2
8-iso-PGE_2	0,0	0,0	0,1	0,1	0,1	0,1	0,7
8-iso-$PGF_{2\alpha}$	0,1	0,0	0,4	0,2	0,1	0,0	0,2
15-keto-PGE_2	0,1	0,0	0,0	0,0	0,0	0,0	0,0
LXA_4	0,0	0,0	0,0	0,0	0,0	0,0	0,0
AT-LXA_4	0,0	0,0	0,0	0,0	0,0	0,0	0,0
LXB_4	0,3	0,0	0,0	0,0	0,0	0,0	0,1
10S,17S-diHDHA	1,0	0,3	0,4	0,2	0,1	0,3	1,2
8S,15S-diHETE	0,9	0,1	0,1	0,1	0,0	0,1	0,4
7S,17S-diHDPA	0,1	0,0	0,0	0,0	0,0	0,0	0,1
14,15-diHETE	0,3	0,0	0,0	0,0	0,0	0,0	0,1
19,20-diHDPA	0,0	0,2	1,2	0,6	0,7	0,3	1,9
TXB_2	2,1	1,1	24,0	19,8	14,2	7,8	95,3
AA	22,7	93,6	279,6	479,1	690,7	488,2	1.037,9
EPA	1,5	4,7	13,3	31,0	49,5	36,7	22,9
DHA	2,1	18,2	106,8	134,2	194,0	156,6	287,3
AdA	2,0	9,5	25,2	52,4	76,2	69,7	164,8
DPA_{n-3}	11,6	13,8	44,7	81,5	125,6	127,4	182,3
ALA/GLA	16,8	20,1	44,3	58,3	91,2	65,4	61,2
LA	322,8	389,5	736,6	711,8	1.005,3	707,8	1.487,9

B

Analyte	Time (hours) 0	2	4	12	24	48	72
AA	1	4,1	12,3	21,1	30,4	21,5	45,6
EPA	1	3,2	9	21,1	33,6	24,9	15,5
DHA	1	8,5	49,7	62,4	90,3	72,9	133,7
AdA	1	4,6	12,3	25,6	37,3	34,1	80,6
DPA_{n-3}	1	1,2	3,8	7	10,8	11	14
ALA/GLA	1	1,2	2,6	3,5	5,4	3,9	3,6
LA	1	1,2	2,3	2,2	3,1	2,2	4,6

C



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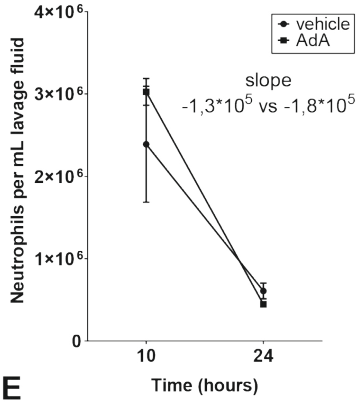

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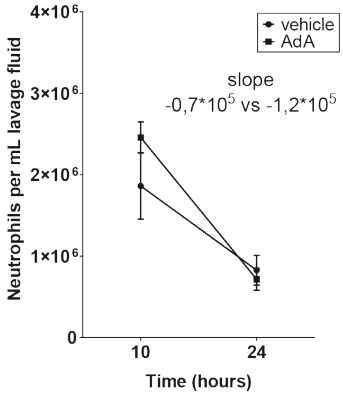


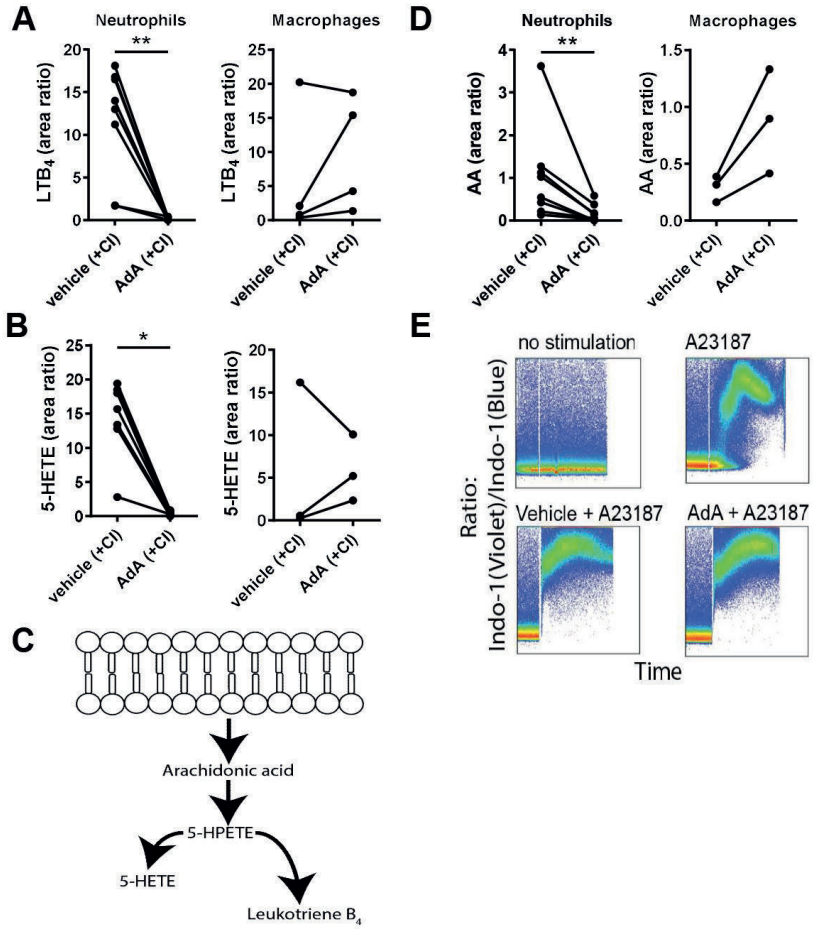


Figure 2. Adrenic acid inhibits leukotriene B_4 production in neutrophils. **(A,B,D)** Peripheral blood neutrophils and macrophages were pre-incubated for 10 minutes or 24 hours with AdA, respectively, before stimulation with calcium ionophore A23187 for 10 minutes. Total free LTB_4, 5-HETE and AA levels relative to internal standard were calculated based on LC-MS/MS signals (see "Materials and Methods"). Statistical significance was established using the Wilcoxon signed-rank test. *, $P<0.05$; **, $P<0.01$. Each line represents one donor, n = 3-8 donors all individual experiments. **(C)** Schematic representation of the LTB_4 production pathway. **(E)** Calcium flux in peripheral blood neutrophils pre-incubated for 10 minutes with either vehicle or AdA and stimulated with calcium ionophore A23187. Experiment was performed once.

Adrenic acid exposed neutrophils have reduced chemoattractant capacity

LTB_4 is a potent chemoattractant for neutrophils, so we next questioned if the downregulation of LTB_4 synthesis in neutrophils could have a functional effect. Indeed, LTB_4 and supernatant of calcium ionophore A23187-stimulated neutrophils attracted neutrophils in a trans-well system (Fig. 3B and Supplemental Fig. 2C). However, migration

was strongly impaired when supernatant of stimulated AdA-exposed neutrophils was used (Fig. 3B). Any residual chemo attraction present is most likely due to incomplete inhibition of LTB_4 production (Fig. 3C) or to the presence of other chemo attractants that are released within 10 minutes of stimulation. Supernatant of unstimulated cells, AdA, or calcium ionophore A23187 alone were not able to induce neutrophil migration (Supplemental Fig. 2C).

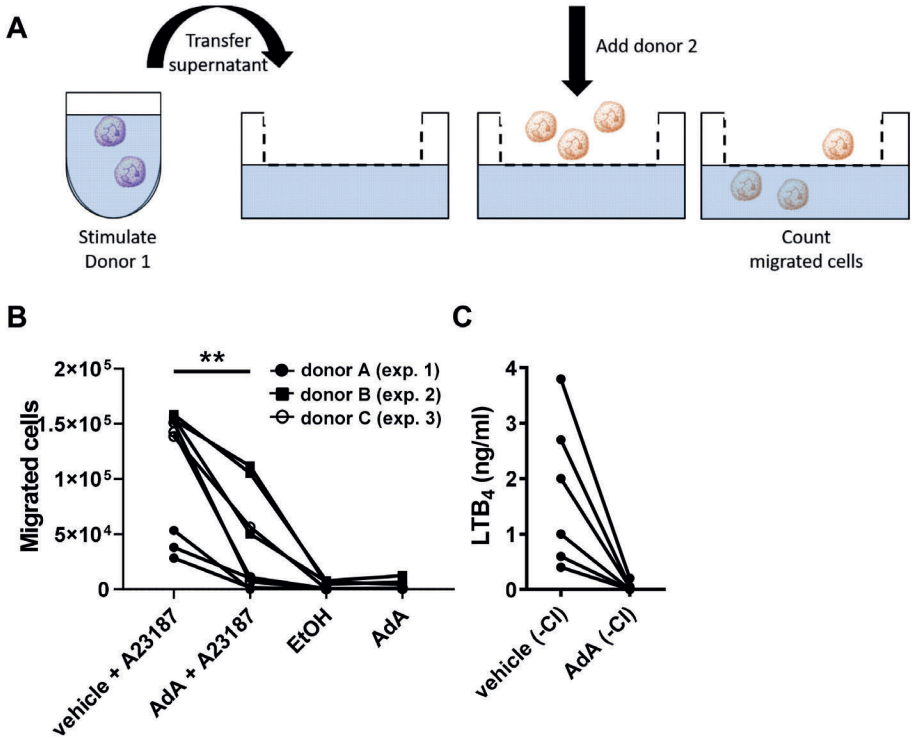


Figure 3. Migration towards soluble factors released by neutrophils activated in the presence of AdA is impaired**. (A)** Schematic overview of the chemotaxis experiment. **(B)** Migration of neutrophils towards supernatant of neutrophils treated with AdA or vehicle (1% EtOH) and stimulated with calcium ionophore A23187 or not. Number of migrated neutrophils was determined after 90 minutes. n=3 donors tested, each with supernatants of three independent donors in three independent experiments. Donors B and C tested with the same set of supernatants. Statistical significance was established using a Wilcoxon signed-rank test. **, P<0.01. **(C)** Concentrations of LTB_4 in the supernatants were determined by LC-MS/MS and quantified using an external calibration line (ranging from 0-5 ng/mL). Controls were performed in triplicate, means of n=6 donors are shown.

Adrenic acid enhances macrophage phagocytosis

As macrophages play a crucial role in the resolution of inflammation, we next questioned whether AdA would also have an effect on macrophages. Several SPMs are able to enhance the phagocytic capacity of macrophages, a process involved in resolution of inflammation(1). We therefore examined whether AdA was able to enhance the phagocytosis of opsonized zymosan particles by THP-1-derived macrophages by 2.5 fold (Fig. 4A). In addition we observed a 10.7 and 11.2 fold increase of efferocytosis of apoptotic HL-60 cells by monocyte derived M0 and M1 macrophages respectively (Fig. 4B).

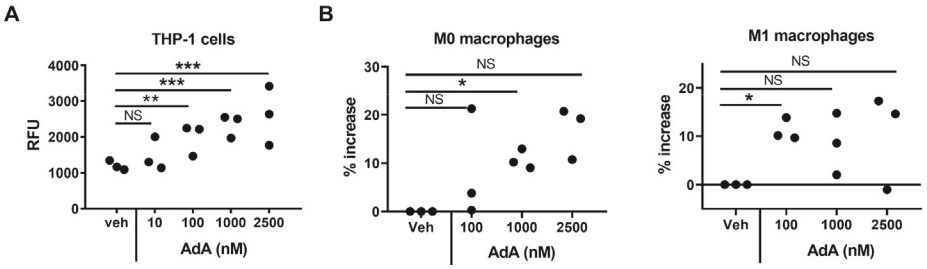


Figure 4. AdA enhances phagocytosis. (**A**) Phagocytosis of opsonized fluorescent zymosan particles by THP-1 derived macrophages. Mean values of n = 3 donors performed in quadruplicate, in three independent experiments. RFU, relative fluorescent units. **(B)** Efferocytosis of apoptotic HL-60 cells by M0 and M1 macrophages. n=3 macrophage donors in three independent experiments. Linear mixed model was performed to show statistical significance. *, P<0.05; **, P<0.01; ***, P<0.001.

Adrenic acid is internalized by neutrophils

We next set out to investigate the molecular mechanisms underlying the anti-inflammatory effects of AdA on neutrophils. Therefore, we studied whether AdA is taken up by the cells. Using GC-MS based analysis, we showed increased total AdA levels in both neutrophils and macrophages upon 10 minute and 24 hour incubation with AdA, respectively, compared to vehicle (Fig. 5A and Supplemental Fig. 3A). Approximately 50% of the added 5 μg AdA was found in the cellular fraction of both cell types after incubation. Next, we determined in which lipid reservoir AdA is stored to obtain an indication about the mechanisms underlying its inhibitory effect on AA release. To this end, we measured the neutrophil lipidome on a commercial lipidomics platform (Lipidyzer™) and showed that AdA is stored in several different lipid reservoirs (Fig. 5B). We could not detect any AdA in lysophospholipids or sphingomyelin lipids in any of the conditions (data not shown). Instead AdA levels increased specifically in cholesteryl esters. AdA accumulation in phosphatidylcholines (PC),diacylglycerides (DAG) and triglycerides (TAG) was only detected after AdA incubation in combination with calcium ionophore A23187 stimulation. AdA did not accumulate in phosphatidylenthanolamines (PE).

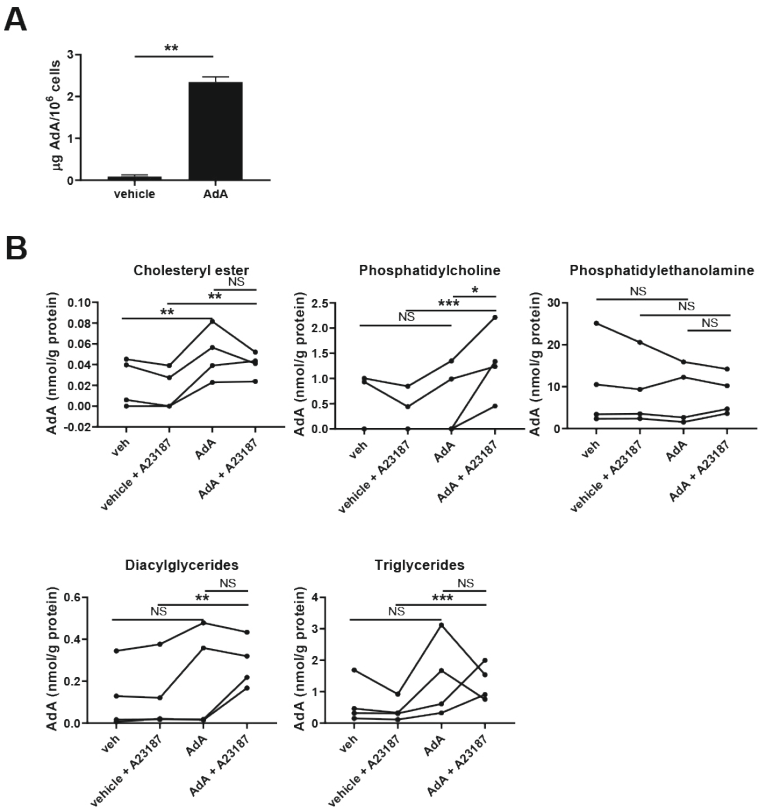


Figure 5. AdA incorporates in different fatty acid reservoirs in neutrophils**. (A)** Neutrophils were incubated with AdA or vehicle for 20 minutes. Total levels of cellular AdA were quantified upon hydrolysis, using GC-MS. Three individual experiments were performed in triplicate, means and SD of n = 3 donors are shown. One-way ANOVA was performed, **, P<0.01. **(B)** Neutrophils were incubated with AdA or vehicle for 10 minutes and were subsequently stimulated with calcium ionophore A23187 or vehicle for 10 minutes. Next, cells were subjected to DMS-MS/MS using the Lipidyzer ™ platform and cellular AdA concentrations in various lipid classes were determined. Experiments were performed in triplicate and each line represents a donor, n = 4 donors in four independent experiments. Matched Two-way ANOVA was performed to compare baseline levels with AdA incubation, to compare the calcium ionophore A23187 stimulated conditions and to compare the effect of calcium ionophore A23187 stimulation after AdA incubation and to baseline, *, P<0.05; **, P<0.01; ***, P<0.001. Values below detection limit were assigned the null value.

No apparent effects of adrenic acid exposure on arrangement of AA in higher order lipids

Since AdA accumulated in phospholipid species such as PC, as well as in DAG and TAG lipids, we hypothesized that AdA could affect the release of AA from these compartments. In addition to cytosolic phospholipase A2 (cPLA$_2$) it has been shown that adipose triglyceride lipase (ATGL) regulates AA release in neutrophils(34). We therefore

investigated the activity of different lipases involved in AA release in these compartments. Surprisingly, AdA did not have an effect on either $cPLA_2$, measured by a phospholipase assay or ATGL activity measured by a pan lipase assay (Fig. 6A). These enzymes release AA from phospholipids and TAGs, respectively. Since AdA does not appear to inhibit AA release by affecting lipase activity, we next sought to investigate in more detail the compartments from which AA is released, using the Lipidyzer™ platform. We were able to confirm the blunted calcium ionophore A23187-mediated AA release in the presence of AdA using this platform (Fig. 6B). This effect was specific for AA as AdA had no effect of free LA or DHA levels (Supplemental Fig. 3B,C) In contrast to the clear effect of AdA exposure on free AA release, we could not detect any build-up or (re-)arrangement of AA in any of the lipid compartments in which we detected AA (Fig. 6 C,D).

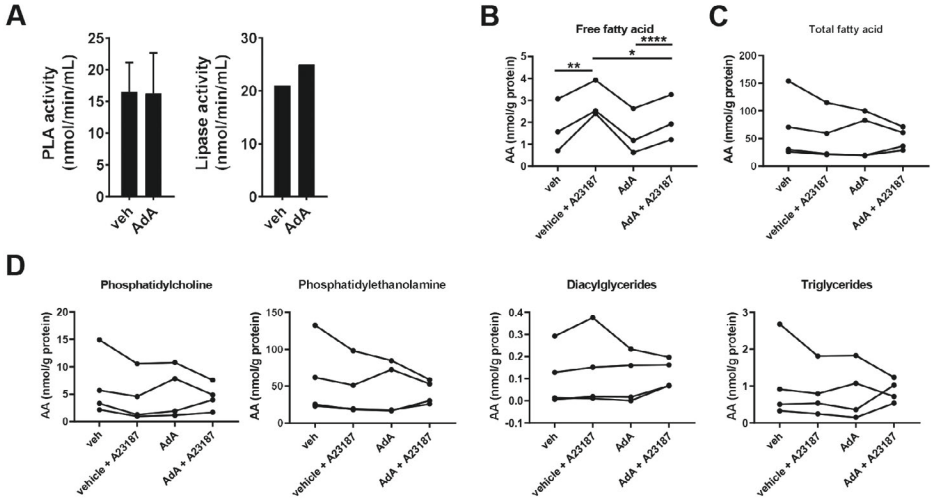


Figure 6. AA concentrations in higher order lipid compartments **(A)** Neutrophils pre-treated with AdA or vehicle for 10 minutes were stimulated with calcium ionophore A23187 for 2 (PLA) or 10 (lipase) minutes. Enzyme activity was determined using activity assays. Means are shown for both assays and SD are shown for PLA activity. $n = 3$ (PLA) $n = 2$ (lipase), all performed in individual experiments. **(B,C,D)** Neutrophils were incubated with AdA or vehicle for 10 minutes. Subsequently, they were stimulated with calcium ionophore A23187 or vehicle for 10 minutes. Next, the cells were subjected to DMS-MS/MS using the Lipidyzer™ platform. Cellular AA levels, free (**B**), total (**C**), and within the PC, PE, DAG, and TAG classes (**D**) are shown. Experiments were performed in triplicate, values below detection limit were assigned the null value. Each line represents one donor, n =4 in four independent experiments. Matched Two-way ANOVA was performed comparing the same conditions as for figure 5.*, P<0.05; **, P<0.01; ***, P<0.001, ****, P>0.0001.

Adrenic acid protects from neutrophil-mediated experimental arthritis

Having observed abrogated LTB_4 synthesis, neutrophil migration and enhanced phagocytosis upon AdA treatment, we investigated the *in vivo* relevance of these findings. Since our results show inhibited neutrophil attraction by neutrophils exposed to AdA, we investigated the anti-inflammatory and pro-resolving effects of AdA in the K/BxN serum transfer arthritis model. This model has been shown to be critically dependent on LTB_4 production by neutrophils(35). In line with those results, we observed that AdA limited the severity of arthritis in two independent experiments carried out in two independent facilities (Fig. 7A). In both experiments, we showed a decreased clinical score in AdA-treated mice, as well as a decrease in ankle thickness measured in one of the experiments (Fig. 7A and Supplemental Fig. 4A). Interestingly, the reduced arthritis severity correlated with lower levels of anti-GPI antibodies in the serum of the AdA-treated mice (Fig. 7C,D). The lower titers were not due to AdA interfering with the ELISA (Supplemental Fig. 4B).

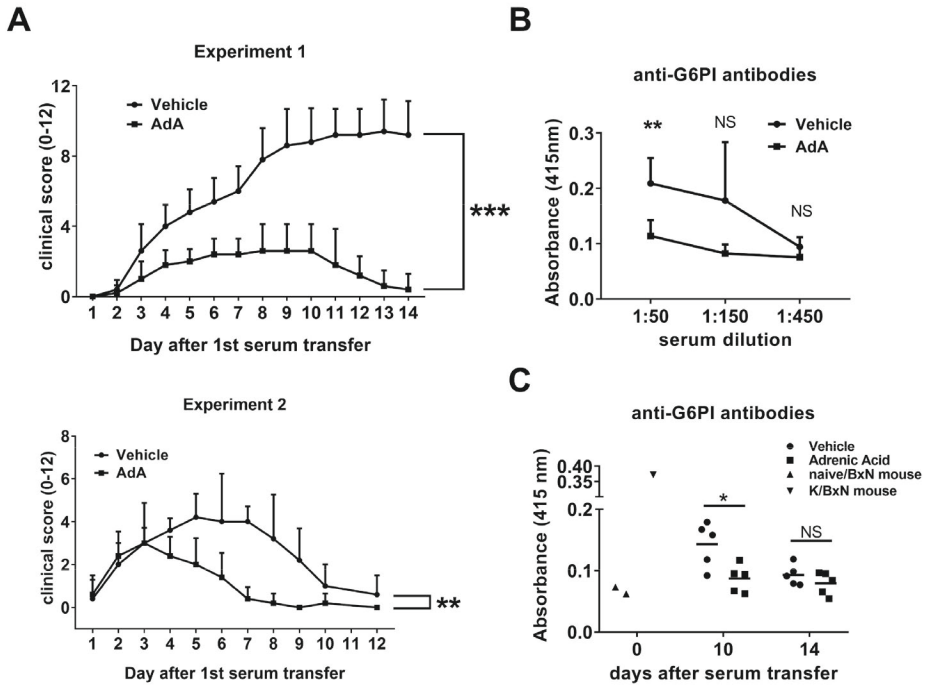


Figure 7. Adrenic acid reduces severity of neutrophils mediated experimental arthritis. **(A)** Clinical arthritis scores are shown for two independent experiments executed independently in two different laboratories**.** Mice were administered AdA from day -3 till the end of the experiment. Mean and SD are shown, *n* = 5 mice/group/experiment. Statistical significance was established using a repeated measures ANOVA as a mixed model. **(B)** Serum anti-GPI antibodies of day 14 measured by ELISA in mice from experiment 1. Mean and SD are shown. **(C)** Anti-GPI antibodies over time of experiment 2 together with controls. Plasma was diluted 1:100. Each dot represents a mouse, mean of duplicates is shown. Statistical significance in (*B*) and (*C*) was established using student-t tests. *, P<0.05; **, P<0.01; ***, P<0.001.

Discussion

In this study, we investigated the kinetics of both n-3 and n-6 PUFAs in the context of resolution of inflammation. We demonstrated an accumulation of the n-6 PUFA AdA in the resolution phase of the self-resolving peritonitis model. Specifically in neutrophils, AdA potently inhibited AA release and LTB_4 production reducing further attraction of neutrophils to the site of inflammation. Furthermore, AdA enhanced phagocytosis and efferocytosis by macrophages *in vitro* and the clearance of neutrophils in the peritonitis model *in vivo*. Finally, AdA clearly showed the potential to prevent inflammation in the K/BxN serum transfer model of arthritis. These findings indicate a novel anti-inflammatory/pro-resolving function for the n-6 PUFA AdA. Our data supports the notion that n-6 PUFAs are not only pro-inflammatory but can have potent anti-inflammatory functions as well and our data set the stage to delineate the mechanisms underlying the biological effects of AdA presented here.

Effects of AdA on AA were already proposed in 1980 by indicating that AdA modulates the inflammatory effects of AA as AdA limited the fall in blood pressure induced by AA injections in rats(16). A few years later, it was shown that this effect of AdA is due to the inhibition of prostaglandin I_2 formation by competing with AA for cyclooxygenase enzymes as well as by reducing the AA content of phosphatidylinositol (PI) structures in human endothelial cells(17, 18). Here, we were unable to show build-up or (re-)arrangement of AA levels in various lipid compartments. However, this is probably due to the relatively small amount of AA which is prevented from becoming free fatty acid compared to the total amount of AA in the cell. Alternatively, AA might be released from lipid species we were technically not able to measure, as for example PI lipids. Since AdA did not affect lipase activity, we hypothesize that AdA might cause a strong activation of acyltransferases, for example the diglycerideacyltransferases (DGAT), could compensate the $cPLA_2$ effects upon calcium ionophore A23187 activation and reverse the balance from free AA to esterified AA. The effect of AdA seems to be specific for AA, as we did not observe this with another n-6 PUFA, LA, nor with the n-3 PUFAs DHA and EPA.

The finding that AdA accumulated specifically inside the cells during the resolution phase strongly points to the activation of a cell-intrinsic mechanism to initiate the resolution phase. The source of AdA might be AA itself, since AA can be converted into AdA by elongases as previously shown in RAW 264.7 macrophages(36). This could be a mechanism by which the cells remove the no longer needed substrate AA.

As we observed potent effects of AdA on LTB_4 production in neutrophils, we have investigated the effect of AdA in an *in vivo* inflammatory model dependent on LTB_4 production by neutrophils(35). In the K/BxN serum-transferred arthritis model, we

expected that AdA would impair the capacity of neutrophils to further amplify the inflammation through the production of LTB_4. Indeed, we observed a potent anti-inflammatory effect of AdA. Unexpectedly, we observed a decrease in the arthritogenic anti-GPI antibodies in the AdA treated mice, in association with the lower clinical arthritis score. Since all mice received the same amount of serum containing the antibodies, we hypothesize that these antibodies might be cleared from the circulation faster than in the untreated mice. Indeed, we show that plasma titers of treated mice are already lower than those of untreated mice at day 10 of the experiment. These results are in line with the enhanced ability of AdA-exposed macrophages to phagocytose, possibly allowing the enhanced clearance of arthritogenic immune complexes by these macrophages. Indeed, it is known that anti-GPI antibodies form immune complexes(37, 38). We therefore propose that, in addition to inhibiting neutrophil derived LTB_4 production, AdA might also enhance the macrophage-mediated clearance of anti-GPI antibodies in this model.

The increase in AdA levels and the disappearance of LTB_4 from the peritoneal cavity at the initiation of the resolution phase is consistent with our finding that neutrophils exposed to AdA inhibit LTB_4 production. However, AA and AA metabolites such as PGE_2 and TXB_2 still increase while AdA levels are also increased. Since there are predominantly macrophages in the peritoneum at this timepoint, the increase of AA and AA metabolites point towards a different pro-resolving activity than what we show in neutrophils. For example, it was shown that macrophage populations sustain PGE_2 production during the late resolution phases in murine peritonitis to prevent autoimmune reactions(39).

Consistent with the data presented here, the same effect on LTB_4 production in neutrophils has been described by others after dietary n-3 supplementation with EPA and DHA(40, 41). However, this is the first time the effect on LTB_4 production is described for an n-6 PUFA and the effect of AdA has never been tested in clinical trials. Although it is likely that the results presented in this study are due to the effects of AdA, we have not investigated whether AdA is enzymatically or non-enzymatically converted to any downstream products such as the 15-LOX metabolite 17-HDoTE(42).

Our data emphasize that n-6 fatty acids should not be categorized as merely pro-inflammatory as they can have potent anti-inflammatory effects as well. Importantly, they highlight a previously unappreciated anti-inflammatory and pro-resolving effect of AdA, offering an emerging therapeutic candidate in inflammatory disease such as atherosclerosis, autoimmune diseases, asthma, and cancer.

References

1. Serhan, C. N. 2014. Pro-resolving lipid mediators are leads for resolution physiology. *Nature* 510: 92–101.
2. Harris, W. S. 2018. The Omega-6:Omega-3 ratio: A critical appraisal and possible successor. *Prostaglandins, Leukot. Essent. Fat. Acids* 132: 34–40.
3. Serhan, C. N., S. Hong, K. Gronert, S. P. Colgan, P. R. Devchand, G. Mirick, and R.-L. Moussignac. 2002. Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals. *J. Exp. Med.* 196: 1025–37.
4. Spite, M., L. V Norling, L. Summers, R. Yang, D. Cooper, N. A. Petasis, R. J. Flower, M. Perretti, and C. N. Serhan. 2009. Resolvin D2 is a potent regulator of leukocytes and controls microbial sepsis. *Nature* 461: 1287–91.
5. Hong, S., K. Gronert, P. R. Devchand, R.-L. Moussignac, and C. N. Serhan. 2003. Novel docosatrienes and 17S-resolvins generated from docosahexaenoic acid in murine brain, human blood, and glial cells. Autacoids in anti-inflammation. *J. Biol. Chem.* 278: 14677–87.
6. Kumar, N., G. Gupta, K. Anilkumar, N. Fatima, R. Karnati, G. V. Reddy, P. V. Giri, and P. Reddanna. 2016. 15-Lipoxygenase metabolites of α-linolenic acid, [13-(S)-HPOTrE and 13-(S)-HOTrE], mediate anti-inflammatory effects by inactivating NLRP3 inflammasome. *Sci. Rep.* 6: 31649.
7. Calder, P. C. 2010. Omega-3 fatty acids and inflammatory processes. *Nutrients* 2: 355–74.
8. Norris, P. C., and E. A. Dennis. 2012. Omega-3 fatty acids cause dramatic changes in TLR4 and purinergic eicosanoid signaling. *Proc. Natl. Acad. Sci. U. S. A.* 109: 8517–22.
9. Oh, D. Y., S. Talukdar, E. J. Bae, T. Imamura, H. Morinaga, W. Fan, P. Li, W. J. Lu, S. M. Watkins, and J. M. Olefsky. 2010. GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. *Cell* 142: 687–98.
10. Serhan, C. N., M. Hamberg, and B. Samuelsson. 1984. Lipoxins: novel series of biologically active compounds formed from arachidonic acid in human leukocytes. *Proc. Natl. Acad. Sci. U. S. A.* 81: 5335–9.
11. Gilroy, D. W., M. L. Edin, R. P. H. De Maeyer, J. Bystrom, J. Newson, F. B. Lih, M. Stables, D. C. Zeldin, and D. Bishop-Bailey. 2016. CYP450-derived oxylipins mediate inflammatory resolution. *Proc. Natl. Acad. Sci. U. S. A.* 113: E3240-9.
12. Ricciotti, E., and G. A. FitzGerald. 2011. Prostaglandins and inflammation. *Arterioscler. Thromb. Vasc. Biol.* 31: 986–1000.
13. Pullman-Mooar, S., M. Laposata, D. Lem, R. T. Holman, L. J. Leventhal, D. DeMarco, and R. B. Zurier. 1990. Alteration of the cellular fatty acid profile and the production of eicosanoids in human monocytes by gamma-linolenic acid. *Arthritis Rheum.* 33: 1526–33.
14. Iversen, L., K. Fogh, G. Bojesen, and K. Kragballe. 1991. Linoleic acid and dihomogammalinolenic acid inhibit leukotriene B4 formation and stimulate the formation of their 15-lipoxygenase products by human neutrophils in vitro. Evidence of formation of antiinflammatory compounds. *Agents Actions* 33: 286–91.
15. Iversen, L., K. Fogh, and K. Kragballe. 1992. Effect of dihomogammalinolenic acid and its 15-lipoxygenase metabolite on eicosanoid metabolism by human mononuclear leukocytes in vitro: selective inhibition of the 5-lipoxygenase pathway. *Arch. Dermatol. Res.* 284: 222–6.
16. Cagen, L. M., and P. G. Baer. 1980. Adrenic acid inhibits prostaglandin syntheses. *Life Sci.* 26: 765–70.

17. Mann, C. J., T. L. Kaduce, P. H. Figard, and A. A. Spector. 1986. Docosatetraenoic acid in endothelial cells: formation, retroconversion to arachidonic acid, and effect on prostacyclin production. *Arch. Biochem. Biophys.* 244: 813–23.

18. Campbell, W. B., J. R. Falck, J. R. Okita, A. R. Johnson, and K. S. Callahan. 1985. Synthesis of dihomoprostaglandins from adrenic acid (7,10,13,16-docosatetraenoic acid) by human endothelial cells. *Biochim. Biophys. Acta* 837: 67–76.

19. Reisman, J., H. M. Schachter, R. E. Dales, K. Tran, K. Kourad, D. Barnes, M. Sampson, A. Morrison, I. Gaboury, and J. Blackman. 2006. Treating asthma with omega-3 fatty acids: where is the evidence? A systematic review. *BMC Complement. Altern. Med.* 6: 26.

20. Miles, E. A., and P. C. Calder. 2012. Influence of marine n-3 polyunsaturated fatty acids on immune function and a systematic review of their effects on clinical outcomes in rheumatoid arthritis. *Br. J. Nutr.* 107 Suppl 2: S171-84.

21. Senftleber, N. K., S. M. Nielsen, J. R. Andersen, H. Bliddal, S. Tarp, L. Lauritzen, D. E. Furst, M. E. Suarez-Almazor, A. Lyddiatt, and R. Christensen. 2017. Marine Oil Supplements for Arthritis Pain: A Systematic Review and Meta-Analysis of Randomized Trials. *Nutrients* 9: 42.

22. Balk, E. M., G. P. Adam, V. Langberg, C. Halladay, M. Chung, L. Lin, S. Robertson, A. Yip, D. Steele, B. T. Smith, J. Lau, A. H. Lichtenstein, and T. A. Trikalinos. 2016. *Omega-3 Fatty Acids and Cardiovascular Disease: An Updated Systematic Review*,.

23. Bowen, K. J., W. S. Harris, and P. M. Kris-Etherton. 2016. Omega-3 Fatty Acids and Cardiovascular Disease: Are There Benefits? *Curr. Treat. Options Cardiovasc. Med.* 18: 69.

24. Farvid, M. S., M. Ding, A. Pan, Q. Sun, S. E. Chiuve, L. M. Steffen, W. C. Willett, and F. B. Hu. 2014. Dietary linoleic acid and risk of coronary heart disease: a systematic review and meta-analysis of prospective cohort studies. *Circulation* 130: 1568–78.

25. Wu, J. H. Y., M. Marklund, F. Imamura, N. Tintle, A. V Ardisson Korat, J. de Goede, X. Zhou, W.-S. Yang, M. C. de Oliveira Otto, J. Kröger, W. Qureshi, J. K. Virtanen, J. K. Bassett, A. C. Frazier-Wood, M. Lankinen, R. A. Murphy, K. Rajaobelina, L. C. Del Gobbo, N. G. Forouhi, R. Luben, K.-T. Khaw, N. Wareham, A. Kalsbeek, J. Veenstra, J. Luo, F. B. Hu, H.-J. Lin, D. S. Siscovick, H. Boeing, T.-A. Chen, B. Steffen, L. M. Steffen, A. Hodge, G. Eriksdottir, A. V Smith, V. Gudnason, T. B. Harris, I. A. Brouwer, C. Berr, C. Helmer, C. Samieri, M. Laakso, M. Y. Tsai, G. G. Giles, T. Nurmi, L. Wagenknecht, M. B. Schulze, R. N. Lemaitre, K.-L. Chien, S. S. Soedamah-Muthu, J. M. Geleijnse, Q. Sun, W. S. Harris, L. Lind, J. Ärnlöv, U. Riserus, R. Micha, D. Mozaffarian, and Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Fatty Acids and Outcomes Research Consortium (FORCE). 2017. Omega-6 fatty acid biomarkers and incident type 2 diabetes: pooled analysis of individual-level data for 39 740 adults from 20 prospective cohort studies. *lancet. Diabetes Endocrinol.* 5: 965–974.

26. Giera, M., A. Ioan-Facsinay, R. Toes, F. Gao, J. Dalli, A. M. Deelder, C. N. Serhan, and O. A. Mayboroda. 2012. Lipid and lipid mediator profiling of human synovial fluid in rheumatoid arthritis patients by means of LC–MS/MS. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* 1821: 1415–1424.

27. Monach, P., K. Hattori, H. Huang, E. Hyatt, J. Morse, L. Nguyen, A. Ortiz-Lopez, H.-J. Wu, D. Mathis, and C. Benoist. 2007. The K/BxN mouse model of inflammatory arthritis: theory and practice. *Methods Mol. Med.* 136: 269–82.

28. Nguyen, L. T., J. Jacobs, D. Mathis, and C. Benoist. 2007. Where FoxP3-dependent regulatory T cells impinge on the development of inflammatory arthritis. *Arthritis Rheum.* 56: 509–520.

29. Bannenberg, G. L., N. Chiang, A. Ariel, M. Arita, E. Tjonahen, K. H. Gotlinger, S. Hong, and C. N. Serhan. 2005. Molecular circuits of resolution: formation and actions of resolvins and protectins. *J. Immunol.* 174: 4345–55.

30. Fredman, G., Y. Li, J. Dalli, N. Chiang, and C. N. Serhan. 2012. Self-Limited versus Delayed Resolution of Acute Inflammation: Temporal Regulation of Pro-Resolving Mediators and MicroRNA. *Sci. Rep.* 2: 639.

31. Bystrom, J., I. Evans, J. Newson, M. Stables, I. Toor, N. van Rooijen, M. Crawford, P. Colville-Nash, S. Farrow, and D. W. Gilroy. 2008. Resolution-phase macrophages possess a unique inflammatory phenotype that is controlled by cAMP. *Blood* 112: 4117–27.

32. Prame Kumar, K., A. J. Nicholls, and C. H. Y. Wong. 2018. Partners in crime: neutrophils and monocytes/macrophages in inflammation and disease. *Cell Tissue Res.* 371: 551–565.

33. Marshall, J., E. Krump, T. Lindsay, G. Downey, D. A. Ford, P. Zhu, P. Walker, and B. Rubin. 2000. Involvement of cytosolic phospholipase A2 and secretory phospholipase A2 in arachidonic acid release from human neutrophils. *J. Immunol.* 164: 2084–91.

34. Schlager, S., M. Goeritzer, K. Jandl, R. Frei, N. Vujic, D. Kolb, H. Strohmaier, J. Dorow, T. O. Eichmann, A. Rosenberger, A. Wolfler, A. Lass, E. E. Kershaw, U. Ceglarek, A. Dichlberger, A. Heinemann, and D. Kratky. 2015. Adipose triglyceride lipase acts on neutrophil lipid droplets to regulate substrate availability for lipid mediator synthesis. *J. Leukoc. Biol.* 98: 837–850.

35. Chen, M., B. K. Lam, Y. Kanaoka, P. A. Nigrovic, L. P. Audoly, K. F. Austen, and D. M. Lee. 2006. Neutrophil-derived leukotriene B_4 is required for inflammatory arthritis. *J. Exp. Med.* 203: 837–842.

36. Guijas, C., A. M. Astudillo, L. Gil-de-Gómez, J. M. Rubio, M. A. Balboa, and J. Balsinde. 2012. Phospholipid sources for adrenic acid mobilization in RAW 264.7 macrophages. Comparison with arachidonic acid. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* 1821: 1386–1393.

37. Binstadt, B. A., P. R. Patel, H. Alencar, P. A. Nigrovic, D. M. Lee, U. Mahmood, R. Weissleder, D. Mathis, and C. Benoist. 2006. Particularities of the vasculature can promote the organ specificity of autoimmune attack. *Nat. Immunol.* 7: 284–292.

38. Maccioni, M., G. Zeder-Lutz, H. Huang, C. Ebel, P. Gerber, J. Hergueux, P. Marchal, V. Duchatelle, C. Degott, M. van Regenmortel, C. Benoist, and D. Mathis. 2002. Arthritogenic monoclonal antibodies from K/BxN mice. *J. Exp. Med.* 195: 1071–7.

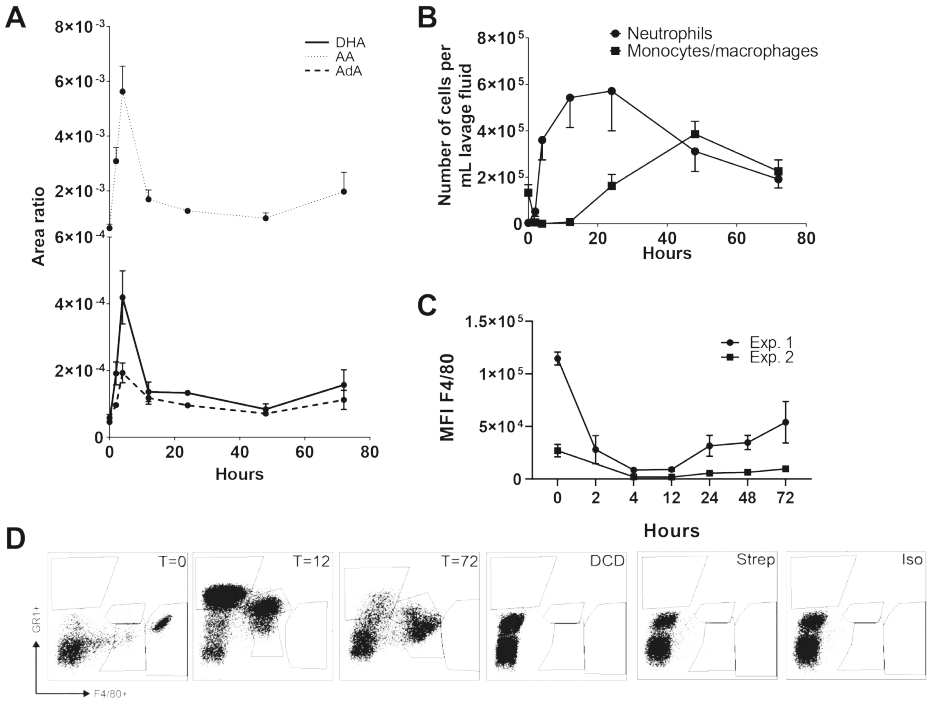
39. Newson, J., M. P. Motwani, A. C. Kendall, A. Nicolaou, G. G. Muccioli, M. Alhouayek, M. Bennett, R. Van De Merwe, S. James, R. P. H. De Maeyer, and D. W. Gilroy. 2017. Inflammatory Resolution Triggers a Prolonged Phase of Immune Suppression through COX-1/mPGES-1-Derived Prostaglandin E 2. *Cell Rep.* 20: 3162–3175.

40. Sperling, R. I., A. I. Benincaso, C. T. Knoell, J. K. Larkin, K. F. Austen, and D. R. Robinson. 1993. Dietary omega-3 polyunsaturated fatty acids inhibit phosphoinositide formation and chemotaxis in neutrophils. *J. Clin. Invest.* 91: 651–660.

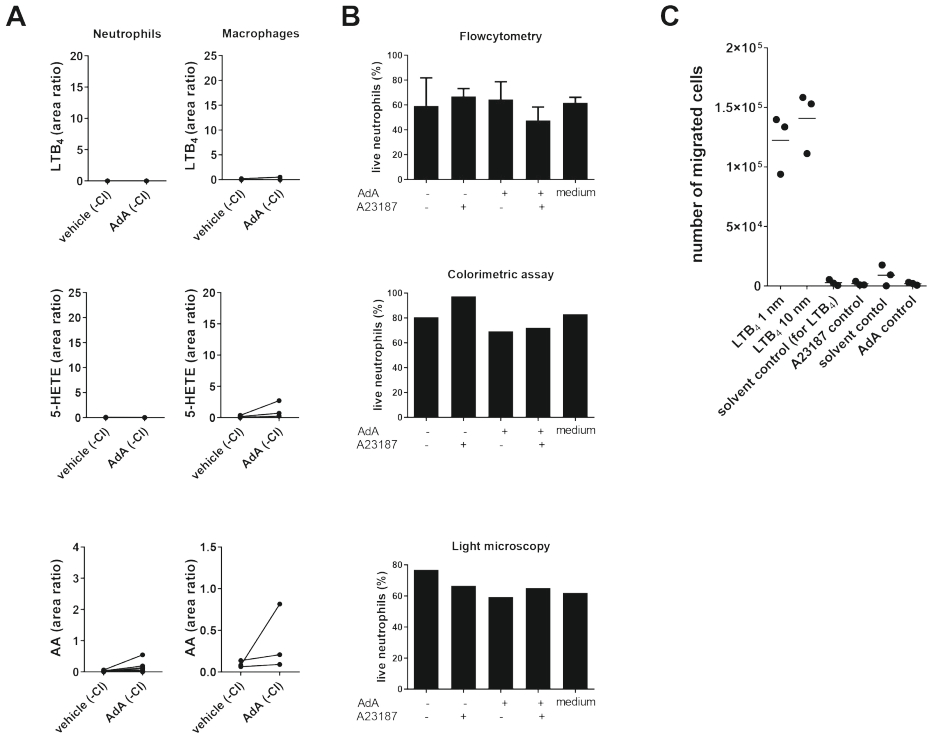
41. Lee, T. H., R. L. Hoover, J. D. Williams, R. I. Sperling, J. Ravalese, B. W. Spur, D. R. Robinson, E. J. Corey, R. A. Lewis, and K. F. Austen. 1985. Effect of Dietary Enrichment with Eicosapentaenoic and Docosahexaenoic Acids on in Vitro Neutrophil and Monocyte Leukotriene Generation and Neutrophil Function. *N. Engl. J. Med.* 312: 1217–1224.

42. Jónasdóttir, H. S., H. Brouwers, J. C. Kwekkeboom, H. M. J. van der Linden, T. Huizinga, M. Kloppenburg, R. E. M. Toes, M. Giera, and A. Ioan-Facsinay. 2017. Targeted lipidomics reveals activation of resolution pathways in knee osteoarthritis in humans. *Osteoarthr. Cartil.* 25: 1150–1160.

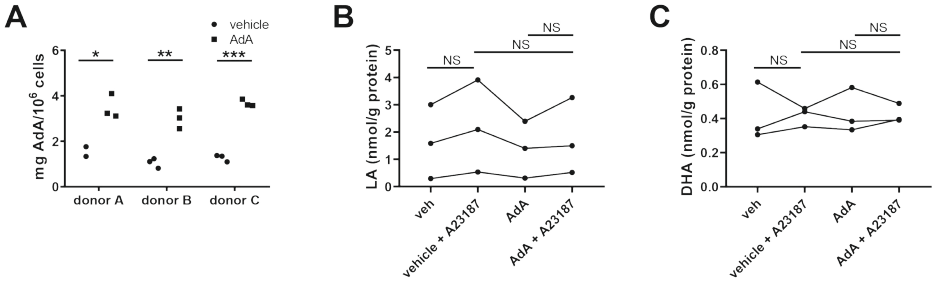
Supplemental figures



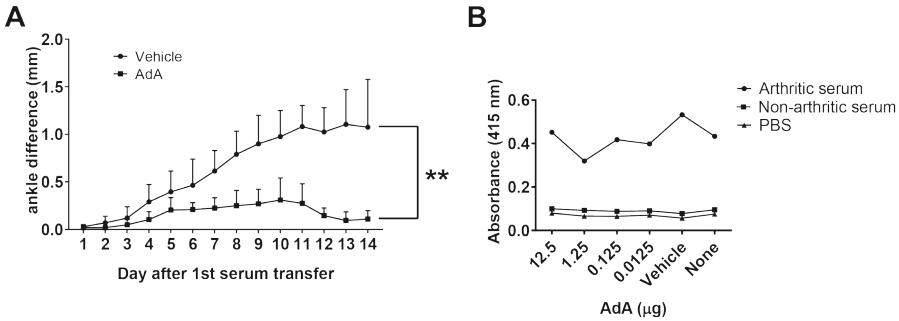
Supplemental Figure 1. Zymosan A peritonitis controls. Mice were administered zymosan and peritoneal lavage was collected at different timepoints. **(A)** Cell free peritoneal lavage fluid was collected and free AA, AdA and DHA were measured using GC-MS. Mean and SEM are shown, n=3 mice per time point. One experiment is depicted. **(B)** Cell numbers are shown for one representative experiment. Mean and SEM are shown, n=3 mice per time point **(C)** F4/80 MFI on the monocyte/macrophage fraction is shown for two independent experiments. Mean and SEM are shown, n=3-8 mice per time point. **(D)** Representative flowcytometry plots of timepoints 0, 12 and 72 hours of experiment 2 depicted in supplementary figure 1C. DCD (dead cell control) shows Dapi positive cells alone, Strep (streptavidin control) is the "secondary antibody only" control for the F4/80 staining and Iso (isotype control) shows isotype background for F4/80-biotin and GR1-FITC. In supplementary figure 1C, the mean MFI of the three F4/80 positive gates is shown.



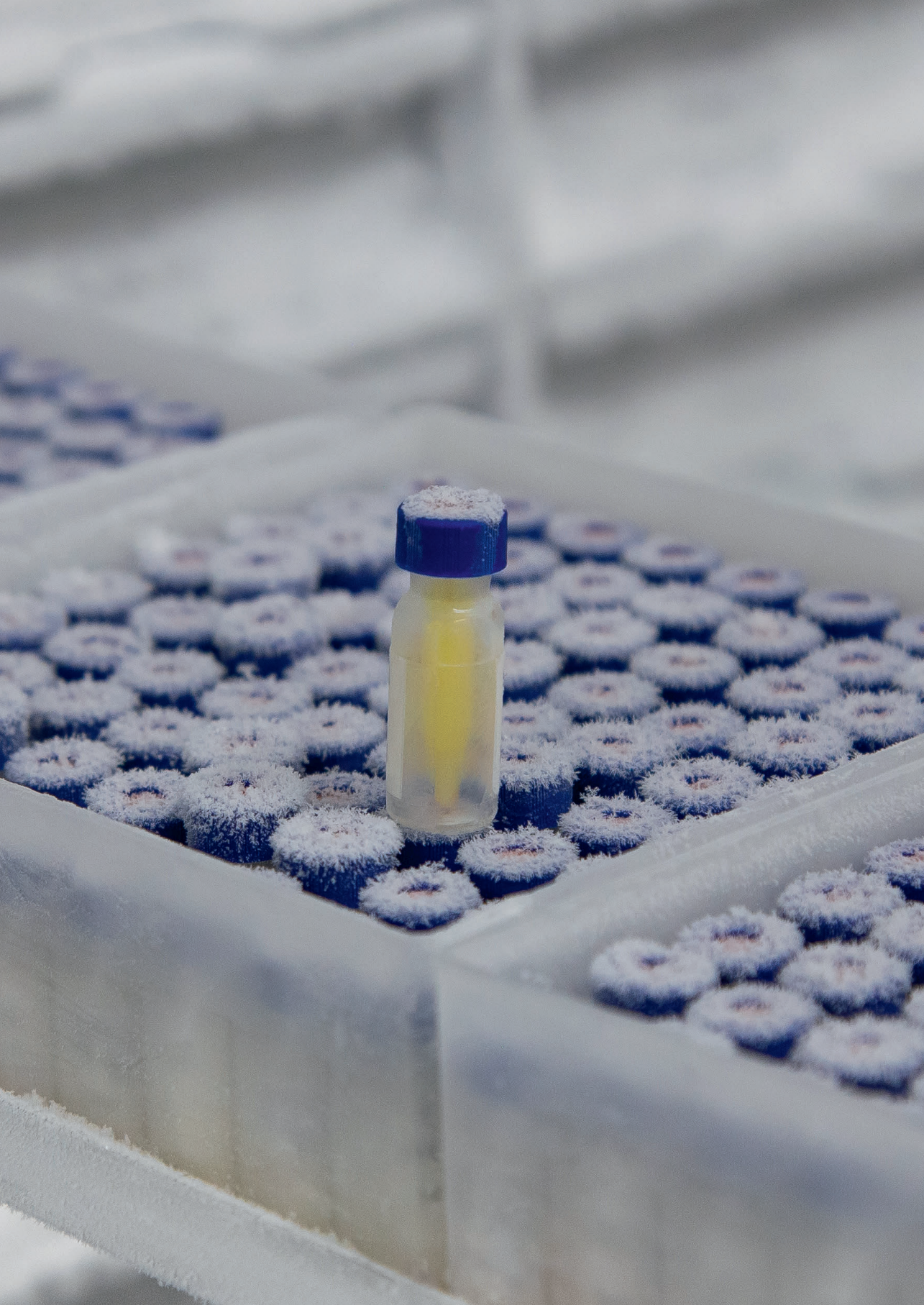
Supplemental Figure 2. Cell assay controls. **(A)** Peripheral blood neutrophils and macrophages were pre-incubated for 10 minutes or 24 hours with AdA, respectively, before stimulation with vehicle (1% EtOH) for 10 minutes. Total free LTB_4, 5-HETE and AA levels relative to internal standard were calculated based on LC-MS/MS signals (see "materials and methods"). Each line represents one donor, n = 3-8 donors all individual experiments. **(B)** Neutrophils were incubated with AdA for 10 minutes before incubation with calcium ionophore A23187 for 10 minutes. Percentage of dead cells was determined by flow cytometry using 1 μg/mL propidium iodide (Molecular Probes) (n=4, SD and mean are shown). Additionally, the CellTiter 96 Aqueous One Solution Cell proliferation Assay (Promega) was used to quantify viable cells, according to the manufacturer's protocol (colorimetric assay, n=1). Alternatively, cells were counted in the presence of 0.2% Trypan Blue (Sigma-Aldrich) (light microscopy, n=1). **(C)** Neutrophil migration towards LTB_4, AdA, calcium ionophore A23187 or vehicle controls in 90 minutes. The means of triplicates of three donors are shown. n=3 experiments.



Supplemental Figure 3. Internalization of AdA in macrophages and free fatty acid levels of Linoleic acid and Docosahexaenoic in neutrophils. **(A)** Macrophages were incubated with AdA for 24 hours. Levels of cellular AdA were quantified after hydrolysis, using GC-MS. Student t-test was performed, *, P<0.05; **, P<0.01; ***, P<0.001. **(B,C)** Neutrophils were incubated with AdA or vehicle for 10 minutes. Subsequently, they were stimulated with calcium ionophore A23187 or vehicle for 10 minutes. Next, the cells were subjected to DMS-MS/MS using the Lipidyzer™ platform. Cellular LA levels of free fatty acid classes are shown for LA *(**B**) and DHA (**C**)* Each line represents one donor, n =4 in four independent experiments. Matched Two-way ANOVA was performed comparing the same conditions as for figure 5.



Supplemental Figure 4. Adrenic acid effects *in vivo*. **(A)** Ankle difference shows the Δ ankle thickness compared to baseline as an average from both ankles. Mice were administered AdA from day -3 till the end of the experiment. Mean and SD are shown, *n*=5 mice per group of two independent experiments. Statistical significance was established using a repeated measures ANOVA as a mixed model, *, P<0.05; **, P<0.01; ***, P<0.001. **(B)** Different amounts of AdA were diluted in serum of either arthritic or non-arthritic mice and levels of anti-G6PI antibodies were determined by ELISA. Mean is represented of samples run in duplicate.



6

Effects of anticoagulants and storage conditions on clinical oxylipid levels in human plasma

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Abstract

Metabolomics and lipidomics are of fundamental importance to personalized healthcare. Particularly the analysis of bioactive lipids is of relevance to a better understanding of various diseases. Within clinical routines, blood derived samples are widely used for diagnostic and research purposes. Hence, standardized and validated procedures for blood collection and storage are mandatory, in order to guarantee sample integrity and relevant study outcomes. We here investigated different plasma storage conditions and their effect on plasma fatty acid and oxylipid levels. Our data clearly indicate the importance of storage conditions for plasma lipidomic analysis. Storage at very low temperature (–80 °C) and the addition of methanol directly after sampling are the most important measures to avoid *ex vivo* synthesis of oxylipids. Furthermore, we identified critical analytes being affected under certain storage conditions. Finally, we carried out chiral analysis and found possible residual enzymatic activity to be one of the contributors to the *ex vivo* formation of oxylipids even at –20 °C.

Keywords: LC-MS/MS, Lipidomics, Storage, Oxylipids, Stability, Plasma

Introduction

Clinical metabolomics and lipidomics stand at the basis of personalized health care as they allow for a detailed molecular assessment of individual metabolite patterns and concentrations. Due to the fact that blood is one of the most widely used biofluids in clinical studies, it is not surprising that it has been used extensively in clinical metabolomics and lipidomics studies (1, 2). A specific sub-field of lipidomics analysis, which is at present rapidly moving towards the clinic, is the analysis of oxylipids, including bioactive lipid mediators (LM) (3, 4). Although several interesting studies in this field have already been published, a surprisingly limited number of investigations has yet studied the influence of sample handling and storage on study outcomes (5). Plasma, collected using different anticoagulants, and serum are the most often used blood components for lipidomics and metabolomics studies. As these blood products are often stored for different lengths of time before analyses, it is highly relevant to identify the proper storage conditions to avoid storage artifacts, i.e. *ex vivo* modification or formation of lipids. Other pitfalls and challenges in lipidomic research are the use of different anti-oxidants, the intrinsic problem of radical-induced lipid autoxidation (6), as well as storage stability (7). All these factors should be systematically studied in order to guarantee high quality data and allow for correct biological interpretation of the results.

Oxylipid analysis is usually carried out using liquid chromatography coupled to tandem mass spectrometry with electrospray ionization (LC-ESI-MS/MS). Matrix effects in LC-ESI-MS/MS are known to affect quantitation of analytes in plasma (8, 9) and different anticoagulants can change the physical properties of the plasma and thus affect the matrix effect. Despite the inevitable need for storage of samples in clinical studies, as of yet there is no general consensus on which anticoagulant is best suited for oxylipid analysis. Moreover, to our knowledge, only one study has addressed the effect of anticoagulants on plasma phospholipids and triglycerides so far (10).

Oxylipids are, chemically speaking, oxidized products of polyunsaturated fatty acids (PUFAs) which contain one or several oxygen functionalities when compared to their PUFA precursor. The molecular oxygen insertion can occur either via enzymatic or non-enzymatic pathways. The non-enzymatic pathways, also referred to as autoxidation and peroxidation due to radical reactions, are suspected to take place *in vivo* in many diseases (11). However, these processes are undesirable *ex vivo,* as they are the main contributors to storage artifacts. Several antioxidants such as vitamins A and E, or butylated hydroxytoluene (BHT) (12) are commonly used to prevent *ex vivo* oxidation and to maintain sample integrity. Their usage includes the coating of blood collection containers (7) and/or addition to the samples (13). It has been known for more than 25

years of research that *ex vivo* radical reactions and peroxidation of PUFAs are of big concern when samples are stored at elevated temperatures, or even at -20 °C (11, 14). Despite that, no dedicated studies have been performed on how such parameters affect oxylipids. The possibility of oxygen insertion via residual enzymatic activity should in this respect also be considered. It is furthermore of crucial importance to assess which timeframe between sample collection/preparation and freezing is considered acceptable, since daily clinical routine samples are usually not processed immediately.

All of the above-mentioned points were addressed in the present study, in which the effects of different storage temperatures, storage time, sample preparation as well as the use of two of the most widely used anticoagulants (15) on levels of oxylipids and PUFAs in human plasma samples were determined. The anticoagulants compared in this study are ethylenediaminetetraacetic acid (K_2EDTA, from here on referred to as EDTA) and sodium heparin (from here on referred to as heparin). We believe that our study is of fundamental importance for the further integration of oxylipid analysis in clinical research as it lays the fundament for proper sample handling and storage, which is critical for the generation of consistent clinical data.

Materials and Methods

Chemicals and materials

K_2EDTA (EDTA) and sodium heparin vacutainers were from BD (Mississauga, ON, Canada)). LC-MS grade methanol (MeOH), glacial acetic acid *pro analysi* (*p.a.),* and LC-MS grade water, were from Sigma Aldrich (Schnelldorf, Germany). Ethanol *p.a.* was from Merck (Darmstadt, Germany). ACN and were from Sigma Aldrich BHT. All substances used as standards were from Cayman Chemicals (Ann Arbor, MI, USA), except 17-hydroxydocosatetraenoic acid (17-HDoTE) which was made in-house *(see Production of 17-HDoTE below).* Sample tubes (1.5 mL) were from Eppendorf (Hamburg, Germany). Autosampler vials, caps and inserts were from Agilent Technologies (Waldbronn, Germany). The internal standard (IS) solution used for oxylipid analysis consisted of LTB_4-d4, 15(*S*)-HETE-d8, PGE_2-d4 50 ng/mL each and 500 ng/mL DHA-d5 in MeOH. The IS solution was prepared beforehand in sufficient amount, aliquotted and the aliquots were stored at -80°C and used for this purpose alone.

Preparation of plasma samples and storage

Blood was collected from 4 healthy, non-fasting volunteers, 2 females and 2 males upon written informed consent. The study was approved by the medical ethical committee of the LUMC. Two EDTA and 2 heparin tubes of blood from each volunteer were collected. The blood was spun at 2100 ×g in order to obtain platelet-poor plasma. Subsequently

a heparin and EDTA plasma pool were prepared and the plasma divided into 200 µL aliquots.

After aliquotting, samples were treated in one of four different ways. 1) Proteins were precipitated with 3-fold volume MeOH w/ 40.8 µg/mL BHT. 2) 4 µL EtOH w/ 15 mg/mL BHT was added. 3) Proteins were precipitated with 3-fold volume MeOH. 4) no additives. After treatment, argon was blown over the samples before storing the aliquots at 4 different storage temperatures, for different amounts of time, see figure 1, resulting in 134 samples.

A baseline for each anticoagulant was established by working-up samples immediately after the aliquotting.

Targeted lipidomics

Targeted lipidomics analysis of the plasma was carried as described previously (3) with some modifications: To plasma aliquots that were stored without prior protein precipitation, 600 µL of MeOH was added, 5.4 µL IS was added to all samples and after vortexing, samples were stored at -20°C for 20 min. The samples were then centrifuged at 4°C for 10 min at 16,100 ×g. From each sample 270 µL MeOH extract was transferred to two glass autosampler vials and dried under a gentle stream of N_2 before reconstituting with 36 µL MeOH, vortexing and sonicating and adding 54 µL H_2O, giving two technical replicates for each sample. After reconstitution, the samples were placed in the autosampler at 6°C for direct analysis.

Liquid chromatography combined with mass spectrometry (LC-MS/MS) analysis was carried out as previously published (3) with some modifications. Briefly: A QTrap 6500 mass spectrometer was used in MRM mode in negative ESI mode (Sciex, Nieuwerkerk aan den Ijssel, The Netherlands), coupled to a LC system employing two LC-30AD pumps, a SIL-30AC autosampler, and a CTO-20AC column oven. (Shimadzu, 's-Hertogenbosch, The Netherlands). The employed column was a Kinetex C_{18} 50 × 2.1 mm, 1.7 μm, protected with a C8 precolumn (Phenomenex, Utrecht, The Netherlands), kept at 50 °C. The following binary gradient of water (A) and MeOH (B) with 0.01% acetic acid was used: 0 min 30% B, held for 1 min, then ramped to 45% at 1.1 min, to 53.5% at 2 min, to 55.5% at 4 min, to 90% at 7 min, and to 100% B at 7.1 min, held for 1.9 min. The injection volume was 40 μL and the flow rate 400 μL/min. The MS was operated under the same conditions as in (3). In addition to the mass transition used for each analyte (see Supplementary Table 1), relative retention times (RRTs) were used for identification. For quantification at baseline, calibration lines, made with standard material for each analyte (see Supplementary Table 1 for range), were used and only peaks with a signal to noise

(S/N) > 10 were quantified. For analytes where no calibration line was used, area ratios were used and S/N > 3 was used as a detection limit. The LC-MS/MS method used does not discriminate between alpha-linolenic acid (ALA) and gamma-linolenic acid (GLA), and therefore the detected fatty acid(s) is listed as ALA/GLA.

Chiral analysis

Chiral analysis was carried out after initial fractionation on our RPLC platform. 300 µL of MeOH extract was dried down, reconstituted in 44 µL of MeOH, vortexed and 66 µL of water added. This sample was then injected on the RP C-18 platform and fractions collected, 6.5–7.5 min for LM analysis and 7.5–8.5 min for HETE analysis. Fractions were dried down under a flow of N2, LM fractions were reconstituted in 28 µL MeOH and 42 µL water and HETE fractions in 40 µL MeOH and 60 µL water. The chiral LC-MS/MS analysis was carried out with the same Shimadzu LC system as the RP analysis, injecting 40 µL on a Chiralpak ® AD-RH (Daicel, Tokyo, Japan) at room temperature with water (A) and ACN (B) with 0.01% acetic acid at 200 µL/min. For HETE analysis an isocratic run with 70%B for 10 min was used. For LM analysis the following gradient was used: 60%B kept constant for 4 min and then linearly increased to 70% at 10 min, kept constant for another 2.5 min.

Data analysis

Peaks were integrated with manual supervision using MultiQuant™ software (Sciex, MA, USA).

Results

Oxylipid analysis was carried out using LC-ESI-MS/MS according to published protocols (3, 16). In order to prevent any possible influence of an extended sample preparation procedure we opted for protein precipitation using MeOH. For every condition an aliquot of pooled plasma obtained from four healthy individuals (two female, two male) was analyzed in technical duplicate. Corrected areas for all analytes in all samples can be found in Supplementary Figures S2-S61. The effects of the following key parameters were studied: i) the anticoagulant, EDTA and heparin; ii) plasma storage temperature, including short-term storage at room temperature and refrigerated; iii) the addition of either the anti-oxidant BHT or immediate protein precipitation using MeOH; and iv) the combined effect of BHT, MeOH and storage temperature. A detailed study design is shown in Figure 1.

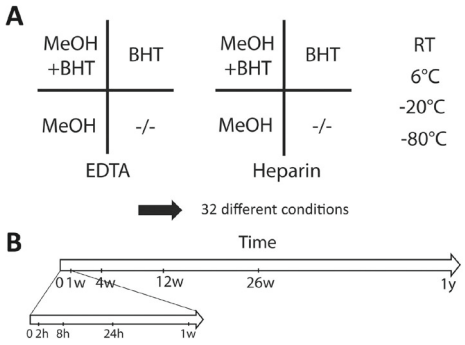

Figure 1. Study design. Blood was collected into both EDTA and heparin tubes and plasma was immediately obtained by centrifugation. **A:** All EDTA and heparin samples were pooled and aliquoted. The aliquots were then split in four groups, MeOH was either added or not, and BHT either added or not (for detailed information see *Materials and Methods*) giving 8 different conditions. These aliquots were then stored at 4 different storage temperatures, resulting in 32 different storage conditions. Abbreviations: BHT butylated hydroxytoluene**,** RT room temperature **B:** In addition to a baseline measurement, samples from the different storage conditions in **A** were analyzed at different time points, spanning from two hours after preparation of the plasma for short-term storage at room temperature and 6°C to long-term storage for one year in -20°C and -80°C freezers.

Effect of anticoagulants at baseline.

Baseline levels of oxylipids and PUFAs (see concentrations in Supplementary Table 1) in both EDTA and heparin plasma directly precipitated with MeOH were determined first. The ratio of EDTA/heparin (see Figure 2) was determined for all analytes detected at baseline. This can also be referred to as a nominal accuracy determination with the values obtained for heparin set to 1.0 (100%). As can be observed in Figure 2A, most of the ratios for PUFAs were between 0.6 and 1.3. Similarly, most ratios for the monohydroxylated fatty acids derived from AA, EPA, DHA, LA and ALA were between 0.7 and 1.2 (Figure 2B and C), except for 5-HETE (ratio 0.3), and 12-HETE (ratio 0.1). Moreover, three other oxylipids, TXB_2 (ratio 0.2) and LTB_4 and LTE_4 (ratio 0.0) were much higher/only present in the heparin samples compared to the EDTA samples (Figure 2D).

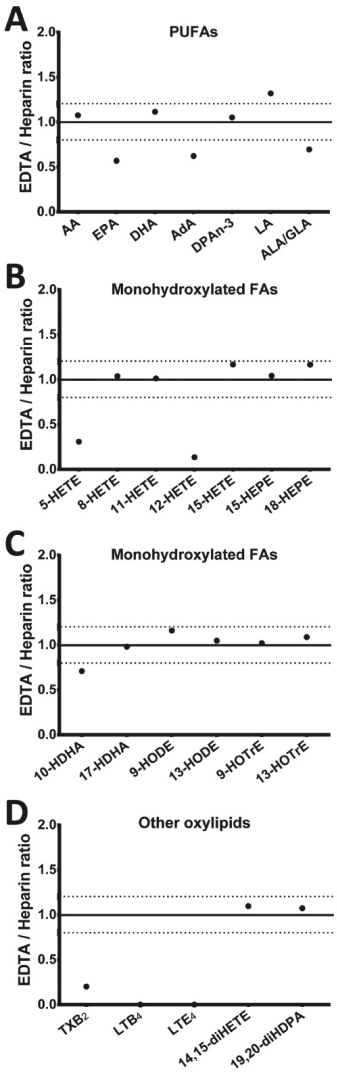

Figure 2. Comparison of lipid analytes in EDTA and heparin plasma at baseline. Heparin and EDTA were added to separate aliquots. The indicated ratios result from dividing the concentration of each analyte in the EDTA sample with the one in the heparin sample. For each analyte the ratio of the average (of technical duplicates) corrected area (to internal standard) from EDTA plasma to the heparin plasma samples is depicted for **A:** PUFAs; **B:** monohydroxylated FA derived from AA and EPA or from **C:** DHA, LA and ALA; and **D:** other oxylipids**.** The dotted line shows the range 0.8-1.2. Abbreviations: AA arachidonic acid, EPA eicosapentaenoic acid, DHA docosahexaenoic acid, AdA adrenic acid, DPA_{n-3} omega-3 docosapentaenoic acid, LA linoleic acid, ALA alpha-linolenic acid, GLA gamma-linolenic acid, HETE hydroxyeicosatetraenoic acid, HEPE hydroxyeicosapentaenoic acid, HDHA hydroxydocosahexaenoic acid, HODE hydroxyoctadecadienoic acid, HOTrE hydroxyoctadecatrienoic acid, TXB_2 thromboxane B2, LTB_4 leukotriene B4, diHETE dihydroxyeicosatetraenoic acid, and diHDPA dihydroxydocosapentaenoic acid.

To confirm that certain eicosanoids and leukotrienes are indeed only detectable in heparin plasma, we repeated this analysis with blood from four (two female, two male) additional volunteers > 1.5 years after the initial measurements (see Supplementary Figure S1). The new measurements confirmed our initial results, indicating that LTB_4, TXB_2, 5-HETE and 12-HETE measurements are particularly sensitive to the anti-coagulant used for samples collection. Our results clearly show that the choice of anticoagulant can significantly affect the reported concentrations of some oxylipids.

Short- and long-term storage

Next, we investigated the effect of short- and long-term storage under different conditions.

After blood collection and plasma preparation, samples are often kept at room temperature or refrigerated for a limited amount of time. To assess for how long samples can be kept at room temperature or refrigerated without changes in measured analyte concentrations, we established oxylipid and PUFA levels after two or eight hours at room temperature and after two, eight, or 24 hours at 6 °C. In addition, levels were established after both 24 hours and one week at -20 °C (see Supplementary Figures S2-S27).

Moreover, since large cohorts are needed in clinical research, samples are sometimes collected over a long period of time and measured at the study endpoint. To assess the effect of long term storage, samples were kept at -20 °C or -80 °C for four weeks, 12 weeks, 26 weeks, or one year before oxylipid and PUFA measurement (see Supplementary Figures S28-S61). The effects of BHT, a known commonly-used anti-oxidant, and of immediate MeOH precipitation on the analyte measurements were assessed. The samples without addition of BHT precipitated with MeOH at baseline were used as reference.

Short-term storage at room temperature

After two hours, no clear differences were observed for any of the PUFAs in any storage condition (Fig.3 and Supplementary Figures S2-S8). However, an increase in levels could be observed for some PUFAs after eight hours compared to baseline. This effect was independent of MeOH precipitation and of the presence of BHT in the EDTA samples (Fig.3A). In the heparin plasma samples, this increase could be prevented by MeOH precipitation, but was independent of BHT-addition (Fig. 3B and Supplementary Figures S2-S8).

Similarly, little differences were observed for monohydroxylated FAs between the different sample treatment conditions (for both anticoagulants) except for 12-HETE in EDTA plasma w/ BHT after a two-hour storage period (Figure 3 and Supplementary Figures

S9-S22). However, a significant increase compared to baseline could be observed after eight hours for several monohydroxylated FAs and this increase was prevented by MeOH precipitation for both anticoagulants (Figure 3). Interestingly, in the case of 12-HETE the addition of BHT significantly affected the measured signal in the unprecipitated samples particularly in EDTA plasma (Figure 3A and Supplementary Figure S12).

For the CYP450 products 14,15-diHETE and 19,20-diHDPA, no clear increase compared to baseline was observed over a period of eight hours, as well as no difference between the storage conditions (Figure 3 and Supplementary Figures S23 and S24). For TXB_2, a significant increase was only observed in the EDTA plasma without MeOH precipitation, especially after 8 h of storage (Figure 3A and Supplementary Figure 25). Neither a change over time nor differences between storage conditions were observed for LTB_4, while for LTE_4 a clear increase was observed in heparin plasma samples without MeOH (Figure 3B and Supplementary Figures S26 and S27).

Short-term storage at 6 °C

Samples kept at 6 °C were worked-up after two, eight, and 24 hours. An increase in PUFA levels was observed already after eight hours for EDTA plasma and after 24 hours for heparin plasma. This increase was prevented by MeOH precipitation only in the heparin plasma samples. Addition of BHT did not have a significant effect on PUFA levels (see Figure 4 and Supplementary Figures S3, S5, and S7).

Except for 12-HETE in EDTA plasma w/ BHT, no significant differences were observed between different storage conditions within the 24 hours storage period for most of the monohydroxylated FAs, the two CYP450 products, and LTB_4 (see Figure 4 and Supplementary Figures S5-S27). Similar to samples stored at room temperature, the measurement of 12-HETE seemed to be affected by BHT addition in the unprecipitated EDTA plasma samples. Moreover, 5-HETE showed accumulation over time in EDTA plasma but not in heparin plasma, and this increase was prevented by MeOH addition (Figure 4A and Suppl. Fig S9). LTE_4 levels in heparin plasma also increased over time and the accumulation was prevented by MeOH addition (Figure 4B and Supplementary Figure S26).

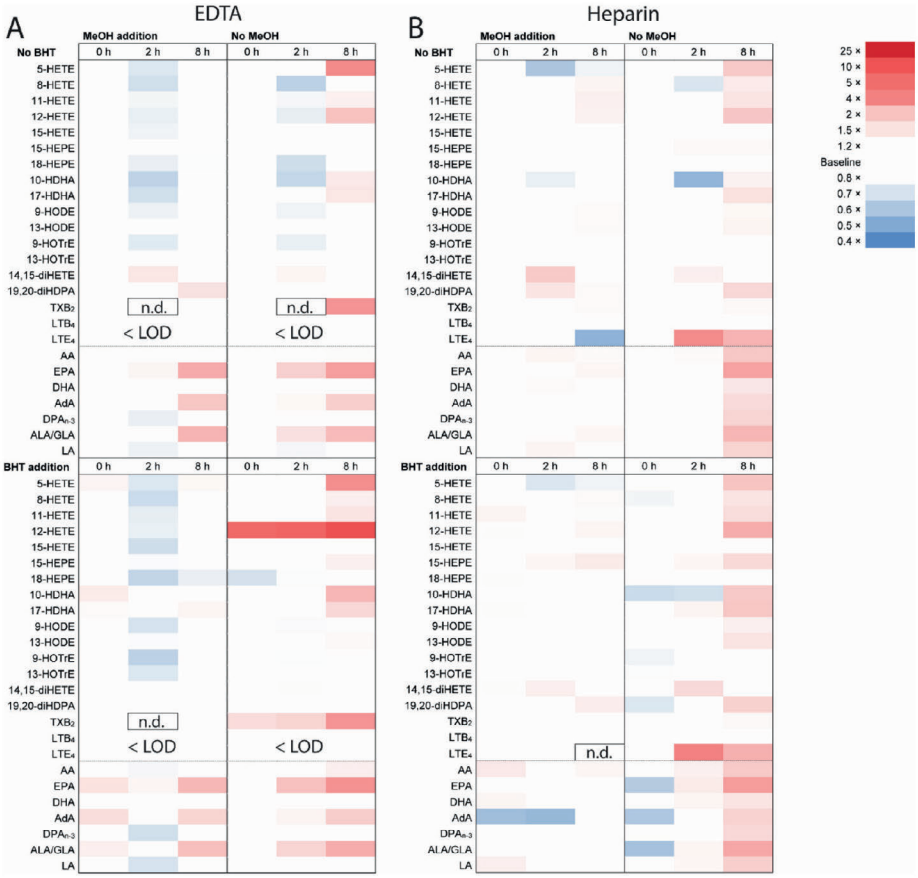

Figure 3. Short-term storage at room temperature. Heatmap showing the fold increase of the corrected area to baseline over time. **A**: EDTA plasma and **B**: heparin plasma. LTB_4 and LTE_4 were not detected in EDTA samples. Abbreviations: n.d. not determined, LOD limit of detection.

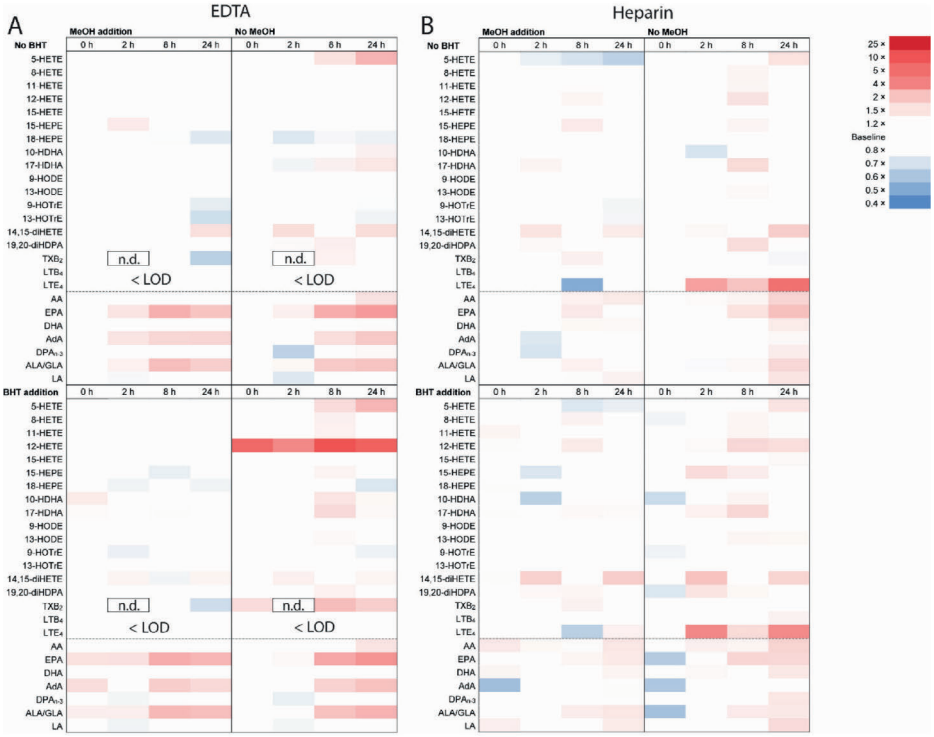

Figure 4. Short-term storage at 6 °C. Heatmap showing the fold increase of the corrected area to baseline over time. **A**: EDTA plasma and **B**: heparin plasma. LTB_4 and LTE_4 were not detected in EDTA samples. Abbreviations: n.d. not determined, LOD limit of detection.

Short-term storage at -20 °C

Samples kept short-term at -20 °C were worked-up after 24 hours and one week. In contrast to room temperature and 6°C conditions, PUFA levels were only marginally affected in heparin plasma after 1 week, while they were already increased after 24 hours in the EDTA plasma. MeOH precipitation and BHT addition had little effect. (Figure 5 and Supplementary Figures S2-S8). Up to 1 week of storage at -20 °C resulted in small changes for most monohydroxylated FAs and CYP450 products in EDTA plasma and was prevented by addition of MeOH, while few changes were observed in heparin plasma (Figure 5 and Supplementary Figures S9-S27). A significant increase in 12-HETE and TXB_2 levels was observed in EDTA plasma and this was prevented by addition of MeOH (Figure 5A and Supplementary Figures S12 and S25. Note the possible outlier in Supplementary Figure S12, see *Discussion*). Similar to samples kept at higher temperatures, LTE_4 levels in heparin plasma had increased after 24 hour storage and addition of MeOH had little effect on this change (Figure 5B and Supplementary Figure S26).

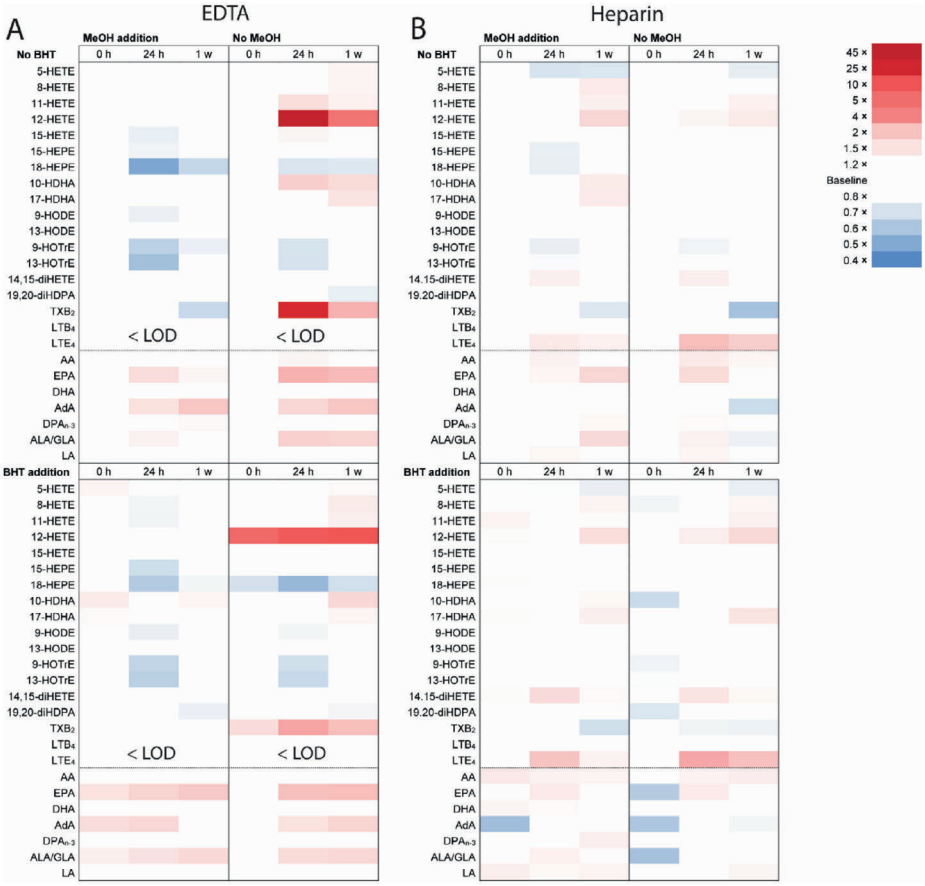

Figure 5. Short-term storage at -20 °C. A heatmap showing the fold increase of the corrected area to baseline over time. **A**: EDTA plasma and **B**: heparin plasma. LTB_4 and LTE_4 were not detected in EDTA samples. Abbreviations: LOD limit of detection. 12-HETE levels without additives in EDTA plasma, possible outlier, see Supplementary Fig S12.

Long-term storage at -80 °C

For PUFAs, only minor differences could be seen between storage conditions during long term storage at -80 °C (Supplementary Figures S28-S34). Compared to baseline, only EPA, AdA, and ALA/GLA levels increased over one year for all conditions (Figure 6 and Supplementary Figures S29, S31, and S33). The addition of MeOH had no effect in the EDTA samples, but seemed to slow down the increase of these PUFA levels when plasma was collected in heparin. For most of the oxylipids, small changes were observed over the time span of one year (Figure 6 and Supplementary Figures S35-S53) and these changes were slowed down by MeOH addition. The increase in 12-HETE and TXB_2 levels in EDTA plasma and LTE_4 in heparin plasma were the most pronounced and

were diminished by the addition of MeOH prior to storage (Figure 6 and Supplementary Figures S38, S51, and S52).

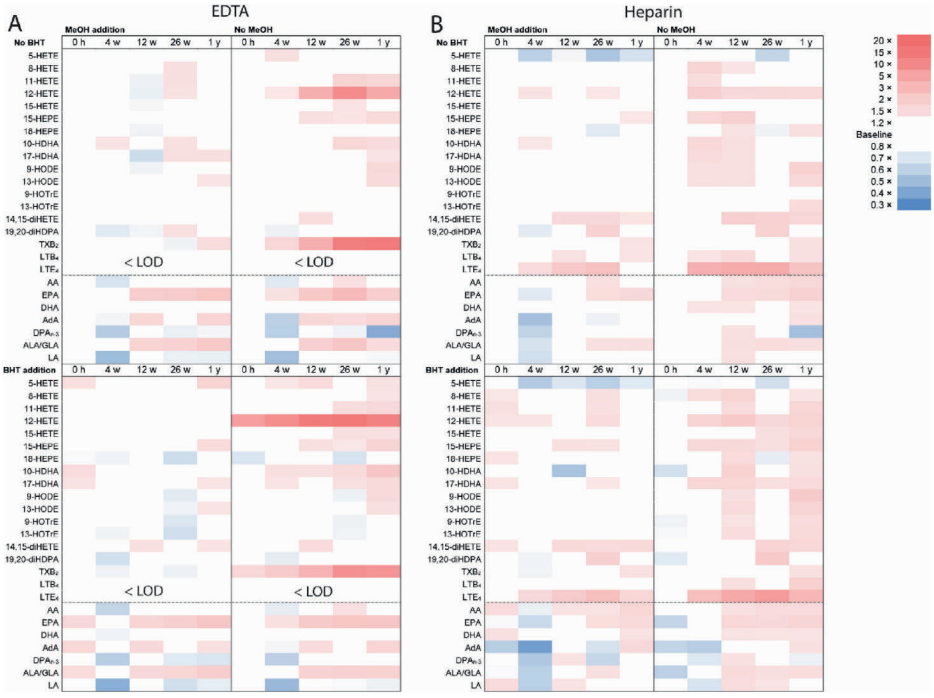

Figure 6. Long-term storage at -80 °C. Heatmap showing the fold increase of average corrected area to baseline over time. **A**: EDTA plasma and **B**: heparin plasma. LTB_4 and LTE_4 were not detected in EDTA samples. Abbreviations: LOD limit of detection.

Long term storage at -20 °C

During a one-year storage period, most PUFAs remained stable (Figure 7 and Supplementary Figure S34). Only for EPA, a significant increase was observed after 12 weeks in EDTA plasma and this was diminished by addition of MeOH(Supplementary Figure S29).

Without MeOH, levels of monohydroxylated FAs were significantly increased by week 4 in EDTA plasma and by week 26 in heparin plasma (Figure 7 and Supplementary Figures S35-S48) and this was diminished by MeOH additon. One year after plasma preparation a clear increase compared to basal levels could be observed for all monohydroxylated FAs even in the samples with MeOH, with the exception of the HODEs and HOTrEs (Figure 7 and Supplementary Figures S35-S48).

The levels of 14,15-diHETE and 19,20-diHDPA without MeOH addition (both anticoagulants) increased less than that of monohydroxylated FAs and the increase was diminished by MeOH addition. (Figure 7 and Supplementary Figures S49 and S50). Levels of TXB_2 were significantly increased only in EDTA plasma without MeOH by week 4 (Figure 7 and Supplementary Figure S51), while LTB_4 and LTE_4 levels were significantly increased in heparin plasma without MeOH by week 26 and week 4 respectively (Figure 7B and Supplementary Figures S52 and S53). As there was no LTB_4 detected at baseline in EDTA plasma, and the figure depicts ratio to basal levels, its fold-increase is not presented in Figure 7A. The addition of BHT did not have a significant effect on lipid levels.

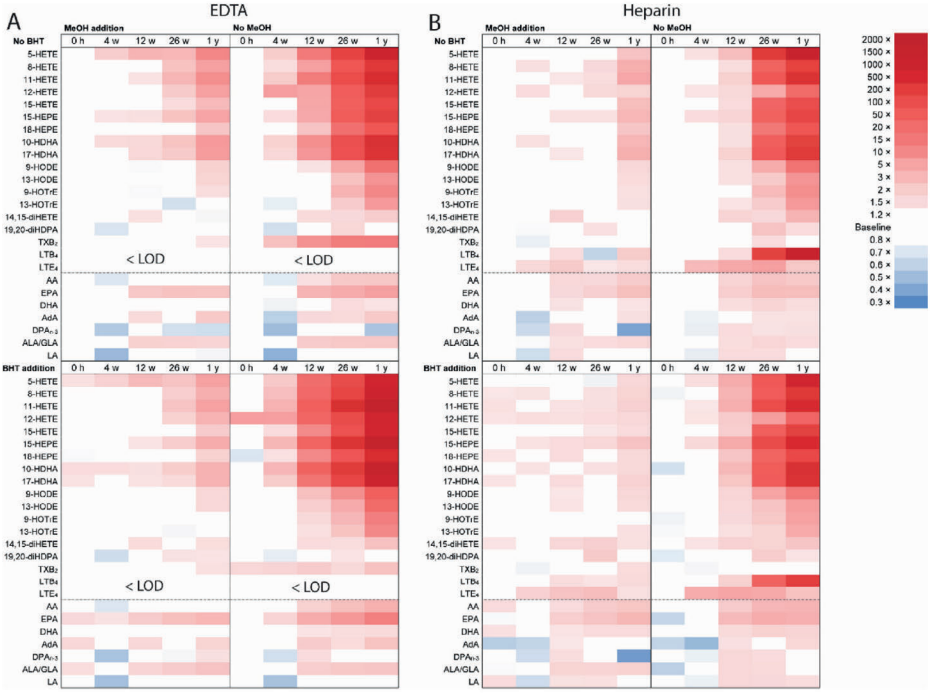

Figure 7. Long-term storage at -20 °C. Heatmap showing the fold increase of corrected area to baseline over time. **A:** EDTA plasma and **B:** heparin plasma. Note the increase in LTB_4 from week 12 is most likely not LTB_4 but the isomer 5*S*,12*S*-diHETE, for more detail see chapter *Appearances of new oxylipids over time*. Abbreviations: LOD limit of detection.

Appearances of new oxylipids over time

In addition to an increase in signal intensity for lipids already detectable at baseline, we also observed the appearance of some lipid species during storage. This was mostly the case in samples stored long-term without MeOH precipitation at -20 °C.

The non-enzymatic products of LTA_4, 6-trans-LTB_4 and 6-trans-12-epi-LTB_4, became detectable after 12 weeks in plasma from both anticoagulants (Supplementary Figures S54 and S55). A small increase in the LTB_4 area ratio in samples without MeOH compared to samples with MeOH, was observed in heparin plasma after 12 weeks, which could be a result of additional 5*S*,12*S*-diHETE that is not distinguishable from LTB_4 due to the very small differences in retention time, as previously identified by our lab (17, 18). After 26 weeks these levels in both EDTA and heparin plasma had increased significantly (Suppl. Figure S53) and the relative retention time (RRT) had clearly shifted towards the 5*S*,12*S*-diHETE value (data not shown). The hydroxylated product of AdA (17-HDoTE) was first detectable after four weeks of storage at -20 °C in plasma from both anticoagulants, and it increased further over time (Supplementary Figure S56). In addition, detectable levels of 7*S*,17*S*-diHDPA ($RvD5_{n\text{-}3\ DPA}$,), 5*S*,15*S*-diHETE, and 8*S*,15*S*-diHETE, were present when samples had been stored at -20°C without MeOH precipitation for 26 weeks (Supplementary Figures S57-S59).

Surprisingly, we also observed the appearance of features that had the characteristics of bioactive lipid mediators (LMs) such as MaR1, PD1, and PDX (10*S*,17*S*-diHDHA), starting at 26 weeks (Supplementary Figures S60 and S61). The obtained chromatographic signals closely matched the relative retention times of purified standard material (within the limit of 0.5% deviation), as well as showing the characteristic fragmentation (Figure 8 and 9). As this finding is highly relevant for the field of bioactive lipid research we further characterized these products. As it has been described that lipid mediators may be accompanied by multiple isomers that share their fragmentation pattern in MS/MS data acquisition, we used a chiral column to obtain orthogonal chromatographic separation. The results of the chiral analysis are discussed in the following section.

Chiral analysis of lipid mediators

Samples that had been kept at -20 °C for 74 weeks with or without MeOH were analyzed on both our standard RP C-18 oxylipid platform (Figures 8A and 9A) and on the chiral platform (Figures 8B and 9B) (see *Materials and Methods*). The signal referring to PDX falls within a window of no more than 0.5% RRT deviation on both chromatographic platforms, when compared to standard material (Figure 8). Therefore, we concluded that the signal appearing during long term storage refers to PDX. For both PD1 and MaR1, chiral analysis revealed that the signals obtained at correct RRT (C-18), presenting genuine MS/MS spectra for both components, do not correspond to the correct analytes for which bioactivity has been assigned.

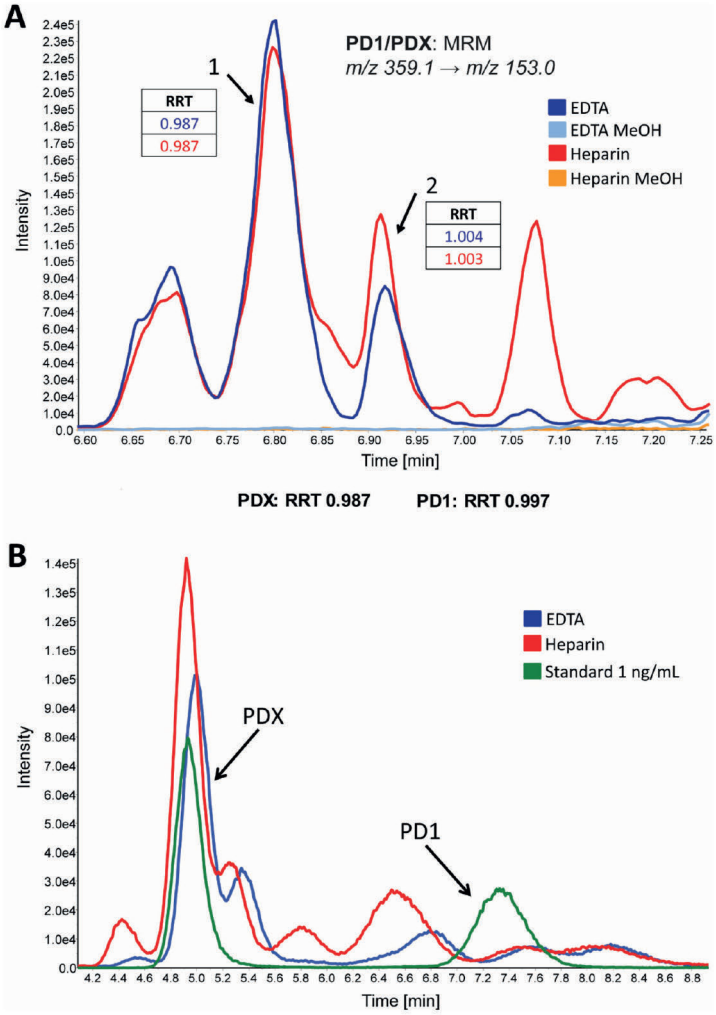

Figure 8. Identification of PD1/PDX (10*S*,17*S*-diHDHA) in samples stored at -20 °C for 74 weeks. **A**: MRM trace of PD1/PDX after separation on RPLC. RRT relative to IS LTB_4-d4 of standard material and unidentified peaks **1** and **2**. **B**: MRM trace of PD1/PDX after separation on chiral column. Dark blue, EDTA plasma without MeOH. Light blue EDTA plasma stored with MeOH, red heparin plasma stored without MeOH, orange heparin plasma stored with MeOH, green standard with 1 ng/mL each PD1 and PDX (For the interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

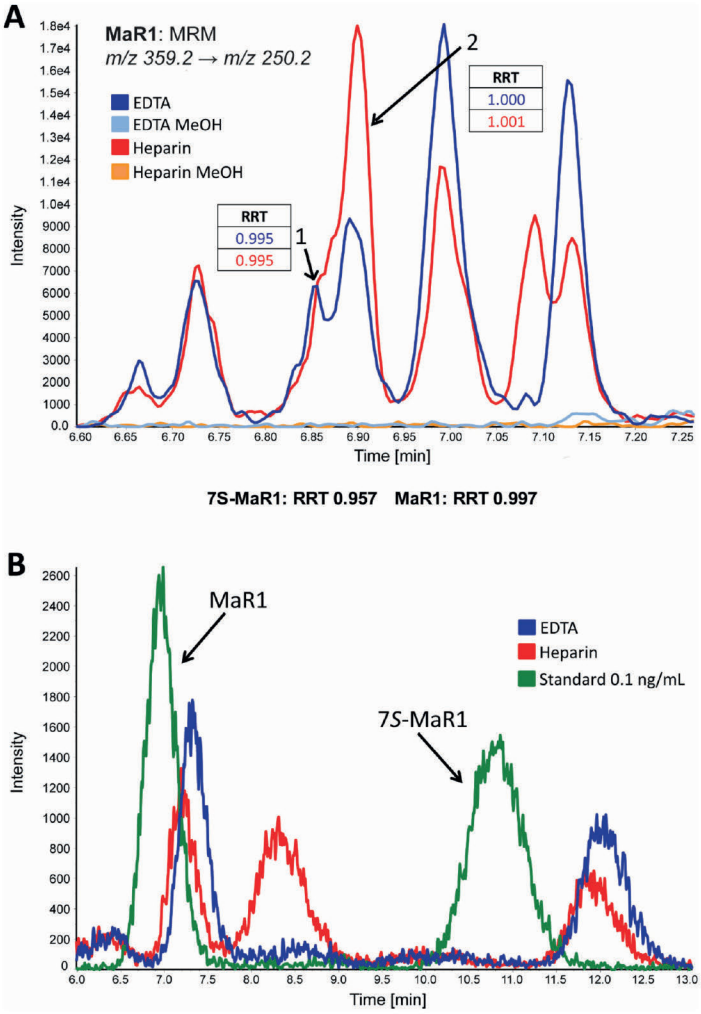

Figure 9. Identification of MaR1 in samples stored at -20 °C for 74 weeks. **A**: MRM trace of MaR1 after separation on RPLC. RRT relative to IS LTB_4-d4 of standard material and unidentified peaks **1** and **2 B**: MRM trace of MaR1 after separation on chiral column. RRT on column to IS LTB_4-d4 of standard material. Dark blue, EDTA plasma without MeOH, light blue EDTA plasma stored with MeOH, red heparin plasma stored without MeOH, orange heparin plasma stored with MeOH, green standard with 0.1 ng/mL each MaR1 and 7*S*-MaR1. (For the interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Chiral analysis of HETEs

In addition to the chiral analysis of lipid mediators, we also investigated the *S* and *R* enantiomers of monohydroxylated FAs over time starting at 12 weeks. Therefore, we analyzed 5-HETE, 12-HETE, and 15-HETE on the chiral platform in samples with or

without MeOH stored at -20 °C or -80 °C for up to 74 weeks. Peak areas of *S* isomers and *R* isomers were corrected to 15*S*-HETE-d8 and *S*/*R* ratios calculated

As can be seen in the supplementary material S62-S64 the levels of 5-HETE and 15-HETE at -80°C remained stable over the 74 weeks storage time. Particularly 12-HETE levels increased during storage at -80°C in EDTA plasma (Figure 6) and showed a high *S/R* ratio. As expected from the results in figure 7, much stronger effects were observed for samples store at -20°C. Particularly for 12-HETE a severe change in the *S/R* ratio occurred over time in the heparin samples. This was also visible for 15-HETE, albeit to a lower extent. In contrast, the *S/R* ratio increased for 5-HETE and this was prevented by the addition of MeOH. For further details please refer to the supplementary material.

Discussion

In this study, we assessed the effect of anti-coagulants, storage temperature and length as well as sample treatment on the levels of PUFAs and PUFA-derived oxylipids in plasma. Our data indicate that 1) EDTA and heparin differently affected the levels of specific lipids both at baseline and after storage, 2) less changes in lipid levels were observed upon storage at lower temperatures and 3) MeOH precipitation prevented changes in lipid levels at all temperatures, while BHT had little effect. Moreover, chiral analyses of mono-hydroxylated lipids suggests that some enzymes displayed a residual activity even at -20°C and this could be prevented by MeOH treatment before storage.

With respect to the employed anticoagulant, particularly platelet-derived oxylipids such as TXB_2 and 12-HETE, and the 5-LOX products 5-HETE, LTB_4 and LTE_4 proved highly affected. These lipids were all higher in heparin than in EDTA plasma. Higher concentration of 12-HETE and TxB_2 (platelet-derived oxylipids) could be explained by heparin activating residual platelets in the platelet-poor plasma used in this study (19), possibly due to re-suspension of the platelets after the centrifugation step. Similarly higher concentration of 5-HETE and the leukotrienes in heparin plasma could be due to residual platelets and possible re-suspension of leukocytes after the centrifugation (20-22) as well as to reduced AA release in EDTA plasma leading to lower residual 5-LOX AA products. In any case, it underlines the importance of careful handling of samples after centrifugation, and special caution should be applied to the interpretation of data obtained for these metabolites, upon long-term storage. Moreover, one must be cautious when comparing results from studies that have used different anticoagulants for plasma collection.

We have also tested the effect of storage temperature for different periods of time on the levels of different lipid classes. For all lipid classes, the levels were stable for longer periods of time at lower storage temperatures. The most affected lipids were the PUFAs EPA, AdA and ALA/GLA, and the oxylipids 5-HETE and 12-HETE, and TXB_2 and LTE_4. The increase in levels is likely due to (residual) enzymatic activity and/or non-enzymatic oxidation. In the case of PUFAs, most increase was observed in the EDTA samples and the increase was marginally affected by MeOH addition, indicating that this is predominantly a non-enzymatic process. In contrast, the increase in oxylipids was significantly reduced by addition of MeOH prior to storage, indicating the involvement of enzymatic processes in the observed changes in levels at all temperatures. To further investigate this, we have performed chiral analysis of the main 5-, 12- and 15-LOX products derived from AA: 5-HETE, 12-HETE and 15-HETE after long-term storage at -20°C and -80°C, as these are the most relevant storage conditions for clinical studies.

The least changes were observed in (oxy)lipid levels after storage at -80°C. Consequently, the effect of MeOH addition prior to storage at -80°C is minor, but still detectable, especially in 12-HETE and TXB_2 levels in EDTA plasma at -80°C. The levels of these compounds after one year of storage were similar to the baseline heparin levels (Supplementary Figures S38 and S51). This could indicate the slow activation of platelets over time in EDTA plasma at -80°C, supported also by a higher *S*/*R*-ratio of 12-HETE in EDTA plasma without additives compared to MeOH addition at 74 weeks at -80 °C (Supplementary Figure 63). At -20°C, the *S*/*R*-ratio in EDTA plasma is relatively constant over time, suggesting that non-enzymatic processes have a higher contribution at this temperature. In heparin, the S/R ratio at -20°C approaches 1 over time, albeit slower in samples with MeOH, indicating the contribution of both enzymatic and non-enzymatic, non-stereospecific pathways to the synthesis of 12-HETE.

At -20°C, most of the oxylipin levels were increased over time, starting with 4 weeks of storage.

Chiral analysis for 5- HETE in heparin shows the increase of *S* over *R* for 5-HETE at -20°C, while for 15-HETE, *S* and *R* increase similarly over time and the *S*/*R* ratio decreases slightly towards 1 at 74 weeks. This indicates that the 5-HETE increase at -20°C is mostly due to enzymatic synthesis of 5*S*-HETE while 15-HETE is likely made in a non-enzymatic, non-stereospecific way. In EDTA, no clear differences were observed in *S/R* ratio over time, indicating the predominant contribution of a non-enzymatic, non-stereospecific production of both 5- and 15-HETE.

The appearance of some other features over time at -20°C (see chapters *Appearances of new oxylipids over time* and *Chiral analysis of lipid mediators*, Figure 8, Figure 9, and Supplementary Figures S53-S61) could then also be explained by the activity of 5-LOX (among other proteins), as this enzyme can be involved in the synthesis of PDX via 17-HpDHA (23); 5*S*,12*S*-diHETE (17), 6-trans-LTB_4 and 6-trans-12-epi-LTB_4 (24,25) via 5-H(p)ETE, and $RvD5_{n\text{-}3\ DPA}$ via 17-$HpDPA_{n\text{-}3}$ (26,27) *in vivo*. In contrast, 5*S*,15*S*-diHETE can be made *in vivo* via 5-LOX (28,29) and/or via 15-LOX and 8*S*,15*S*-diHETE via 15-LOX(34) from 8*S*-H(p)ETE. For PDX a double oxygenation catalyzed by 15-LOX might be an alternative pathway as shown for soybean 15-LOXby Chen et al (30).

However, as is apparent in Figures 8 and 9, multiple isomers have formed during long-term storage at -20°C, so to assign the formation of these oxylipids to enzyme activity rather than non-enzymatic, non-stereospecific radical reactions would require a more detailed investigation.

Interestingly, BHT addition in general had very little effect on analyte levels, even in the EDTA samples, in which the contribution of non-enzymatic, non-stereospecific reactions seems to be predominant. However, it strongly affected the 12-HETE levels in EDTA at baseline, which could be explained by a possible interference with the measurement method, activation of platelets (31-33) or contamination of the baseline EDTA samples with erythrocytes and/or platelets. It is conceivable that the combination of BHT in particular with EDTA results in activation of residual platelets in plasma samples. As described by Ruzzene et al. (31), BHT can activate phospholipase C *via* protein kinase C, while EDTA has been described to cause irreversible damage to platelets (35). The combination of these two processes could lead to an increased production of 12-HETE and TXB2, in particular when proteins have not been precipitated with MeOH.

To prevent the protein-catalyzed oxidation process in samples where proteins have not been precipitated with MeOH, we added a 10- fold increased amount of BHT. However, our data indicate that even these high concentrations of BHT were not able to significantly limit the production of oxylipids. Therefore, we deem BHT addition to plasma samples an unnecessary effort in oxylipid analysis that, in combination with EDTA, might even be counterproductive in the analysis of 12-
HETE and TXB2.

To conclude, oxylipid datasets from plasma prepared with different anticoagulants cannot be directly compared, especially for platelet-derived metabolites and leukotrienes. For long-term studies, samples should be stored at -80 °C, but MeOH addition to plasma prior to storage can maintain sample integrity at least for 12 (EDTA) to 26 (heparin) weeks at

-20°C. For some analytes, like 12-HETE and TXB_2 in EDTA plasma and LTE_4 in heparin plasma, addition of MeOH prior to storage at -80 °C appears necessary to prevent *ex vivo* accumulation over time. In addition, for measuring of EPA, AdA, and ALA/GLA, addition of MeOH to heparin plasma kept at -80 °C is recommended.

For short-term, samples can be kept for two hours at room temperature or refrigerated for eight hours without significant changes on levels, except for LTE_4 in heparin and EPA, AdA and ALA/GLA in EDTA. For oxylipids, MeOH addition prolongs the time at which a sample can be kept at these temperatures, but does not affect the increase of EPA, AdA and ALA/GLA in EDTA plasma. These PUFA levels also increase at -20°C in EDTA plasma (slightly less with MeOH than without), so for PUFA analysis heparin should be the preferred choice.

Author contribution

HB and HSJ carried out the experiments and were responsible for data analysis. AIF and MG designed and supervised the study. All authors contributed to the critical assessment of the data and manuscript preparation.

Additional information

The authors declare no competing interests. The study was approved by the Medical Ethical Committee of the Leiden University Medical Center. All methods were performed in accordance with the relevant guidelines and regulations outlined by the Medical Ethical Committee of the Leiden University Medical Center.

Transparency document

The Transparency document associated with this article can be found, in online version.

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References

1. B.J. de Lange-Brokaar, M. Kloppenburg, S.N. Andersen, A.L. Dorjee, E. Yusuf, L. Herb-van Toorn, H.M. Kroon, A.M. Zuurmond, V. Stojanovic-Susulic, J.L. Bloem, R.G. Nelissen, R.E. Toes, A. Ioan-Facsinay, Characterization of synovial mast cells in knee osteoarthritis: association with clinical parameters, Osteoarthritis Cartilage, 24 (2016) 664-671.
2. T. Hyötyläinen, M. Orešič, Bioanalytical techniques in nontargeted clinical lipidomics, Bioanalysis, 8 (2016) 351-364.
3. H.S. Jónasdóttir, H. Brouwers, J.C. Kwekkeboom, H.M.J. van der Linden, T. Huizinga, M. Kloppenburg, R.E.M. Toes, M. Giera, A. Ioan-Facsinay, Targeted lipidomics reveals activation of resolution pathways in knee osteoarthritis in humans, Osteoarthritis and Cartilage, 25 1150-1160.
4. C.N. Serhan, Discovery of specialized pro-resolving mediators marks the dawn of resolution physiology and pharmacology, Molecular Aspects of Medicine, (2017).
5. I. Willenberg, A.I. Ostermann, N.H. Schebb, Targeted metabolomics of the arachidonic acid cascade: current state and challenges of LC–MS analysis of oxylipins, Analytical and Bioanalytical Chemistry, 407 (2015) 2675-2683.
6. J.-M. Galano, Y.Y. Lee, C. Oger, C. Vigor, J. Vercauteren, T. Durand, M. Giera, J.C.-Y. Lee, Isoprostanes, neuroprostanes and phytoprostanes: An overview of 25years of research in chemistry and biology, Progress in Lipid Research, 68 (2017) 83-108.
7. A.E. Barden, E. Mas, K.D. Croft, M. Phillips, T.A. Mori, Minimizing artifactual elevation of lipid peroxidation products (F2-isoprostanes) in plasma during collection and storage, Analytical Biochemistry, 449 (2014) 129-131.
8. P.J. Taylor, Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography–electrospray–tandem mass spectrometry, Clinical Biochemistry, 38 (2005) 328-334.
9. B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Strategies for the Assessment of Matrix Effect in Quantitative Bioanalytical Methods Based on HPLC–MS/MS, Analytical Chemistry, 75 (2003) 3019-3030.
10. V. Gonzalez-Covarrubias, A. Dane, T. Hankemeier, R.J. Vreeken, The influence of citrate, EDTA, and heparin anticoagulants to human plasma LC–MS lipidomic profiling, Metabolomics, 9 (2013) 337-348.
11. C. Vigor, J. Bertrand-Michel, E. Pinot, C. Oger, J. Vercauteren, P. Le Faouder, J.-M. Galano, J.C.-Y. Lee, T. Durand, Non-enzymatic lipid oxidation products in biological systems: Assessment of the metabolites from polyunsaturated fatty acids, Journal of Chromatography B, 964 (2014) 65-78.
12. C.R. Lambert, H.S. Black, T. George Truscott, Reactivity of butylated hydroxytoluene, Free Radical Biology and Medicine, 21 (1996) 395-400.
13. A.R. Magnusardottir, G.V. Skuladottir, Effects of storage time and added antioxidant on fatty acid composition of red blood cells at –20°C, Lipids, 41 (2006) 401.
14. G.L. Milne, Q. Dai, L.J. Roberts Ii, The isoprostanes—25 years later, Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids, 1851 (2015) 433-445.
15. N.P. Sadagopan, W. Li, J.A. Cook, B. Galvan, D.L. Weller, S.T. Fountain, L.H. Cohen, Investigation of EDTA anticoagulant in plasma to improve the throughput of liquid chromatography/tandem mass spectrometric assays, Rapid Communications in Mass Spectrometry, 17 (2003) 1065-1070.

16. M. Schlegel, D. Köhler, A. Körner, T. Granja, A. Straub, M. Giera, V. Mirakaj, The neuroimmune guidance cue netrin-1 controls resolution programs and promotes liver regeneration, Hepatology, 63 (2016) 1689-1705.

17. M. Giera, A. Ioan-Facsinay, R. Toes, F. Gao, J. Dalli, A.M. Deelder, C.N. Serhan, O.A. Mayboroda, Lipid and lipid mediator profiling of human synovial fluid in rheumatoid arthritis patients by means of LC–MS/MS, Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids, 1821 (2012) 1415-1424.

18. H.S. Jónasdóttir, C. Papan, S. Fabritz, L. Balas, T. Durand, I. Hardardottir, J. Freysdottir, M. Giera, Differential Mobility Separation of Leukotrienes and Protectins, Analytical Chemistry, 87 (2015) 5036-5040.

19. B. Porro, P. Songia, I. Squellerio, E. Tremoli, V. Cavalca, Analysis, physiological and clinical significance of 12-HETE: A neglected platelet-derived 12-lipoxygenase product, Journal of Chromatography B, 964 (2014) 26-40.

20. V. Evangelista, A. Celardo, G. Dell'Elba, S. Manarini, A. Mironov, G.d. Gaetano, C. Cerletti, Platelet contribution to leukotriene production in inflammation: in vivoevidence in the rabbit, Thromb. Haemost. 81 (1999) 442–448.

21. T.M. Laidlaw, J.A. Boyce, Platelets in aspirin-exacerbated respiratory disease, J. Allergy Clin. Immunol. 135 (2015) 1407–1415.

22. J.A. Maclouf, R.C. Murphy, Transcellular metabolism of neutrophil-derived leukotriene A4 by human platelets. A potential cellular source of leukotriene C4, J. Biol.

Chem. 263 (1988) 174–181.

23. C.N. Serhan, N.A. Petasis, Resolvins and Protectins in Inflammation-Resolution, Chemical reviews, 111 (2011) 5922-5943.

24. C.A. Rouzer, T. Matsumoto, B. Samuelsson, Single protein from human leukocytes possesses 5-lipoxygenase and leukotriene A4 synthase activities, Proceedings of the National Academy of Sciences of the United States of America, 83 (1986) 857-861.

25. T. Shimizu, T. Izumi, Y. Seyama, K. Tadokoro, O. Rådmark, B. Samuelsson, Characterization of leukotriene A4 synthase from murine mast cells: evidence for its identity to arachidonate 5-lipoxygenase, Proceedings of the National Academy of Sciences of the United States of America, 83 (1986) 4175-4179.

26. J. Dalli, R.A. Colas, C.N. Serhan, Novel n-3 Immunoresolvents: Structures and Actions, Scientific Reports, 3 (2013) 1940.

27. T. Gobbetti, J. Dalli, R.A. Colas, D. Federici Canova, M. Aursnes, D. Bonnet, L. Alric, N. Vergnolle, C. Deraison, T.V. Hansen, C.N. Serhan, M. Perretti, Protectin D1(n-3 DPA) and resolvin D5(n-3 DPA) are effectors of intestinal protection, Proceedings of the National Academy of Sciences of the United States of America, 114 (2017) 3963-3968.

28. C. Chavis, I. Vachier, P. Chanez, J. Bousquet, P. Godard, 5(S),15(S)-dihydroxyeicosatetraenoic acid and lipoxin generation in human polymorphonuclear cells: dual specificity of 5-lipoxygenase towards endogenous and exogenous precursors, The Journal of Experimental Medicine, 183 (1996) 1633.

29. E. Thomas, J.L. Leroux, F. Blotman, C. Chavis, Conversion of endogenous arachidonic acid to 5,15-diHETE and lipoxins by polymorphonuclear cells from patients with rheumatoid arthritis, Inflammation Research, 44 (1995) 121-124.

30. P. Chen, B. Fenet, S. Michaud, N. Tomczyk, E. Vericel, M. Lagarde, M. Guichardant, Full characterization of PDX, a neuroprotectin/protectin D1 isomer, which inhibits blood platelet aggregation, FEBS Lett. 583 (2009) 3478–3484.

31. M. Ruzzene, M. Francesconi, A. Donella-Deana, A. Alexandre, R. Deana, The antioxidant butylated hydroxytoluene (BHT) inhibits the dioctanoylglycerol-evoked platelet response but potentiates that elicited by Ionomycin, Archives of Biochemistry and Biophysics, 294 (1992) 724-730.

32. S.F. Mohammad, W.H. Anderson, J.B. Smith, H.Y. Chuang, R.G. Mason, Effects of heparin on platelet aggregation and release and thromboxane A2 production, The American Journal of Pathology, 104 (1981) 132-141.

33. A. Greinacher, Platelet activation by heparin, Blood, 117 (2011) 4686.

34. S. Mulugeta, T. Suzuki, N.T. Hernandez, M. Griesser, W.E. Boeglin, C. Schneider, Identification and absolute configuration of dihydroxy-arachidonic acids formed by oxygenation of 5S-HETE by native and aspirin-acetylated COX-2, Journal of Lipid Research, 51 (2010) 575-585.

35. J.G.W.G. Escolar, EDTA-induced changes in platelet structure and function: adhesion and spreading, Platelets 11 (2000) 56–61.

Supplementary material

Analyte name	Average baseline concentration in EDTA plasma, n=2 [ng/mL]	Average baseline concentration in heparin plasma, n=2 [ng/mL]
5-HETE	0.042	0.166
8-HETE	0.040	0.039
11-HETE	0.017*	0.017
12-HETE	0.072	0.595
15-HETE	0.082	0.068
15-HEPE	0.012*	0.011*
18-HEPE	0.041	0.034
10-HDHA	0.008*	0.014*
17-HDHA	0.049	0.051
14,15-diHETE	0.095	0.087
19,20-diHDPA	0.473	0.442
TXB2	0.032, n=1	0.138
LTB4	n.d	0.020
LTD4	n.d	0.027, n=1
LTE4	n.d	0.012
AA	843+	785+
EPA	49.8	87.3
DHA	598+	536+
AdA	33.3	53.3
DPAn-3	223	213
ALA/GLA	624+	903+
LA	4754+	3607+

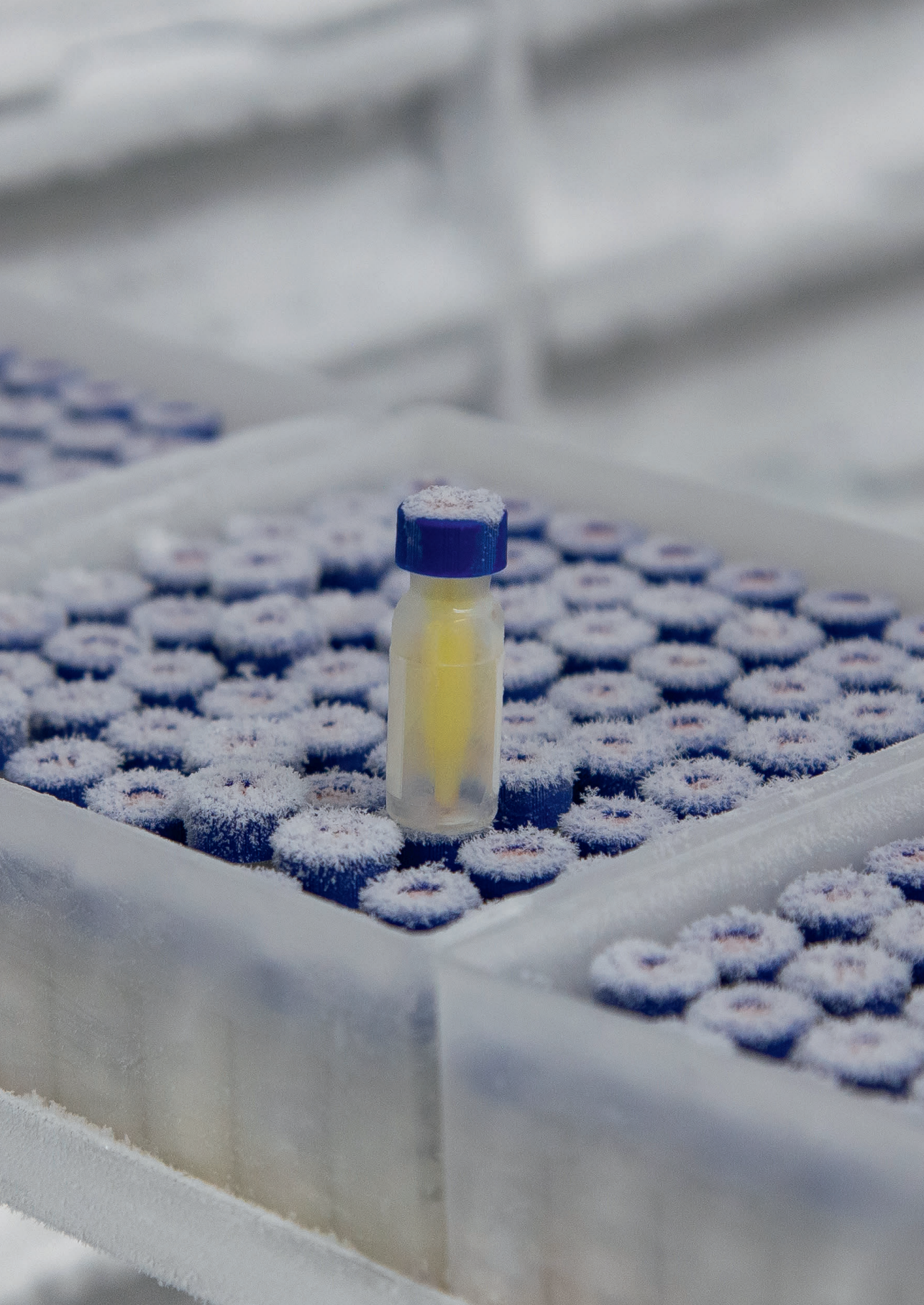
Supplementary Table 1. Calculated concentrations of oxylipids and PUFAs at baseline. Pooled plasma directly precipitated with MeOH after aliquotting. Average of the concentration of two technical replicates for each anticoagulant unless otherwise specified.

* Concentration below lowest standard

+ Concentration above highest standard

Abbreviations: AA arachidonic acid, EPA eicosapentaenoic acid, DHA docosahexaenoic acid, AdA adrenic acid, DPAn-3 omega-3 docosapentaenoic acid, LA linoleic acid, ALA alpha-linolenic acid, GLA gamma-linolenic acid, HETE hydroxyeicosatetraenoic acid, HEPE hydroxyeicosapentaenoic acid, HDHA hydroxydocosahexaenoics acid, TXB2 thromoboxane B2, LTB4 leukotriene B4, diHETE dihydroxyeicosatetraenoic acid, and diHDPA dihydroxydocosapentaenoic acid

Supplementary figures S1-S64 can be found in the online version of this article.



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Hyaluronidase treatment of synovial fluid is required for accurate detection of inflammatory cells and soluble mediators

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Abstract

Background: Synovial fluid (SF) is commonly used for diagnostic and research purposes, as it is believed to reflect the local inflammatory environment. Owing to its complex composition and especially the presence of hyaluronic acid, SF is usually viscous and non-homogeneous. In this study we investigated the importance of homogenization of the total SF sample before subsequent analysis.

Methods: SF was obtained from the knee of 29 arthritis patients (26 rheumatoid arthritis, 2 osteoarthritis and 1 juvenile idiopathic arthritis patient) as part of standard clinical care. Synovial fluid was either treated with hyaluronidase as a whole or after aliquoting to determine whether the concentration of soluble mediators is evenly distributed in the viscous synovial fluid. Cytokine and IgG levels were measured by ELISA or Luminex and a total of seven fatty acid and oxylipin levels were determined using LC-MS/MS in all aliquots. For cell analysis, synovial fluid was first centrifuged and the pellet was separated from the fluid. The fluid was subsequently treated with hyaluronidase and centrifuged to isolate remaining cells. Cell numbers and phenotype were determined using flow cytometry.

Results: In all patients, there was less variation in IgG, 17-HDHA, leukotriene B_4 (LTB_4) and prostaglandin E_2 (PGE_2) levels when homogenization was performed before aliquoting the SF sample. There was no difference in variation for cytokines, 15-HETE, and fatty acids arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Between 0.8-70% of immune cells (median 5 %) remained in suspension and were missing in subsequent analyses when the cells were isolated from untreated SF. This percentage was higher for T and B cells: 7-85% (median 22%) and 7-88% (median 23 %), respectively.

Conclusions: Homogenization of the entire SF sample leads to less variability in IgG and oxylipin levels and prevents erroneous conclusions based on incomplete isolation of synovial fluid cells.

Keywords: Synovial fluid; Hyaluronidase; Rheumatoid arthritis

Background

Synovial inflammation is a symptom of many rheumatic musculoskeletal diseases such as rheumatoid arthritis (RA), juvenile idiopathic arthritis (JIA), spondyloarthritis (SpA), osteoarthritis (OA) and systemic lupus erythematosus (lupus). Synovial joints contain a synovial lining (synovium) consisting primarily of synovial fibroblasts, and a synovial cavity containing SF.

During inflammation, immune cells such as macrophages, lymphocytes and neutrophils infiltrate the joint and SF accumulates. The cells and soluble mediators present at these local sites of inflammation are of interest to unravel disease pathophysiology and are therefore often studied.

Synovial fluid cytokines have been of interest for many years and more recently, lipid derived inflammatory mediators were studied in RA and OA(1–6). Next to the interest in soluble mediators, frequencies and phenotype of specific T – and B cell subsets, monocytes and NK cells were determined in a wide range of rheumatic musculoskeletal diseases(7–23). In addition to experimental research, synovial fluid is also used for diagnostic purposes to obtain information about antigen specific antibody levels or white blood cell (WBC) count(24–26).

Synovial fluid is often aspirated from the inflamed joints of arthritis patients to reduce discomfort and to make room to inject therapeutics or to perform diagnostics, as part of standard clinical care. Therefore, it is easier accessible for research than synovium, which can only be obtained via a more invasive procedure such as arthroplasty or synovial biopsy. Synovial fluid provides lubrication to the joint and acts as a transport medium for nutrients and cells. It consists of an ultrafiltrate of plasma containing many different proteins including high amounts of hyaluronan. Hyaluronan is produced by the synovial fibroblasts and is present in a 10^5 higher concentration in synovial fluid compared to plasma(27). Hyaluronan forms dense mesh networks in the synovial fluid, which make the fluid viscous. Due to this viscosity, handling synovial fluid in a laboratory setting is challenging. To overcome this problem, synovial fluid can be treated with hyaluronidase, which breaks down the dense mesh network of hyaluronan fibers. Indeed, a few reports, analyzing equine SF or SF from non-inflammatory conditions, have shown that technical issues can occur when analyzing untreated SF (28–30). For instance, it was shown that treating SF with hyaluronidase before cytokine analysis improves cytokine recovery in a polystyrene, but not magnetic bead, Luminex assay (30). In addition, hyaluronidase treatment is routinely performed before proteomic and metabolomic analysis as this is required to prevent clogging of the mass spectrometers (5,33). However, to date there

are no reports on the importance of hyaluronidase treatment as part of the standard processing protocol for SF and this treatment is therefore not routinely used in arthritis research. For example, hyaluronidase treatment is not mentioned in more than half of the above-mentioned studies investigating SF immune cells in inflammatory joint diseases(1,3,6–10,12,14,15,20,31,32). In daily practice, SF is collected, aliquoted and stored before analysis to be able to analyze soluble mediators in multiple patients at once. Subsequently, one aliquot per patient is then used to measure soluble mediators. However, it is unclear whether the levels of soluble mediators are comparable between the aliquots, when the aliquots were taken and stored before hyaluronidase treatment. Moreover, the effect of hyaluronidase treatment on the recovery of cells from SF is unclear and there is no consensus whether inflammatory cells should be analyzed before or after hyaluronidase treatment.

In this study, we investigated whether homogenization of SF sample is required for the reproducibility of IgG, cytokine and lipid *measurements*. In addition we performed flow cytometric analysis on cells isolated from treated and untreated SF.

Methods

Patients

Synovial fluid was obtained via knee aspiration from 29 arthritis patients (26 rheumatoid arthritis and 1 juvenile idiopathic arthritis patient) visiting the rheumatology outpatient clinic of the LUMC as part of standard clinical care. Written informed consent was obtained from all these donors. Anonymized leftover synovial fluid of two osteoarthritis patients was collected using a syringe before knee-replacement surgery performed at the departments of orthopedic surgery at the LUMC and the Alrijne hospital in Leiden. The study was approved by the local ethical committee.

Processing of synovial fluid for molecular measurements

On the same day of knee aspiration, the fluid was divided in two sets of 3-5 aliquots ranging between 300 μL and 1 mL to be able to fit the fluid in a heat block with vortex modus. 1 mg/mL hyaluronidase from bovine testis (Sigma-Aldrich) was added in a 1:11 ratio. Hyaluronidase was suspended just before use in phosphate buffered saline (PBS, B.Braun). SF was vortexed for 5 minutes and subsequently incubated for 25 minutes at 37 °C. Next, the first set of aliquots (set 1) was directly centrifuged for 10 minutes at 931 x g, while the second set (set 2) was first pooled together before centrifugation. After centrifugation, the pooled set was divided again in 3-5 individual aliquots. Of each of the aliquots in set 1 and set 2, 100 μL supernatant was transferred to a glass vial (Agilent Technologies) together with 294 μL methanol (Fluka LC-MS CHROMASOLV grade,

Sigma-Aldrich) and 6 μL internal standards (50 ng/mL leukotriene B_4-d4, prostaglandin E_2-d4, 15-HETE-d8 and 500 ng/mL of docosahexaenoic acid-d5, all from Cayman Chemicals). Argon gas was added and the samples were stored at -80 °C. Similarly, 200 μL supernatant was also stored at -20 °C for immunoglobulin and IL-8 analysis. For IL-6, IL-10, CXCL1, CXCL5 and TNFα analysis, SF was centrifuged for 10 minutes at 931 x g on the day of knee aspiration and supernatant was stored directly at -80°C. At the day of analysis, SF was thawed to room temperature and treated as described above for the fresh SF.

ELISA

Interleukin 8 (IL-8) and IgG ELISA were performed according to manufacturer's protocol (Invitrogen, BD Biosciences and Bethyl Laboratories respectively).

Luminex

Bio-Plex pro reagent kit III and Bio-Plex Pro Human IL-6 set (171BK29MR2), IL-10 set (171BK32MR2), TNF-α (171BK55MR2), CXCL1 (171BK22MR2) and CXCL5 (171BK14MR2) were purchased from Bio-rad. The cytokines and chemokines were determined according to the manufacturer's protocol. SF samples were measured on the Bio-Plex 200 system (Bio-Rad) and analysis was done using Bio-plex Manager 6.2 Software (Bio-Rad).

Lipidomic analysis of synovial fluid

LC-MS/MS based lipid mediator and oxylipin profiling was carried out as described elsewhere(4). A QTrap 6500 mass spectrometer (Sciex) was used, coupled to a Shimadzu Nexera LC30-system including auto-sampler and column oven (Shimadzu). The column was a Kinetex C18 50 × 2.1 mm, 1.7 μm, protected with a C8 pre-column (Phenomenex). LC-MS/MS peaks were integrated with manual supervision and the areas were corrected to corresponding IS using MultiQuant™ 2.1 (Sciex). For quantitation, the multiple reaction monitoring (MRM) transitions and collision energies (CE) were used together with calibration lines for quantification. Calibration lines were constructed using 15-HETE, 17R-HDHA, leukotriene B_4, prostaglandin E_2, arachidonic acid, docosahexaenoic acid and eicosapentaenoic (Cayman Chemicals)

Flow cytometry analyses of synovial fluid cells

For general analysis, the fluid was centrifuged for 10 minutes at 931 x g and cells were collected and analyzed. The supernatant was treated with hyaluronidase as stated above and subsequently centrifuged for 10 minutes at 931 x g to isolate remaining cells. For the effect of hyaluronidase treatment on cell marker expression, the SF was diluted 20x in PBS and divided in two. One sample was treated with hyaluronidase (treated) and the other sample was treated similarly, but without the addition of hyaluronidase

(untreated). Isolated cells were resuspended in PBS and filtered through a 70 μm cell strainer before flow cytometry. For general cell characterization, cells were stained with anti-CD3 (AF700, clone UCHT1), anti-CD14 (FITC, clone M5E2), anti-CD15 (APC, clone HI98), anti-CD16 (PE, clone B73.1), anti-CD19 (PerCp/Cy5.5, clone SJ25C1) and anti-CD45(APC/Cy7, clone 2D1). The gating strategy is depicted in supplementary figure 1. For additional characterization into $CD4^+$ T cells and $CD8^+$ T cells, cells were stained with anti-CD3 (PE, clone SK7), anti-CD4 (APC, clone SK3), anti-CD8 (FITC, clone SK1). For the effect of hyaluronidase treatment on cell marker expression, cells were stained with anti-CD3 (Pacific Blue, clone SK7), anti-CD4 (APC, clone SK3), anti-CD8 (FITC, clone SK1), anti-CD19 (APC/Cy7, clone SJ25C1), anti-CD44 (PE/Cy7, clone G44-26), anti-CD69 (PE/CF594, clone FN50). Positivity for CD44 and CD69 was determined using isotype controls IgG2bk (PE/Cy7, clone 27-35) and IgG1 (PE/CF594, clone X40). For additional characterization into naïve B cells, memory B cells and plasmablasts/cells, cells were stained with anti-CD3 (Pacific Blue, clone SK7), anti-CD14 (Pacific Blue, clone M5E2), anti-CD19 (APC/Cy7, clone SJ25C1), anti-CD20 (AF 700, clone 2H7) and anti-CD27 (PE/Cy7, clone M-T271). For gating strategy for the T cell and B cell subsets see supplementary figure 2.

All antibodies were from BD except for the anti-CD20 which was from Sony Biotechnology (USA, CA, San Jose). For all analysis, dead cells were excluded using DAPI (Molecular Probes) and cells were quantified using Flow count Fluorospheres (Beckman Coulter). Cells were measured on a LSR Fortessa (BD) and were analyzed with FACSDiva Software (BD).

Statistical analyses and calculations.
Wilcoxon signed rank tests were performed to evaluate significance between groups in all figures. Coefficient of variation (CV) was calculated by dividing the standard deviation of a group of measurements by the mean and expressing this as a percentage.

Results

Effect of hyaluronidase on soluble mediator measurements
To test the whether the concentration of inflammatory soluble mediators is evenly distributed in viscous synovial fluid, we treated each synovial fluid as depicted in figure 1A. We also treated set 1 with hyaluronidase to exclude the possibility that the viscosity hampers soluble mediator detection as was shown before(30). We measured CXCL1, CXCL5 IL-6, IL-8, IL-10, TNFα and total IgG levels in the aliquots of set 1 and set 2 in eight arthritis patients. Levels of the cytokines differed considerably between patients (figure 2A-E). The data of CXCL5 is not shown as this mediator was only detected in two out

of eight patients. To assess intra-assay variation, the CV was calculated for each set of aliquots of the patients with detectable soluble mediator levels. There was no difference in CV between the two sets of samples for CXCL1, IL-6, IL-8, IL-10, TNFα (figure 2B). IgG levels were stable between the different patients and in contrast to the cytokines, hyaluronidase treatment before aliquoting resulted in lower CVs for IgG measurements (figure 2F and 2L). In addition to measurements of large protein structures as cytokines and antibodies, we investigated small molecules, such as fatty acids and oxidized lipids (figure 3A-C). Hyaluronidase treatment before aliquoting did not improve CV values for AA, DHA and EPA (figure 3D). These fatty acids can be converted to monohydroxylated fatty acids such as 15-HETE and 17-HDHA, derived from AA and DHA, respectively. Hydroxylated products can in turn be converted to highly bioactive oxylipids like PGE_2 and LTB_4. In contrast to the CV values of fatty acids, the values for 17-HDHA and LTB_4 improved significantly when fluids were treated with hyaluronidase before aliquoting (figure 3E, 3F).

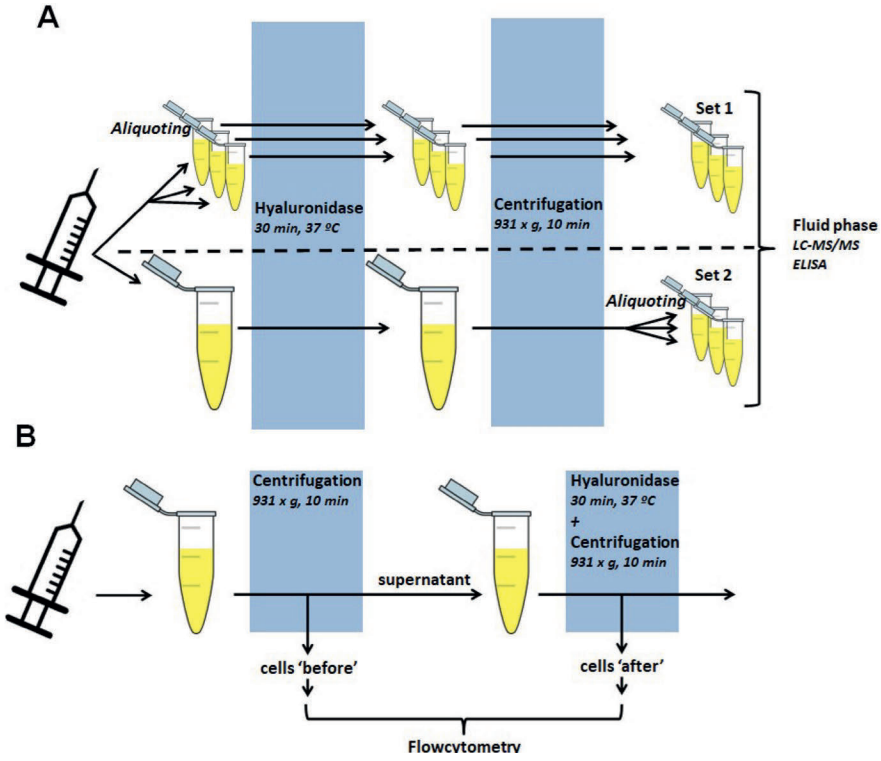


Figure 1. Schematic overview of the experimental setup. A) Half of the Synovial fluid was divided into aliquots while the other half was kept as a whole. The SF was treated with hyaluronidase after which it was centrifuged. Set 1 was kept as separate aliquots during the whole procedure and set 2 was divided in aliquots directly after centrifugation. B) Synovial fluid was centrifuged and the pelleted cells are the 'before' cells. The supernatant was treated with hyaluronidase and subsequently centrifuged. The pelleted cells are the 'after' cells.

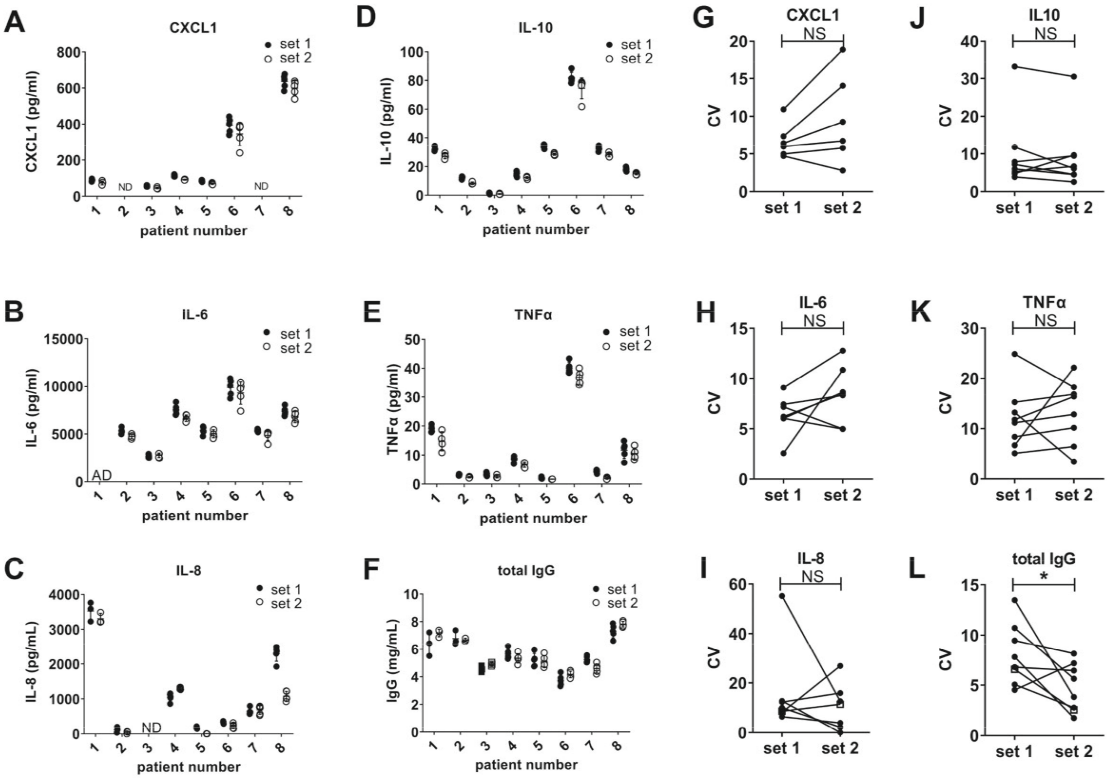


Figure 2. Hyaluronidase treatment lowers variability in IgG levels. A-F) CXCL1, IL-6, IL-8, IL-10, TNFα and total IgG levels measured by ELISA or Luminex. Dots are RA patients, the OA patient is represented by squares. Each point represents one aliquot. Means with SD are shown. IL-8 and total IgG were analyzed in a separate set of 8 patients compared to CXCL1, IL-6, IL-10 and TNFα. ND = not detected. AD = above detection limit. G-L) Coefficients of variation (CV) are depicted for each set of aliquots. Each line represents one patient. n=6-8 patients. Dots are RA patients, the OA patient is represented by open squares Wilcoxon signed rank test was performed. *, $P<0.05$.

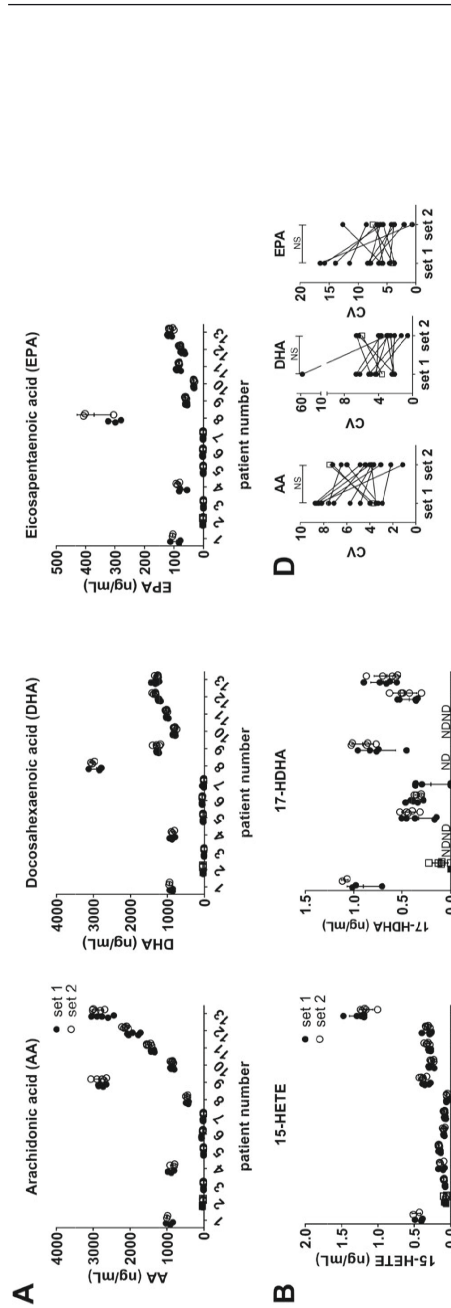


Figure 3. Reproducibility of lipid measurements improves by aliquoting after hyaluronidase treatment. The concentrations for each set of aliquots are depicted for (A) fatty acids and (B) monohydroxylated products and (C) oxylipins. The CVs for each set of aliquots are depicted for (D) fatty acids and (E) monohydroxylated products and (F) oxylipins. n=13 patients and each line represents one patient. Dots are RA patients, the OA patient is represented by squares. No line or ND means not detected. Wilcoxon signed rank test was performed. *, $P<0.05$; **, $P<0.01$.

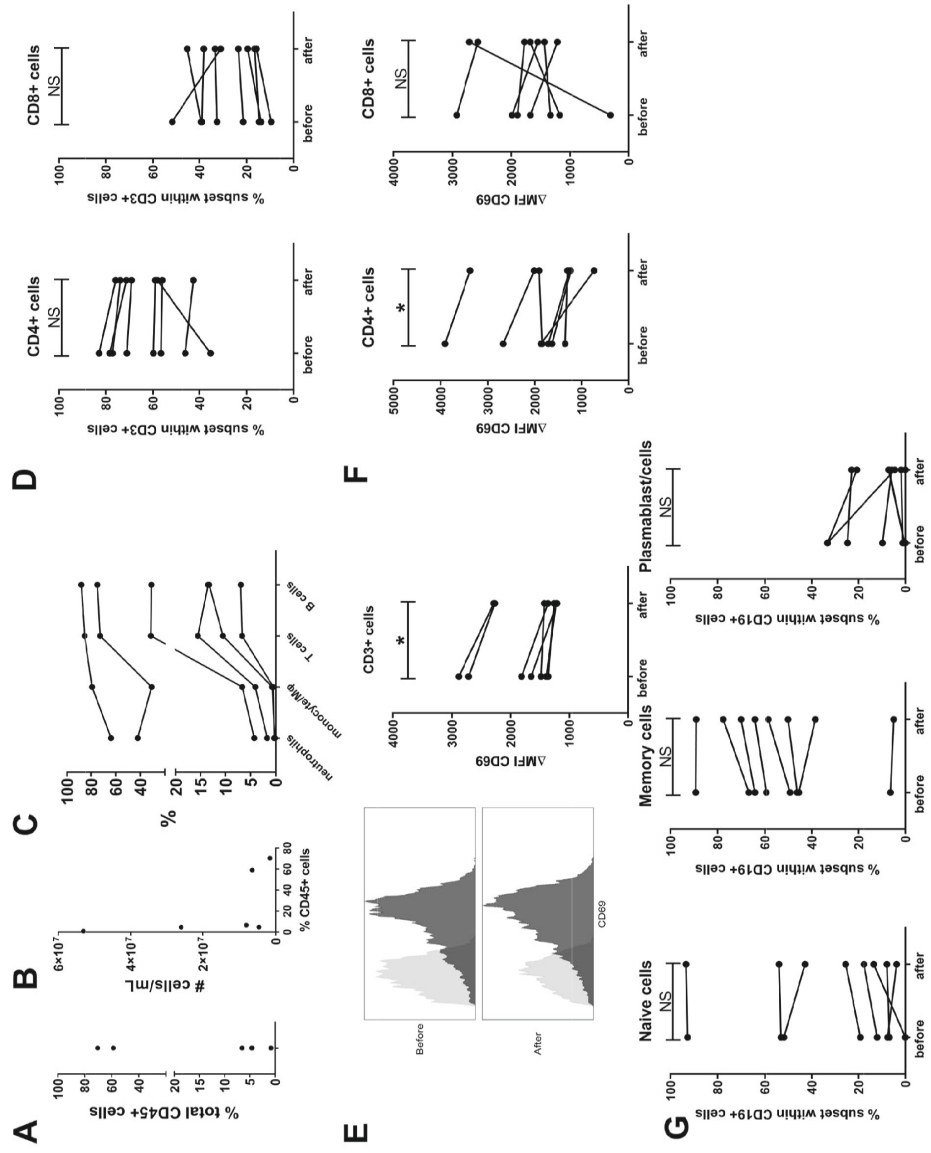
Cellular measurements benefit from hyaluronidase treatment.

We hypothesize that the viscosity of SF impairs the recovery of immune cells during isolation. To test this, readily-accessible cells (before fraction) were first isolated from SF by centrifugation. Thereafter, the supernatant, i.e. hyaluronan-rich fraction (after fraction), was treated with hyaluronidase and centrifuged again, to isolate remaining cells (figure 1B). Depending on the patient, up to 70% of the $CD45^+$ cells could be isolated from the supernatant after hyaluronidase treatment (figure 4A). This percentage did not correlate with the total number of cells present in the fluid (figure 4B). Interestingly, mostly T and B cells were recovered from the hyaluronan-rich (after) fraction (figure 4C). Further characterization of T cell subsets revealed no difference in $CD4^+$ or $CD8^+$ T cell percentages between the before and after fractions (figure 4D). Surprisingly, however, the cells in the after fraction displayed a less activated phenotype, reflected by a lower expression of $CD69^+$ (Figure 4E). This effect was primarily observed in the $CD4^+$ T cells (Figure 4F). There was no difference in the percentage of $CD69^+$ cells in $CD3^+$, $CD4^+$ or $CD8^+$ T cells between the before and after samples (data not shown). The hyaluronidase treatment itself does not effect CD69 expression (Supplementary figure 3B). We could not detect any differences in either naïve B cell, memory B cell or plasmablast/plasmacell percentages between the fractions (Figure 4G).

Smaller immune cells are retained in the hyaluronan-rich supernatant

To gain more insight into the possible mechanism underlying the retention of certain lymphocytes in the hyaluronan mesh, we investigated expression of the hyaluronan receptor CD44 and the size of the immune cells in the SF supernatant (13). We hypothesized that cells with a higher expression of CD44 could be preferentially retained in the untreated SF. However, there was no difference in CD44 expression on lymphocytes and neutrophils (Figure 5A-C). In control experiments, the hyaluronidase treatment itself did not affect CD44 expression (Supplementary figure 3A). Interestingly, smaller cells are preferentially retained in the untreated SF (Figure 5D).

> Figure 4. Hyaluronidase treatment is essential for an unbiased analysis of the cellular composition of synovial fluid. **A and C)** Percentage of cells obtained after hyaluronidase treatment relative to total (before hyaluronidase + after hyaluronidase) number of cells. Cell subsets are defined as follows: neutrophils are $CD15^+CD16^+$, monocyte/macrophages are $CD15^-CD14^+$, T cells are $CD3^+$, B cells are $CD19^+$. Each dot or line represents one patient. n=5-6 patients. **B)** Percentages from A plotted against the number of live cells per mL in the total SF (before hyaluronidase + after hyaluronidase) SF determined using flow count beads. **D)** Percentages of $CD4^+$ and $CD8^+$ cells present within the $CD3^+$ cell population obtained before hyaluronidase treatment (before) or after (after). **E)** CD69 expression on $CD3^+$ cells. Flowcytometry plot of one representative donor is shown together with the summary of all patients. The expression of the cellular marker is indicated by ΔMFI which is defined as MFI of the antibody staining (dark grey) – MFI of the isotype control (light grey). **F)** CD69 expression on $CD4^+$ and $CD8^+$ cells. **G)** Percentage of naïve ($CD20^+CD27^-$) and memory ($CD20^+CD27^+$) B cells, as well as plasmablast / plasma cells ($CD20^-CD27^+$) present within the $CD19^+$ population before and after hyaluronidase are depicted. Each line is one patient. n=7-8 patients. Wilcoxon signed rank test was performed.


A
% total CD45+ cells
B
cells/mL
% CD45+ cells
C
%
neutrophils
monocytes/Mφ
T cells
B cells
D
CD4+ cells
CD8+ cells
NS
% subset within CD3+ cells
before
after
E
Before
After
CD69
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CD3+ cells
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CD8+ cells
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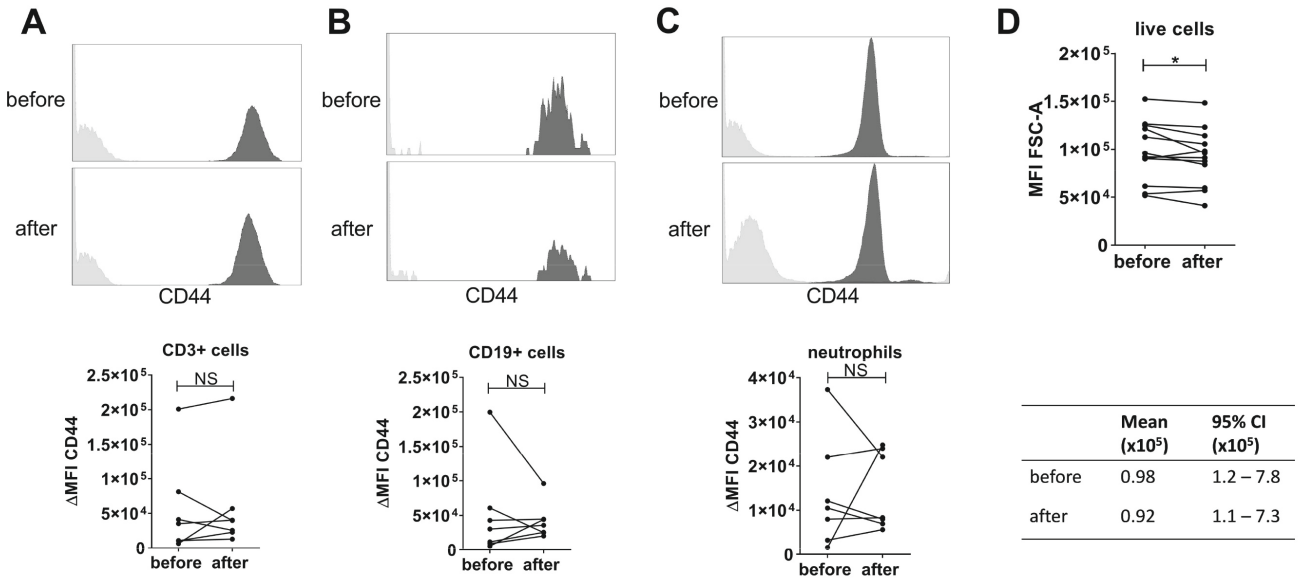
Memory cells
Plasmablast/cells
% subset within CD19+ cells



	Mean ($x10^5$)	95% CI ($x10^5$)
before	0.98	1.2 – 7.8
after	0.92	1.1 – 7.3

Figure 5. Smaller immune cells remain in SF after centrifugation without hyaluronidase**. A-C)** Treatment of SF and the analysis of cells were performed as described in figure 4, except for neutrophils, which were identified based on FSC/SSC. CD44 expression on $CD4^+$ and $CD8^+$ T cells, $CD19^+$ B cells and neutrophils. Flowcytometry plot of one representative donor is shown together with the summary of all patients. Each line represents a patient. n=7 patients. Wilcoxon signed rank test was performed. **D)** MFI values FCS-A of live cells determined by flowcytometry as described in figure 4. Each dot represents one patient. n= 12 patients. Mean and 95% shown in lower panel. Wilcoxon signed rank test was performed.

Discussion

Hyaluronidase treatment of SF is not routinely used in rheumatology research, however our results show the critical importance of homogenization of the synovial fluid sample to obtain reproducible and accurate immunological measurements. We show that IgG titers, 17-HDHA levels and pro-inflammatory LTB_4 levels become significantly less variable when SF is treated with hyaluronidase before aliquoting. In addition, hyaluronidase treatment of SF prior to cell isolation highly increased isolated cell numbers and therefore positively affects the accuracy of subsequent cell analysis.

Several joint diseases are characterized by the presence of auto-antibodies such as RF and ACPA in RA and ANA and anti-dsDNA antibodies in SLE(24,25). However, the mere presence of the autoantibodies in circulation does not directly indicate a role for these antibodies in joint inflammation or destruction. Therefore, researchers are investigating presence, relative abundance and specificity of these antibodies in the synovial fluid of e.g. arthritis patients.(35–37). In these experiments, it is important to accurately determine the presence and specificity of autoantibodies in the joint. Our data shows that total IgG levels are not evenly distributed throughout a synovial fluid sample as measurements were less variable when taking aliquots from homogenized synovial fluid compared to when they were taken from untreated synovial fluid. We did not analyze presence or abundance of antibodies of certain specificity, but as they are present in much lower concentrations than total IgG, higher variability between aliquots might more easily result in erroneous significant differences between patient samples.

In addition to total IgG levels, 17-HDHA and pro-inflammatory LTB_4 levels were also less variable when SF was aliquoted after homogenization. Although we could not show a significant reduction of CVs of PGE_2 concentrations, as PGE_2 was only detected in 3 out of 13 patients, the 3 positive patients show a similar trend as for LTB_4. Fatty acid concentrations were stable between the two sets of aliquots, this could be due to a high abundance compared to oxylipins (100-3000 ng/mL versus 0.1-1.5 ng/mL) or better dispersion in SF as we hypothesize that the oxylipins might interact more with their environment(5).

It was shown before that reproducibility of IL-6 and IL-8 measurements in SF by multiplex (Luminex) analysis are affected by hyaluronidase treatment(30). In contrast, we show that the reproducibility of IL-6, IL-8 and also CXCL1, IL-10 and TNFα measurements is equal between aliquots taken before or after hyaluronidase treatment of SF. However, this difference could easily be explained by the differences in experimental set-up. We have treated both sets of aliquots with hyaluronidase (figure 1A) wherease Jayadev *et al*.

compared untreated versus treated SF. In that study, accurate measurements of IL-6 and IL-8 might easily be hampered by the viscousity of the SF explaining their finding. They indeed show that the viscosity of the untreated aliquots hampers cytokine measurements in the Luminex assay.

Since immune cells in local tissues are of great interest in understanding disease pathology, we have compared cell populations isolated from untreated and treated SF. Up to a surprisingly high percentage (70%) of cells are often excluded from analyses when cells are isolated from untreated SF. Since specifically T and B cells are excluded, resulting data are likely highly biased. Moreover, we show that within the $CD4^+$ T cell populations additional bias is introduced within the $CD69^+$ cells. Based on our results, we hypothesize that specifically T and B cells are excluded based on their cellular size, since we could not confirm any increased interaction with the SF environment compared to other cell types. However, this hypothesis could be further tested through analysis of additional cell adhesion molecules. In addition we could perform experiments using different sized beads to support our hypothesis of the involvement of cellular size.

Conclusions

In summary, we show that homogenization of SF using hyaluronidase leads to less variability in oxylipin and IgG level measurements. In addition, homogenization of SF improves the recovery of immune cells during cell isolation. Based on our data, we advise researchers to perform hyaluronidase treatment on SF samples before aliquoting and subsequent analyses. Implementing hyaluronidase treatment in protocols using SF will likely result in improved reproducibility of soluble mediator measurements, new insights in the cell type ratios in joint diseases and even in the discovery of previously unidentified cell types in SF.

Abbreviations

AA:	arachidonic acid
CV:	coefficient of variation
DHA:	docosahexaenoic acid
EPA:	eicosapentaenoic acid
IL:	Interleukin
JIA:	juvenile idiopathic arthritis
LTB_4:	leukotriene B_4
OA:	osteoarthritis
PGE_2:	prostaglandin E_2
RA:	rheumatoid arthritis
SF:	synovial fluid
SpA:	spondyloarthritis
WBC:	white blood cell

Declarations

Ethics approval and consent to participate

Written informed consent was obtained from all the LUMC patients. The leftover materials of the Alrijne patients was received anonymized. The study was approved by the local ethical committee.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HB	Conducted the experiments, analyzed and interpreted the data and wrote the manuscript.
JH	Conducted some last experiments
EL	Included LUMC patients
RB	Included Alrijne patients
MK	included LUMC patients, interpreted the data and supervised the project
RT	interpreted the data and supervised the project
MG	his laboratory conducted the LC-MS/MS measurements
AI	interpreted the data and supervised the project
All	authors read and approved the final manuscript

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References

1. Snelling SJB, Bas S, Puskas GJ, Dakin SG, Suva D, Finckh A, et al. Presence of IL-17 in synovial fluid identifies a potential inflammatory osteoarthritic phenotype. PLoS One [Internet]. 2017 Apr 1 [cited 2020 Aug 30];12(4). Available from: /pmc/articles/PMC5388337/?report=abstract
2. Altobelli E, Angeletti PM, Piccolo D, De Angelis R. Synovial Fluid and Serum Concentrations of Inflammatory Markers in Rheumatoid Arthritis, Psoriatic Arthritis and Osteoarthitis: A Systematic Review. Curr Rheumatol Rev [Internet]. 2017 May 2 [cited 2020 Aug 30];13(3). Available from: https://pubmed.ncbi.nlm.nih.gov/28460627/
3. Mustonen AM, Käkelä R, Lehenkari P, Huhtakangas J, Turunen S, Joukainen A, et al. Distinct fatty acid signatures in infrapatellar fat pad and synovial fluid of patients with osteoarthritis versus rheumatoid arthritis. Arthritis Res Ther [Internet]. 2019 May 22 [cited 2020 Aug 30];21(1). Available from: https://pubmed.ncbi.nlm.nih.gov/31118103/
4. Giera M, Ioan-Facsinay A, Toes R, Gao F, Dalli J, Deelder AM, et al. Lipid and lipid mediator profiling of human synovial fluid in rheumatoid arthritis patients by means of LC–MS/MS. Biochim Biophys Acta - Mol Cell Biol Lipids [Internet]. 2012 Nov 1 [cited 2018 Jul 2];1821(11):1415–24. Available from: https://www.sciencedirect.com/science/article/pii/S1388198112001461?via%3Dihub
5. Jónasdóttir HS, Brouwers H, Kwekkeboom JC, van der Linden HMJ, Huizinga T, Kloppenburg M, et al. Targeted lipidomics reveals activation of resolution pathways in knee osteoarthritis in humans. Osteoarthr Cartil [Internet]. 2017 Jul 1 [cited 2018 Jul 11];25(7):1150–60. Available from: https://www.sciencedirect.com/science/article/pii/S1063458417308397?via%3Dihub#appsec1
6. Barden AE, Moghaddami M, Mas E, Phillips M, Cleland LG, Mori TA. Specialised pro-resolving mediators of inflammation in inflammatory arthritis. Prostaglandins Leukot Essent Fat Acids [Internet]. 2016 Apr 1 [cited 2020 Aug 30];107:24–9. Available from: https://pubmed.ncbi.nlm.nih.gov/27033423/
7. Tan LC, Mowat AG, Fazou C, Rostron T, Roskell H, Dunbar PR, et al. Specificity of T cells in synovial fluid: High frequencies of CD8+ T cells that are specific for certain viral epitopes. Arthritis Res [Internet]. 2000 [cited 2020 Aug 30];2(2):154–64. Available from: /pmc/articles/PMC17809/?report=abstract
8. Church LD, Filer AD, Hidalgo E, Howlett KA, Thomas AM, Rapecki S, et al. Rheumatoid synovial fluid interleukin-17-producing CD4 T cells have abundant tumor necrosis factor-alpha co-expression, but little interleukin-22 and interleukin-23R expression [Internet]. 2010 [cited 2020 Aug 30]. Available from: http://arthritis-research.com/content/12/5/R184
9. Schmidt T, Berthold E, Arve-Butler S, Gullstrand B, Mossberg A, Kahn F, et al. Children with oligoarticular juvenile idiopathic arthritis have skewed synovial monocyte polarization pattern with functional impairment—a distinct inflammatory pattern for oligoarticular juvenile arthritis. Arthritis Res Ther [Internet]. 2020 Dec 12 [cited 2020 Aug 30];22(1):186. Available from: https://arthritis-research.biomedcentral.com/articles/10.1186/s13075-020-02279-9
10. Yoon BR, Yoo SJ, Choi YH, Chung YH, Kim J, Yoo IS, et al. Functional phenotype of synovial monocytes modulating inflammatory T-cell responses in rheumatoid arthritis (RA). PLoS One [Internet]. 2014 Oct 17 [cited 2020 Aug 30];9(10):109775. Available from: /pmc/articles/PMC4201467/?report=abstract

11. Jaime P, García-Guerrero N, Estella R, Pardo J, García-Álvarez F, Martinez-Lostao L. CD56+/CD16– Natural Killer cells expressing the inflammatory protease granzyme A are enriched in synovial fluid from patients with osteoarthritis. Osteoarthr Cartil [Internet]. 2017 Oct 1 [cited 2018 Jul 20];25(10):1708–18. Available from: https://www.sciencedirect.com/science/article/pii/S1063458417310555?via%3Dihub

12. Yamin R, Berhani O, Peleg H, Aamar S, Stein N, Gamliel M, et al. High percentages and activity of synovial fluid NK cells present in patients with advanced stage active Rheumatoid Arthritis. Sci Rep. 2019 Dec 1;9(1).

13. Rao DA, Gurish MF, Marshall JL, Slowikowski K, Fonseka CY, Liu Y, et al. Pathologically expanded peripheral T helper cell subset drives B cells in rheumatoid arthritis. Nature. 2017 Feb 1;542(7639):110–4.

14. Bondeson J, Wainwright SD, Lauder S, Amos N, Hughes CE. The role of synovial macrophages and macrophage-produced cytokines in driving aggrecanases, matrix metalloproteinases, and other destructive and inflammatory responses in osteoarthritis. Arthritis Res Ther [Internet]. 2006 [cited 2018 Jul 20];8(6):R187. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17177994

15. Shen H, Goodall JC, Hill Gaston JS. Frequency and phenotype of T helper 17 cells in peripheral blood and synovial fluid of patients with reactive arthritis. J Rheumatol. 2010 Oct;37(10):2096–9.

16. Möttönen M, Heikkinen † J, Mustonen † L, Isomäki P, Luukkainen ¶ R, Lassila O. Clinical and Experimental Immunology 360 CD 4 + CD 25 + T cells with the phenotypic and functional characteristics of regulatory T cells are enriched in the synovial fluid of patients with rheumatoid arthritis. Clin Exp Immunol. 2005;140:360–7.

17. Rosshirt N, Hagmann S, Tripel E, Gotterbarm T, Kirsch J, Zeifang F, et al. A predominant Th1 polarization is present in synovial fluid of end-stage osteoarthritic knee joints: analysis of peripheral blood, synovial fluid and synovial membrane. Clin Exp Immunol. 2018;195:395–406.

18. Moradi B, Schnatzer P, Hagmann S, Rosshirt N, Gotterbarm T, Kretzer JP, et al. CD4 + CD25 +/high CD127 low/– regulatory T cells are enriched in rheumatoid arthritis and osteoarthritis joints-analysis of frequency and phenotype in synovial membrane, synovial fluid and peripheral blood [Internet]. 2014 [cited 2020 Aug 30]. Available from: http://arthritis-research.com/content/16/2/R97

19. Newson J, Motwani MP, Kendall AC, Nicolaou A, Muccioli GG, Alhouayek M, et al. Inflammatory Resolution Triggers a Prolonged Phase of Immune Suppression through COX-1/mPGES-1-Derived Prostaglandin E 2. Cell Rep [Internet]. 2017 Sep [cited 2019 Jul 7];20(13):3162–75. Available from: https://linkinghub.elsevier.com/retrieve/pii/S2211124717312585

20. Illges H, Braun M, Peter HH, Melchers I. Reduced expression of the complement receptor type 2 (CR2, CD21) by synovial fluid B and T lymphocytes. Clin Exp Immunol [Internet]. 2000 [cited 2020 Aug 30];122(2):270–6. Available from: /pmc/articles/PMC1905766/?report=abstract

21. Pelzek AJ, Grönwall C, Rosenthal P, Greenberg JD, McGeachy M, Moreland L, et al. Arthritis Rheumatol. 2017;69(6):1176–86.

22. Amara K, Clay E, Yeo L, Ramsköld D, Spengler J, Sippl N, et al. B cells expressing the IgA receptor FcRL4 participate in the autoimmune response in patients with rheumatoid arthritis. J Autoimmun [Internet]. 2017 Jul 1 [cited 2020 Aug 30];81:34–43. Available from: /pmc/articles/PMC5473332/?report=abstract

23. Zhao Q, Jung LK. Frequency of CD19+CD24hiCD38hi regulatory B cells is decreased in peripheral blood and synovial fluid of patients with juvenile idiopathic arthritis: a preliminary study. Pediatr Rheumatol Online J [Internet]. 2018 Jul 4 [cited 2018 Jul 20];16(1):44. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29973221

24. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, et al. 2010 Rheumatoid arthritis classification criteria: An American College of Rheumatology/European League Against Rheumatism collaborative initiative. ARTHRITIS Rheum [Internet]. 2010 [cited 2019 Oct 22];62(9):2569–81. Available from: www.arthritisrheum.organdwww.interscience.wiley.com

25. Aringer M, Costenbader K, Daikh D, Brinks R, Mosca M, Ramsey-Goldman R, et al. 2019 European League Against Rheumatism/American College of Rheumatology classification criteria for systemic lupus erythematosus. Ann Rheum Dis. 2019 Sep;78(9):1151–9.

26. Altman R, Asch E, Bloch D, Bole G, Borenstein D, Brandt K, et al. Development of criteria for the classification and reporting of osteoarthritis: Classification of osteoarthritis of the knee. Arthritis Rheum [Internet]. 1986 [cited 2020 Aug 30];29(8):1039–49. Available from: https://pubmed.ncbi.nlm.nih.gov/3741515/

27. Goldring SR, Goldring MB. Biology of the Normal Joint. Kelley Firestein's Textb Rheumatol [Internet]. 2017 Jan 1 [cited 2018 Jul 20];1-19.e4. Available from: https://www.sciencedirect.com/science/article/pii/B9780323316965000012

28. Moreno MJ, Clayburne G, Schumacher HR. Processing of noninflammatory synovial fluids with hyaluronidase for cytospin preparations improves the accuracy of differential counts. Diagn Cytopathol. 2000;22(4):256–8.

29. Ekmann A, Rigdal ML, Gröndahl G. Automated counting of nucleated cells in equine synovial fluid without and with hyaluronidase pretreatment. Vet Clin Pathol. 2010;39(1):83–9.

30. Jayadev C, Rout R, Price A, Hulley P, Mahoney D. Hyaluronidase treatment of synovial fluid to improve assay precision for biomarker research using multiplex immunoassay platforms. J Immunol Methods [Internet]. 2012;386(1–2):22–30. Available from: http://dx.doi.org/10.1016/j.jim.2012.08.012

31. Jaime P, García-Guerrero N, Estella R, Pardo J, García-Álvarez F, Martinez-Lostao L. CD56+/CD16- Natural Killer cells expressing the inflammatory protease granzyme A are enriched in synovial fluid from patients with osteoarthritis. Osteoarthr Cartil [Internet]. 2017 [cited 2019 Oct 21];25(10):1708–18. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28668542

32. Rao DA, Gurish MF, Marshall JL, Slowikowski K, Fonseka CY, Liu Y, et al. Pathologically expanded peripheral T helper cell subset drives B cells in rheumatoid arthritis. Nature [Internet]. 2017 [cited 2018 Jul 20];542(7639):110–4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28150777

33. Mateos J, Lourido L, Fernández-Puente P, Calamia V, Fernández-López C, Oreiro N, et al. Differential protein profiling of synovial fluid from rheumatoid arthritis and osteoarthritis patients using LC–MALDI TOF/TOF. J Proteomics [Internet]. 2012 Jun 6 [cited 2018 Jul 20];75(10):2869–78. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22245418

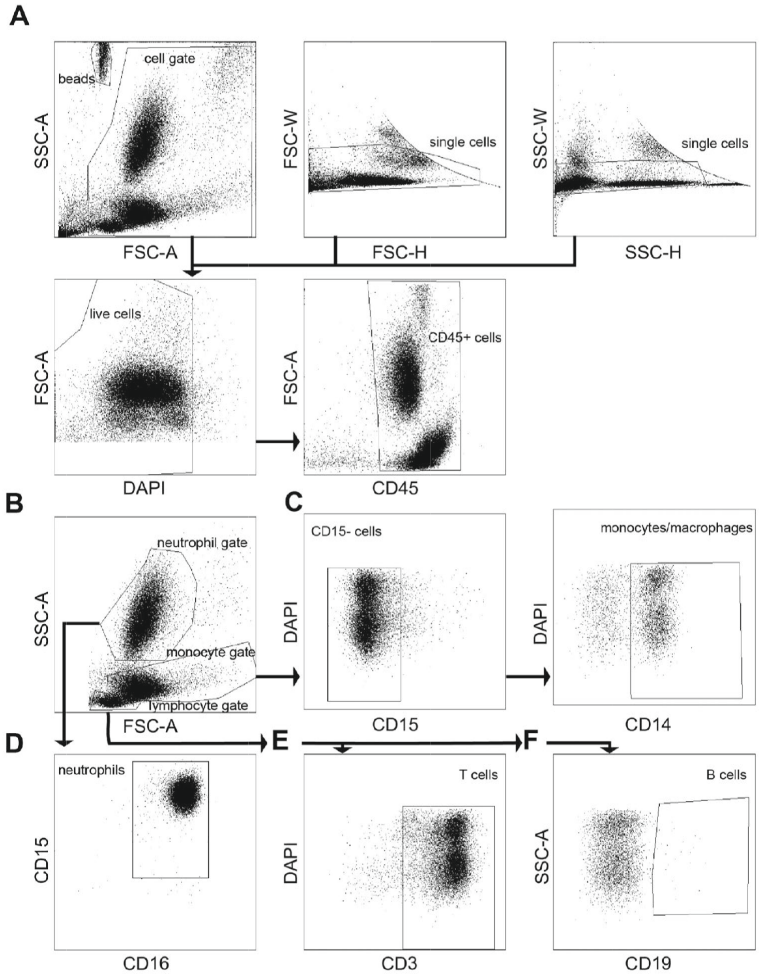
34. Naor D, Sionov RV, Ish-Shalom D. CD44: Structure, function, and association with the malignant process. Vol. 71, Advances in Cancer Research. Academic Press Inc.; 1997. p. 241–319.

35. Masson-Bessière C, Sebbag M, Durieux JJ, Nogueira L, Vincent C, Girbal-Neuhauser E, et al. In the rheumatoid pannus, anti-filaggrin autoantibodies are produced by local plasma cells and constitute a higher proportion of IgG than in synovial fluid and serum. Clin Exp Immunol [Internet]. 2000 Mar [cited 2020 May 12];119(3):544–52. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10691929

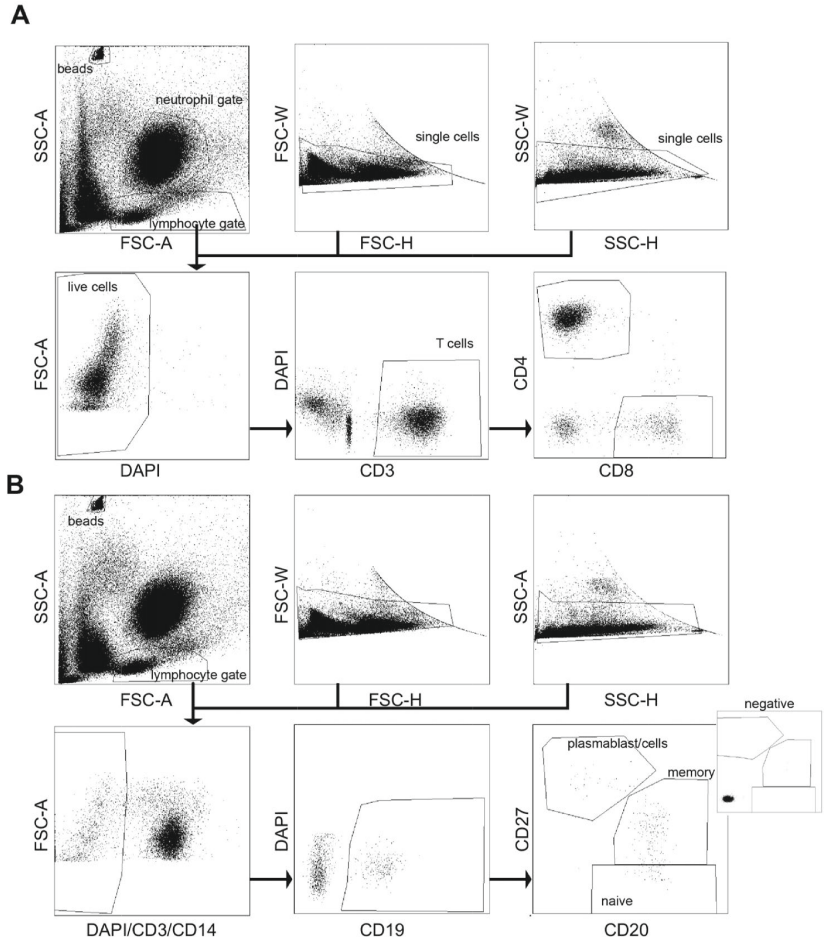
36. Polgár A, Falus A, Koó E, Ujfalussy I, Seszták M, Szuts I, et al. Elevated levels of synovial fluid antibodies reactive with the small proteoglycans biglycan and decorin in patients with rheumatoid arthritis or other joint diseases. Rheumatology (Oxford) [Internet]. 2003 Apr [cited 2020 May 12];42(4):522–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12649398

37. Willemze A, Shi J, Mulder M, Stoeken-Rijsbergen G, Drijfhout JW, Huizinga TWJ, et al. The concentration of anticitrullinated protein antibodies in serum and synovial fluid in relation to total immunoglobulin concentrations. Ann Rheum Dis [Internet]. 2013 Jun [cited 2020 May 12];72(6):1059–63. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23486413

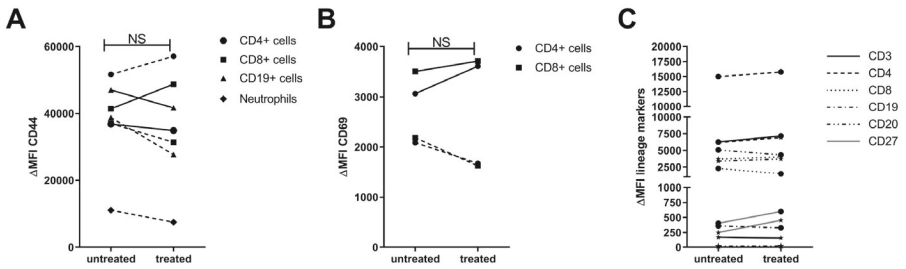
Supplemental figures



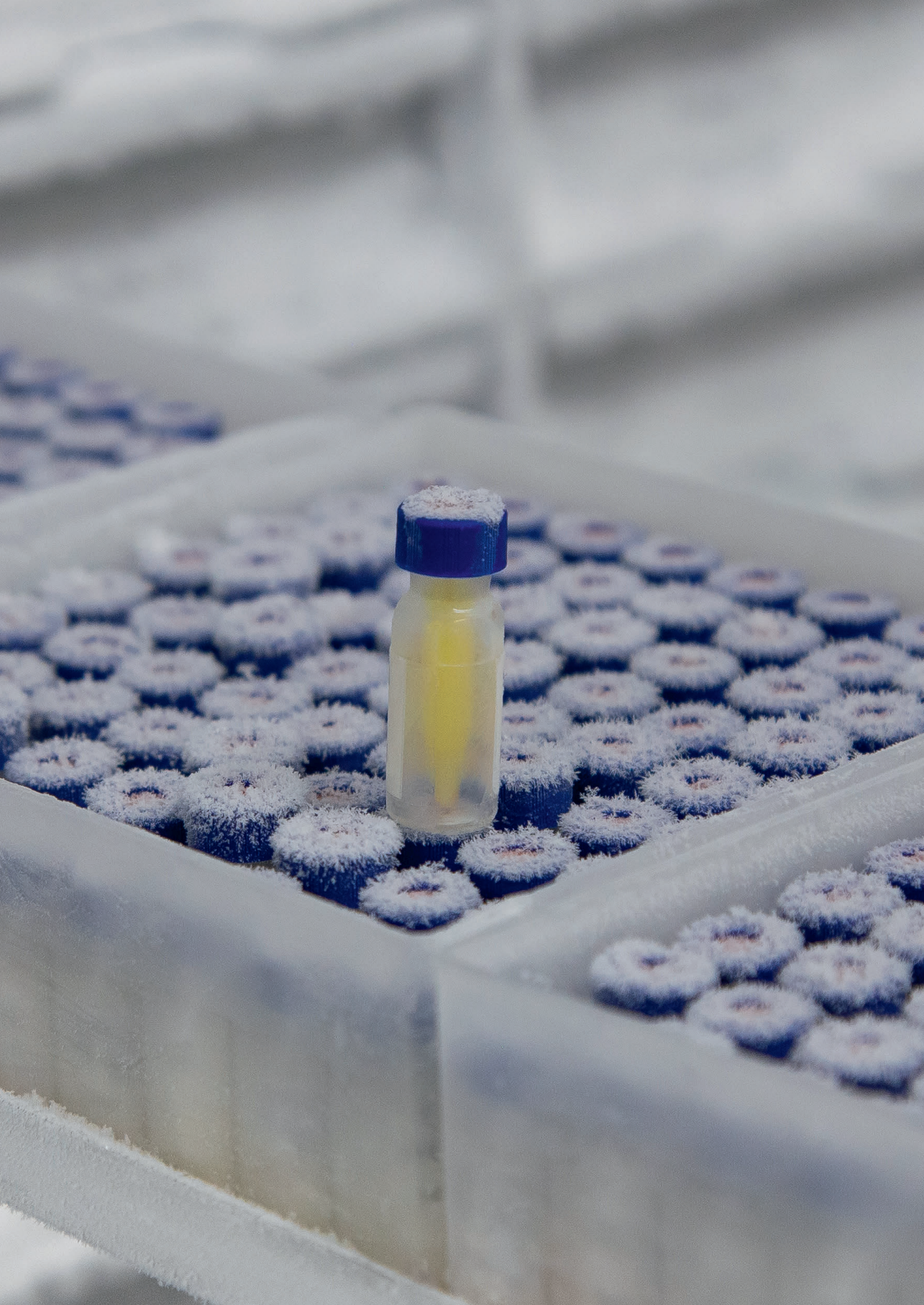
Supplementary figure 1. Gating strategy of experiments shown in figure 4A-C. **A)** Cells were gated separately from flow count beads on FSC-A/SSC-A and doublet cells were excluded by setting gates in FCS-W/FCS-H and SSC-W/SSC-H plots. Dapi staining was used to exclude dead cells and CD45+ cells were gated based on antibody staining. **B)** Neutrophil gates, monocyte gates and lymphocyte gates were set based on morphology on the FCS-A/SSC-A. **C)** The monocyte gate was plotted and contaminating CD15+ neutrophils were excluded after which CD14+ monocyte/macrophages were gated. **D)** The neutrophil gate is plotted and the neutrophils are characterized by CD15+ and CD16+ positivity The lymphocytes were plotted in panel **E)** and **F)** to be able to gate CD3+ T cells and CD19+ B cells respectively based on antibody staining.



Supplementary figure 2. Gating strategy of experiments shown in figure 4D and 4E**. A)** Flow count beads, neutrophil gates and lymphocyte gates were set based on morphology on the FCS-A/SSC-A. Doublet cells were excluded by setting gates in FCS-W/FCS-H and SSC-W/SSC-H plots. Dapi staining was used to exclude dead cells and the live cell gate was plotted to gate CD3+ cells based on antibody staining. The CD3+ cells were further analyzed for CD4 and CD8 expression. B) Gates for Flow count beads, lymphocyte gate and single cells were set the same was a in panel A. Dead cells, CD3+ cells and CD14+ cells were excluded by gating the negative cells. These Dapi/CD3/CD14 negative cells were plotted and analyzed for CD19. CD19+ B cells were further analyzed for CD27 and CD20 to evaluate naïve B cell, memory B cell and plasmablast/plasmacell numbers.



Supplementary figure 3. Hyaluronidase treatment does not affect cell marker expression. Synovial fluid was diluted 20x in PBS and divided in two. One sample was treated with hyaluronidase (treated) and the other sample was treated similar but without the addition of hyaluronidase (untreated). Cells were isolated by centrifugation and CD44 (**A**) and CD69 (**B**) expression was analyzed on various cell types in two donors (dashed versus closed line). **C**) Lineage marker expression is shown in two donors (stars versus dots). ΔMFI is calculated using the isotype control. Wilcoxon signed rank test was performed. n=2 donors.



8

General Discussion

Introduction

Lipids are a class of organic compounds which are insoluble in water. Lipids have a wide range of functions in the (human) body: they are the basis for cellular structure, represent a vital energy source and are involved in inflammatory processes. Steroids for example, are potent anti-inflammatory lipids. In contrast to steroids, are specialized pro resolving mediators (SPM) a class of free fatty acid derived molecules which are believed to have pro resolving mechanisms in a large variety of diseases. SPM are synthesized during periods of inflammation and are believed to be pivotal in the return to homeostasis. They were shown to enhance phagocytosis by macrophages, a mechanism by which macrophages remove dead cells and tissue debris caused by tissue inflammation. In addition, SPM were shown to inhibit neutrophil migration, a mechanism by which neutrophils are attracted to a site of inflammation as part of the innate immune system. Rheumatoid arthritis (RA), and to a lesser extent osteoarthritis (OA), are both diseases in which inflammation is present. The aim of the work presented in this thesis was to investigate whether SPM could have therapeutic potential in rheumatoid arthritis (RA) and osteoarthritis (OA). In addition, the studies in this thesis have focused on optimal processing methods for the detection and analysis of the cellular and molecular mediators investigated in these studies.

Resolution in the context of arthritis

Every (bio)medical (science) student will learn the meaning of words dolor, calor, tumor and rubor in the first months of their study. These Latin words describe the four classic signs of inflammation, namely pain, warmth, swelling and redness. Rheumatoid arthritis patients will recognize these symptoms directly as one or more joints in their body will be painful, swollen, red and warm. In contrast, OA patients have very painful joints but the warmth, swelling and redness are much less pronounced. The inflammation in RA patients is chronic. RA patients will have elevated systemic inflammatory markers, high numbers of innate immune cells as well as adaptive immune cells in the joints (**chapter 3**) and antigen specific immune responses indicated by the presence of (auto)antibodies. In OA patients on the other hand, structural damage to the joints is evident but there are only signs of low grade inflammation and this is localized to the joints . Both diseases might benefit from enhancing the resolution phase of inflammation.

To find evidence for the activity of pro-resolution pathways, in **chapter 2** we reviewed the published evidence on the potential role for fatty acids and fatty acid derived lipids in RA and OA. It became evident that indeed fatty acids and their derivatives can be found in serum, plasma and synovial fluid (SF) of both RA and OA patients, however, supplementation of dietary omega-3 fatty acids did not clearly affect disease outcome measures such as inflammation or pain and only two studies reported the detection of

SPM in arthritis patients(1, 2). We followed this up with a study in which we analyzed RA and OA SF and cells for the presence of pro-resolving pathways(3, 4) in **chapter 3**. In this study, we failed to detect SPM in the soluble fraction of synovial fluid (SF) of RA and OA patients. We did detect Resolvin D en Resolvin E precursors 17-HDHA and 18-HEPE, which were shown to have pro-resolving capacities (5, 6).

Detection of SPM in body fluids of arthritis patients

Detection of SPM has proven quite complex due to various reasons such as their low abundance, the presence of interfering matrix molecules and the presence of different stereoisomers. To overcome their low abundance, it is important to use a sensitive detection method such as mass spectrometry and if necessary concentrate the sample. Interfering matrix molecules can be eliminated using various methods such as breaking down the material using hyaluronidase in the case of hyaluronic acid which is present in SF. In addition, proteins can be precipitated and the lipid species can be specifically extracted from the sample by various extraction methods such as solid-phase extraction (SPE) where you can specifically enrich for specific lipid species. Furthermore the detection of SPM can be wrongfully interpreted as stereoisomers are not always easily distinguished. For example, LTB4 and 5*S*,12*S*-diHETE have the exact same retention time and cannot easily be distinguished by LC-MS/MS(1, 7). Importantly, the configuration of lipids determines the bioactivity and stability. For instance lipoxin A4 and its isomer lipoxin B4 were shown to have differential effects in vitro and the LXA4 epimer 15-epi-lipoxin is more stable than its counterpart(8). Moreover, a study performed by Skarke et al. described the detection of PD1 and Mar1 in humans after fish oil supplementation, however the chromatograms are different from the chromatograms shown in **chapter 6**(9). The different stereoisomers are the result of the various pathways involved in SPM synthesis. In 2014 a method for the detection of SPM was published resulting in a robust but sensitive method to detect SPM in body fluids(10). This method was used for all studies described in this thesis. However, despite the use of a highly sensitive method, previous published findings could not be reproduced. A possible explanation for the different results could be a different work-up of the samples. In **chapter 3** the SF was treated with hyaluronidase and centrifuged whereafter the soluble fraction was measured. Both previous reports do not mention a hyaluronidase treatment step, which we show is important to do when handling SF in the study described in **chapter** 7(11). It is therefore likely that both previously described studies had cellular material present in the SF samples during storage. Cellular material and perhaps extracellular matrix products are a source of fatty acids, fatty acids derivatives and phospholipase (PLA), lipoxygenase (LOX) and cyclooxygenase (COX) enzymes. From the results of the study described in **chapter 6**, it is evident that proteins should be precipitated before storage of samples

for lipidomic analysis because our data indicates residual enzyme activity and free fatty acid release during storage(12).

The role of SPM in arthritis

The evidence for the effect of SPM on arthritis tissue cells is scarce (**chapter 2**) and we therefore studied the effect of lipoxin A4, lipoxin B4, Maresin-1 and Resolvin E2 on cells isolated from OA patients(**chapter 4**). Both chondrocytes and cells isolated from synovium (synoviocytes) expressed some SPM receptors on the cell surface, which was also shown by others. However, there was no robust effect on IL-6 production by either CD14+ synovial macrophages or synovial fibroblasts as only in very specific conditions, there was an inhibition of IL-6 production by synovial fibroblasts (**chapter 4**). This decrease was only detected in the situation where a pool of SPM at a concentration of 10 nM was supplemented and the effect was not there at the 100 nM concentration. In addition, the in vivo treatment of high fat diet induced experimental OA with lipoxin A4, lipoxin B4, Maresin-1 did not result in decreased disease activity. However, in these experiments only one dosage was tested and the SPM were injected systemically. We did not investigate whether the SPM could be detected locally which is most likely needed to exert the hypothesized effects on decreased disease activity. Later, others showed that intra-articular injection of RvD1 could limit synovitis and cartilage damage in a surgically induced OA model(13). In addition to adjustment of the admission route, a different type of SPM, such as D series resolvins or their precursor 17-HDHA could also be investigated in future in vivo and in vitro studies.

The production of SPM is a multi-step process involving several enzymes. It is shown by some that SPM can be produced by a single cell type while others propose the subsequent involvement of different cell types(5, 14–18). However, several cell types were stimulated in the various studies described in this thesis, but neither synovial fluid cells, synoviocytes, monocyte derived macrophages or neutrophils produced SPM (**chapters 3 and 5**). The data from the study described in **chapter 3** shows that the intermediate hydroxylated products are indeed available extracellular for potential take up and conversion into SPM by other cell types. However, the actual SPM RvD2 can only be detected in the insoluble fraction of the SF. These data might indicate that the SPM are trapped in the extracellular matrix or bound to the cells and that their production and effects might be only present between cells in very close proximity. Indeed, as isolated cells either from blood or from tissues did not produce SPM, it could be that elements of the tissue microenvironment are vital in the production and effects of endogenous SPM. The effect of SPM in cocultures or tissue pieces remains to be investigated. Furthermore, local administration of SPM in experimental mouse models might be a better approach then systemic administration. Finally, for most SPM stable analogs exist, such as aspirin

triggered SPM (AT-SPM)(17, 19–21). Aspirin acetylates COX enzymes which can produce AT-SPM. AT-SPM are so called R epimers, which are more resistant to (auto)oxidation and therefore have much longer half-lives(22). Incubation of cells with exogenous SPM or administration of exogenous SPM might lead to rapid inactivation and therefore no biological effects. Finally, RvE1 and Mar-1 were shown to have tissue regenerative properties(23–26). Furthermore, RvD1 was even shown to have cartilage protective properties in vitro and in vivo(27, 28). It would therefore be interesting to investigate the tissue regenerative properties on joint tissues instead of only focusing on inflammatory processes. As RvD2 was detected in OA SF (**chapter 3**), the Resolvin D series receptor was detected on OA joint tissue cells (**chapter 4**) and cartilage protective effects were reported previously, further research should focus on therapeutic properties of D series resolvins in OA.

The role for fatty acids in arthritis

Especially omega-3 fatty acids have gained the popular status of being anti-inflammatory and protective especially in cardiovascular disease and cardiovascular disease related mortality(29). The evidence is unambiguous, intake of omega-3 fatty acids drastically decreases incidence, mortality and disease severity. Already for decades government health institutes, such as the Dutch voedingscentrum, advise citizens to consume foods containing omega-3 fatty acids such as fatty fish. It is no surprise that the effect of omega-3 fatty acids was also studied in a wide range of other diseases including RA and OA, but dietary intake of fish oils does not prevent or limit the development of human arthritis disease (**chapter 2**)(30–32). The difference in therapeutic potential of fatty acids between the two disease types is easily explained. Fatty acids are present in the blood vessels and the ratio of unsaturated and saturated fatty acids is very important in remaining the blood vessels healthy. Changes is this ratio towards more saturated fatty acids directly leads to increased fatty acid deposits and therefore increased chance of blood clots leading to cardiovascular events. Such a direct role for fatty acids in arthritis is not present, however, fatty acid derived lipid mediators such as prostaglandin E2 (PGE2) and leukotriene B4 (LTB4) are important pro-inflammatory mediators in RA. Surprisingly, the study described in **chapter 5** describes an pro-resolving role for an omega-6 fatty acid, as adrenic acid (AdA) potently inhibits the production of LTB4 by neutrophils(33). Furthermore, AdA was able to reduce arthritis disease development in mice suffering from K/BxN serum transfer induced arthritis. As the joints of human RA patients contain large numbers of neutrophils,(**chapter 3**) inhibiting the production of the potent neutrophil chemoattractant LTB4 by treatment with AdA might be beneficial to these patients.

Conclusions and future perspectives

The studies described in this thesis provides the field with valuable data on the potential therapeutic effects of fatty acids and SPM in RA and OA. The omega-6 fatty acid AdA shows potent pro-resolving effects on the production of pro-inflammatory chemoattractant LTB4 with great promise to limit RA disease progression. In contrast to the promising potential therapeutic effects of AdA in RA, the evidence for pro-resolving effects in OA is still scarce. The results of the studies from this thesis show that neither LXA4, LXB4, RvE2 or Mar-1 were able to reduce OA disease activity in the experimental set-up we used. Further research is necessary to determine whether SPM have therapeutic potential in OA by investigating different dosages, admission routes and a larger range of SPM subtypes. In addition, Future research into biological effects of SPM should focus on the total local microenvironment opposite to the effect on isolated cells and in vivo treatment as our data indicate that SPM are present locally and as they have no effects on isolated cells using the various cell types and experimental set-ups we used.. Finally, the studies described in this thesis show the utmost critical importance of the right sample preparation and storage for the intended subsequent analysis.

References

1. Giera, M., A. Ioan-Facsinay, R. Toes, F. Gao, J. Dalli, A. M. Deelder, C. N. Serhan, and O. A. Mayboroda. 2012. Lipid and lipid mediator profiling of human synovial fluid in rheumatoid arthritis patients by means of LC–MS/MS. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* 1821: 1415–1424.
2. Barden, A. E., M. Moghaddami, E. Mas, M. Phillips, L. G. Cleland, and T. A. Mori. 2016. Specialised pro-resolving mediators of inflammation in inflammatory arthritis. *Prostaglandins Leukot. Essent. Fat. Acids* 107: 24–29.
3. Brouwers, H., J. Von Hegedus, R. Toes, M. Kloppenburg, and A. Ioan-Facsinay. 2015. Lipid mediators of inflammation in rheumatoid arthritis and osteoarthritis. *Best Pract. Res. Clin. Rheumatol.* 29: 741–755.
4. Jónasdóttir, H. S., H. Brouwers, J. C. Kwekkeboom, H. M. J. van der Linden, T. Huizinga, M. Kloppenburg, R. E. M. Toes, M. Giera, and A. Ioan-Facsinay. 2017. Targeted lipidomics reveals activation of resolution pathways in knee osteoarthritis in humans. *Osteoarthr. Cartil.* 25: 1150–1160.
5. von Hegedus, J. H., A. S. Kahnt, R. Ebert, M. Heijink, R. E. M. Toes, M. Giera, and A. Ioan-Facsinay. 2020. Toll-like receptor signaling induces a temporal switch towards a resolving lipid profile in monocyte-derived macrophages. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* 1865: 158740.
6. Endo, J., M. Sano, Y. Isobe, K. Fukuda, J. X. Kang, H. Arai, and M. Arita. 2014. 18-HEPE, an n-3 fatty acid metabolite released by macrophages, prevents pressure overload–induced maladaptive cardiac remodeling. *J. Exp. Med.* 211: 1673–1687.
7. Jónasdóttir, H. S., C. Papan, S. Fabritz, L. Balas, T. Durand, I. Hardardottir, J. Freysdottir, and M. Giera. 2015. Differential Mobility Separation of Leukotrienes and Protectins. *Anal. Chem.* 87: 5036–5040.
8. Serhan, C. N. 2005. Lipoxins and aspirin-triggered 15-epi-lipoxins are the first lipid mediators of endogenous anti-inflammation and resolution. *Prostaglandins, Leukot. Essent. Fat. Acids* 73: 141–162.
9. Skarke, C., N. Alamuddin, J. A. Lawson, X. Li, J. F. Ferguson, M. P. Reilly, and G. A. FitzGerald. 2015. Bioactive products formed in humans from fish oils. *J. Lipid Res.* 56: 1808.
10. Jónasdóttir, H. S., A. Ioan-Facsinay, J. Kwekkeboom, H. Brouwers, A. M. Zuurmond, R. Toes, A. M. Deelder, and M. Giera. 2014. An Advanced LC–MS/MS Platform for the Analysis of Specialized Pro-Resolving Lipid Mediators. *Chromatographia* 78: 391.
11. A, B., M. E, C. KD, P. M, and M. TA. 2014. Short-term n-3 fatty acid supplementation but not aspirin increases plasma proresolving mediators of inflammation. *J. Lipid Res.* 55: 2401–2407.
12. Jonasdottir, H. S., H. Brouwers, R. E. M. Toes, A. Ioan-Facsinay, and M. Giera. 2018. Effects of anticoagulants and storage conditions on clinical oxylipid levels in human plasma. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* 1863: 1511–1522.
13. Sun, A. R., X. Wu, B. Liu, Y. Chen, C. W. Armitage, A. Kollipara, R. Crawford, K. W. Beagley, X. Mao, Y. Xiao, and I. Prasadam. 2019. Pro-resolving lipid mediator ameliorates obesity induced osteoarthritis by regulating synovial macrophage polarisation. *Sci. Rep.* 9.
14. Werz, O., J. Gerstmeier, S. Libreros, X. De la Rosa, M. Werner, P. C. Norris, N. Chiang, and C. N. Serhan. 2018. Human macrophages differentially produce specific resolvin or leukotriene signals that depend on bacterial pathogenicity. *Nat. Commun. 2017 91* 9: 1–12.

15. Serhan, C. N., and K. A. Sheppard. 1990. Lipoxin formation during human neutrophil-platelet interactions: Evidence for the transformation of leukotriene A4 by platelet 12-lipoxygenase in vitro. *J. Clin. Invest.* 85: 772–780.

16. Norris, P. C., D. Gosselin, D. Reichart, C. K. Glass, and E. A. Dennis. 2014. Phospholipase A2 regulates eicosanoid class switching during inflammasome activation. *Proc. Natl. Acad. Sci. U. S. A.* 111: 12746–12751.

17. Clària, J., and C. N. Serhan. 1995. Aspirin triggers previously undescribed bioactive eicosanoids by human endothelial cell-leukocyte interactions. *Proc. Natl. Acad. Sci. U. S. A.* 92: 9475–9479.

18. ClÃ ria, J., M. H. Lee, and C. N. Serhan. 1996. Aspirin-triggered lipoxins (15-epi-LX) are generated by the human lung adenocarcinoma cell line (A549)-neutrophil interactions and are potent inhibitors of cell proliferation. *Mol. Med.* 2: 583.

19. Gilligan, M. M., A. Gartung, M. L. Sulciner, P. C. Norris, V. P. Sukhatme, D. R. Bielenberg, S. Huang, M. W. Kieran, C. N. Serhan, and D. Panigrahy. 2019. Aspirin-triggered proresolving mediators stimulate resolution in cancer. *Proc. Natl. Acad. Sci.* 116: 6292–6297.

20. J, D., W. JW, C. RA, A. H, C. CY, C. N, P. NA, and S. CN. 2013. Resolvin D3 and aspirin-triggered resolvin D3 are potent immunoresolvents. *Chem. Biol.* 20: 188–201.

21. Serhan, C. N., J. F. Maddox, N. A. Petasis, I. Akritopoulou-Zanze, A. Papayianni, H. R. Brady, S. P. Colgan, and J. L. Madara. 2002. Design of Lipoxin A4 Stable Analogs That Block Transmigration and Adhesion of Human Neutrophils. *Biochemistry* 34: 14609–14615.

22. Serhan, C. N. 2014. Pro-resolving lipid mediators are leads for resolution physiology. *Nature* 510: 92–101.

23. Albuquerque-Souza, E., F. Schulte, T. Chen, M. Hardt, H. Hasturk, T. E. Van Dyke, M. Holzhausen, and A. Kantarci. 2020. Maresin-1 and Resolvin E1 Promote Regenerative Properties of Periodontal Ligament Stem Cells Under Inflammatory Conditions. *Front. Immunol.* 0: 2509.

24. H, H., K. A, G.-S. E, B. A, A. C, S. CN, and V. D. TE. 2007. Resolvin E1 regulates inflammation at the cellular and tissue level and restores tissue homeostasis in vivo. *J. Immunol.* 179: 7021–7029.

25. Wang, C. W., S. H. Yu, T. Fretwurst, L. Larsson, J. V Sugai, J. Oh, K. Lehner, Q. Jin, and W. V Giannobile. 2020. Maresin 1 Promotes Wound Healing and Socket Bone Regeneration for Alveolar Ridge Preservation. *J. Dent. Res.* 22034520917903.

26. Serhan, C. N., J. Dalli, S. Karamnov, A. Choi, C. Park, Z. Xu, R. Ji, M. Zhu, and N. A. Petasis. 2012. Macrophage proresolving mediator maresin 1 stimulates tissue regeneration and controls pain. *FASEB J.* 26: 1755–1765.

27. Norling, L. V., S. E. Headland, J. Dalli, H. H. Arnardottir, O. Haworth, H. R. Jones, D. Irimia, C. N. Serhan, and M. Perretti. 2016. Proresolving and cartilage-protective actions of resolvin D1 in inflammatory arthritis. *JCI Insight* 1.

28. Sun, A. R., X. Wu, B. Liu, Y. Chen, C. W. Armitage, A. Kollipara, R. Crawford, K. W. Beagley, X. Mao, Y. Xiao, and I. Prasadam. 2019. Pro-resolving lipid mediator ameliorates obesity induced osteoarthritis by regulating synovial macrophage polarisation. *Sci. Reports 2019 91* 9: 1–13.

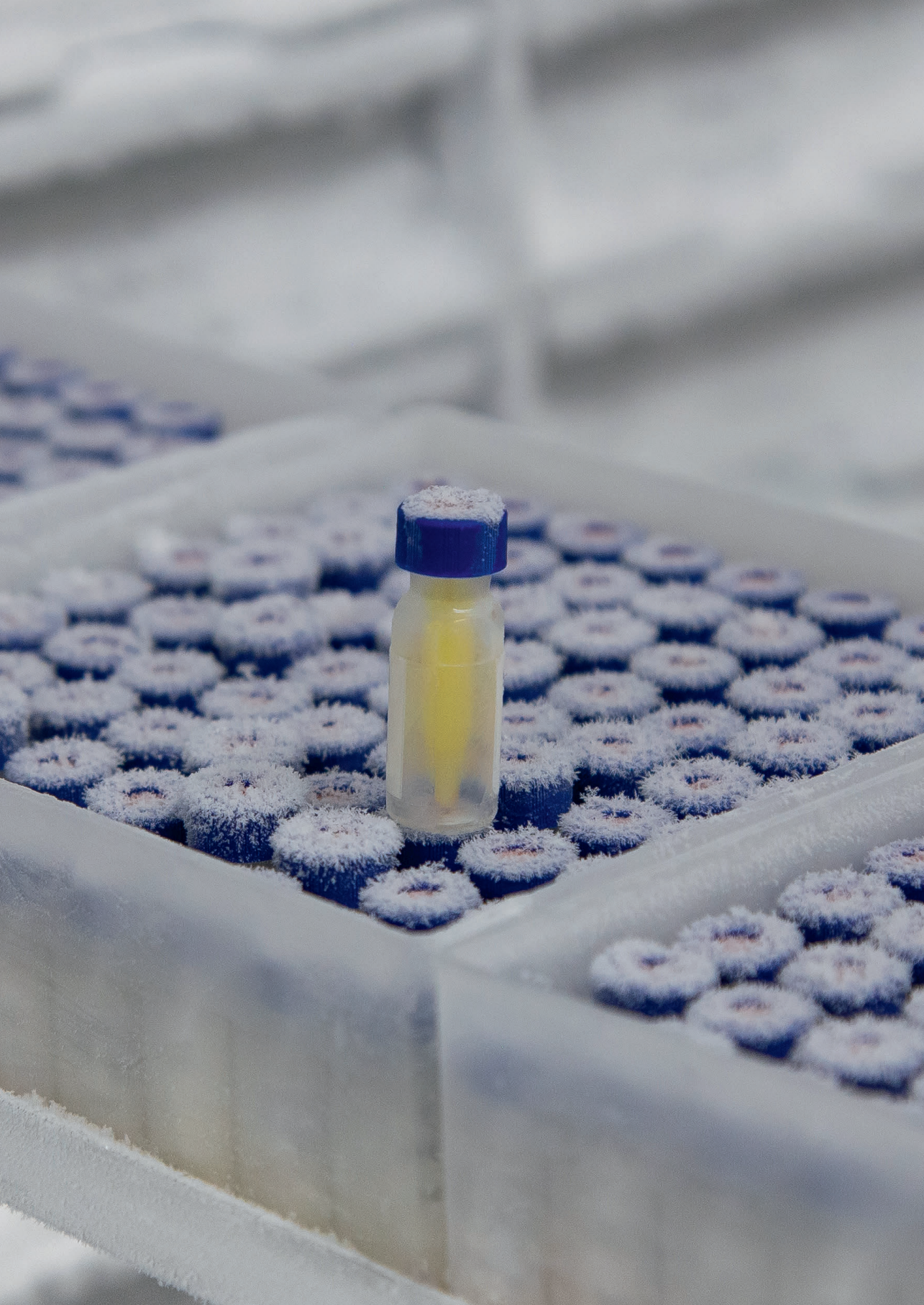
29. Sacks, F. M., A. H. Lichtenstein, J. H. Y. Wu, L. J. Appel, M. A. Creager, P. M. Kris-Etherton, M. Miller, E. B. Rimm, L. L. Rudel, J. G. Robinson, N. J. Stone, and L. V. Van Horn. 2017. Dietary Fats and Cardiovascular Disease: A Presidential Advisory From the American Heart Association. *Circulation* 136: e1–e23.

30. Frommer, K. W., R. Hasseli, A. Schäffler, U. Lange, S. Rehart, J. Steinmeyer, M. Rickert, K. Sarter, M. M. Zaiss, C. Culmsee, G. Ganjam, S. Michels, U. Müller-Ladner, and E. Neumann. 2019. Free Fatty Acids in Bone Pathophysiology of Rheumatic Diseases. *Front. Immunol.* 10: 2757.

31. Loef, M., J. W. Schoones, M. Kloppenburg, and A. Ioan-Facsinay. 2019. Fatty acids and osteoarthritis: different types, different effects. *Jt. Bone Spine* 86: 451–458.

32. Ioan-Facsinay, A., and M. Kloppenburg. 2018. Bioactive lipids in osteoarthritis. *Curr. Opin. Rheumatol.* 30: 108–113.

33. Brouwers, H., H. S. Jónasdóttir, M. E. Kuipers, J. C. Kwekkeboom, J. L. Auger, M. Gonzalez-Torres, C. López-Vicario, J. Clària, J. Freysdottir, I. Hardardottir, J. Garrido-Mesa, L. V. Norling, M. Perretti, T. W. J. Huizinga, M. Kloppenburg, R. E. M. Toes, B. Binstadt, M. Giera, and A. Ioan-Facsinay. 2020. Anti-Inflammatory and Proresolving Effects of the Omega-6 Polyunsaturated Fatty Acid Adrenic Acid. *J. Immunol.* 205: 2840–2849.



Appendices

Osteoartritis (OA), ook wel artrose genoemd, en reumatoïde artritis (RA) zijn beide aandoeningen die effect hebben op de gewrichten. Artrose komt voor bij ongeveer 1,5 miljoen Nederlanders en vooral bij ouderen. Reumatoïde artritis komt minder voor, maar toch hebben op dit moment zo'n 270.000 mensen deze diagnose in Nederland. Het ziekteverloop en de symptomen verschillen wel van elkaar. Bij RA patiënten worden hoge ontstekingswaarden gemeten en zij kunnen meestal met succes behandeld worden met medicijnen die actief het immuun systeem onderdrukken. Op radiografische beelden zoals MRI worden bij artrose patiënten ook signalen van ontsteking gemeten, maar wordt het beeld vooral gedomineerd door grote schade aan de gewrichten. Het kraakbeen is versleten en het bot is niet meer mooi glad maar vormt osteofyten, dit zijn uitstulpingen van het bot. Artrose patiënten hebben hierdoor vaak veel pijn in de gewrichten en nemen hiervoor pijnstillers zoals paracetamol. Er is nog veel onduidelijk over het ontstaan van artrose en over hoe we deze ziekte in een vroeg stadium kunnen behandelen zodat grote schade aan de gewrichten wordt voorkomen.

Rond het jaar 2000 werd er door een groep wetenschappers in Amerika een interessante ontdekking gedaan. Een bepaald type vetten, afkomstig van onverzadigde vetzuren, zouden eigenschappen hebben die ervoor zorgen dat een ontsteking op tijd wordt beëindigd, zodat grote schade aan het weefsel voorkomen wordt. Dit proces wordt resolutie genoemd en de vetten kregen de naam pro-resolving mediators (SPM), ofwel mediatoren die pro-resolutie eigenschappen hebben. De vetzuren werden in 4 categorieën verdeeld op basis van hun molecuulformule, namelijk de lipoxins, maresins, protectins en resolvins. In de studies die beschreven staan in dit proefschrift hebben we onderzocht of deze SPM de potentie hebben om te dienen als therapie in artrose. Daarnaast hadden een tweetal studies beschreven in dit proefschrift het doel om te onderzoeken op welke manier het lichaamsmateriaal behandeld en opgeslagen moet worden voordat het gebruikt wordt om metingen in te verrichten.

We zijn dit onderzoek begonnen door in een literatuurstudie te onderzoeken wat er al bekend was over de aanwezigheid van deze SPM en daaraan gerelateerde vetten in RA en OA **(hoofdstuk 2).** We keken voornamelijk naar de SPM zelf en de vetten waaruit zij geproduceerd worden (precursor vetzuren), de omega-3 en omega-6 onverzadigde vetten. De literatuurstudie liet zien dat de precursor vetzuren aanwezig zijn in het bloed van RA en OA patiënten maar ook in het gewrichtsvocht (synoviaal vocht). Één van de vetzuren, het omega-6 vetzuur arachidonzuur kan worden omgezet naar pro-ontstekings vetten zoals prostaglandines en leukotrienes. Vooral deze pro-ontstekings vetten waren meetbaar in het gewrichtsvocht in RA en in mindere mate in dat van artrose patiënten. De daadwerkelijk werden maar door één studie gerapporteerd en alleen in RA gewrichtsvocht.

Omdat het bewijs voor de aanwezigheid van SPM in RA en artrose patiënten zo schaars was, besloten we zelf de aanwezigheid van deze vetten te onderzoeken in gewrichtsvocht (**hoofdstuk 3**). Hiervoor gebruikten we een techniek genaamd tandem massaspectometrie welke ontwikkeld is binnen het Leids Universitair Medisch Centrum (LUMC). In de studie beschreven in **hoofdstuk 3** hebben we gewrichtsvocht gemeten van RA en artrose patienten. Gewrichtsvocht is heel stroperig en het bevat immuun cellen en onoplosbare deeltjes. Om te voorkomen dat de stroperige structuur, de cellen en de onoplosbare deeltjes storen in de meting, behandelden we het gewrichtsvocht eerst met het enzym hyaluronidase. Na deze behandeling wordt het gewrichtsvocht heel hard gecentrifugeerd, waardoor alleen het vloeibare vocht overblijft. Na deze behandeling, konden we 37 verschillende vetten detecteren, variërend van de precursor onverzadigde vetzuren, tussenproducten zoals 17-HDHA en 15-HETE en pro-ontstekings vetten zoals prostaglandines en leukotrines. Maar ondanks de zeer gevoelige techniek, konden we geen SPM detecteren, behalve als we de meting verrichtte op het onoplosbare gedeelte van het gewrichtsvocht, en ook dan konden we maar één SPM detecteren, Resolvin D2. In **hoofdstuk 3** laten we verder zien dat de geïsoleerde cellen uit het gewrichtsvocht verschillende vetten kan produceren als ze worden geactiveerd. Hiervoor gebruiken ze dezelfde enzymen die nodig zijn als de productie van SPM, maar de daadwerkelijke SPM werden in deze experimenten niet geproduceerd.

Zoals aan het begin van deze samenvatting beschreven staat, zijn signalen van ontsteking zichtbaar op radiografische beelden van gewrichten van artrose patienten. Onze hypothese is dat deze ontsteking bijdraagt aan de schade die we zien aan de gewrichten zoals het verdwijnen van kraakbeen en het ontstaan van osteofyten. Als de SPM inderdaad in staat zijn om resolutie te bevorderen, zou er met het verdwijnen van de ontsteking ook minder schade moeten ontstaan in de gewrichten. Dit wilden we graag onderzoeken in een diermodel, het hoog vet geïnduceerde artrose muismodel. In de studie beschreven in **hoofdstuk 4** laten we zien dat wanneer je muizen voed met een hoog vet dieet, deze muizen ontsteking en schade in de gewrichten ontwikkelen. We hebben deze muizen behandeld met een mix van verschillende SPM, namelijk Maresin 1, Lipoxin A4 en Lipoxin B4, maar deze behandeling had geen effect op de ontsteking en schade. Ook zagen we geen effect van deze SPM op de productie van ontstekingsfactor interleukin-6, welke gemaakt wordt door verschillende cellen in het gewricht.

Vervolgens onderzochten we in **hoofdstuk 5** hoe de resolutie fase precies verloopt in een diermodel waarin een muis buikvliesontsteking krijgt door het inspuiten van gistdeeltjes. Deze muis wordt in de eerste 12 uur erg ziek, waarna de muis zichzelf herstelt, een effectieve resolutie fase dus. Hier ontdekten we dat een omega-6 vetzuur, adreenzuur, zich opstapelt in de buikholte tijdens de resolutie fase. We tonen daarna in deze studie

aan dat adreenzuur pro-resolutie eigenschappen heeft, zoals het inhiberen van de migratie van immuun cellen en het bevorderen van het opruimen van dode immuun cellen door macrofagen. Daarnaast inhibeert adreenzuur de productie van de pro-ontstekings vet leukotriene B4 door verschillende typen immuun cellen. Als laatste tonen we in deze studie aan dat de aanwezigheid van adreenzuur tijdens de inductie van artritis ervoor zorgt dat er veel minder ontsteking ontstaat in deze muizen dan wanneer ze niet worden blootgesteld aan adreenzuur.

De laatste twee studies in dit proefschrift beschrijven hoe belangrijk het is om het lichaamsmateriaal dat onderzoekers gebruiken in hun studie op een juiste manier te behandelen en te bewaren. In de studie in **hoofdstuk 6** hebben we de effecten onderzocht van verschillende methodes die te maken hebben met de verzameling, verwerking en opslag van bloedplasma, op vetzuurmetingen. De aanleiding voor dit onderzoek was het resultaat van de studie beschreven in **hoofdstuk 3**, waar we geen SPM konden vinden in gewrichtsvocht van RA en artrose patienten terwijl zij in eerder onderzoek wel waren gedetecteerd. In deze studie hebben we bloedplasma verzameld en in een deel daarvan op dezelfde dag, zo snel mogelijk, de verschillende vetten geanalyseerd. De rest van het bloedplasma hebben we op kamertemperatuur, 4°C, -20°C of -80°C bewaard en na verschillende opslagperiodes hebben we de vetten geanalyseerd. De resultaten laten zien dat de tijd en temperatuur tot aan het invriezen, als ook de vriezertemperatuur zeer belangrijk is. Het grootste verschil tussen de nulmeting zagen we bij opslag bij -20°C voor een periode langer dan een half jaar. De SPMs protectin DX and Resolvin D5 werden meetbaar in het materiaal na een opslagperiode van een half jaar ondanks dat deze vetten niet in de nulmeting gedetecteerd konden worden.

De studie beschreven in **hoofdstuk 7** had als doel om te onderzoeken of gewrichtsvocht een voorbehandeling nodig heeft om betrouwbare resultaten te verkrijgen in een aantal veelgebruikte analysetechnieken. Zoals hierboven beschreven hebben we deze behandeling met hyaluronidase ook toegepast op de gewrichtsvochten die we analyseerden voor de studie beschreven in **hoofdstuk 3**. Hyaluroniase is een enzym dat hyaluronzuur afbreekt, de stof die het gewrichtsvocht stroperig maakt. We laten zien dat het vooral van belang is als de immuun cellen in het gewrichtsvocht worden onderzocht. In één van de vochten bleef zelfs 70% van de immuun cellen ‘hangen’ in het vocht wanneer deze niet werd behandeld met hyaluronidase. Deze achtergebleven cellen werden daardoor gemist in de flowcytometrie analyse, dit is een techniek waarbij cellen worden gelabeld met fluorescerende markers en vervolgens worden aangestraald met gekleurde lasers. Deze techniek wordt veel gebruikt voor het karakteriseren van aanwezige immuun cellen om op die manier iets te kunnen zeggen over het type immuun respons.

Samengenomen tonen de studies beschreven in dit proefschrift aan dat SPM wellicht niet de potentie hebben om als therapie te worden ingezet voor artrose. Het vetzuur adreenzuur daarentegen heeft veelbelovende pro-resolutie effecten op immuun cellen en in een artritis muismodel. Verder onderzoek is nodig om het mechanisme van adreenzuur te ontrafelen en om te onderzoeken of het pro-resolutie effect ook in patienten standhoudt. De laatste studies beschreven in dit proefschrift laten zien dat de manier van verwerken en het bewaren van lichaamsmateriaal, effect heeft op de resultaten van bepaalde analyses. Grotere bewustwording hiervan zal leiden tot kwalitatief hoogwaardiger onderzoek.

Curriculum vitae

Hilde Brouwers was born in Alkmaar on the 22nd of June in 1990. In 2008 she graduated from the Stedelijk Dalton College (VWO) in Alkmaar and directly proceeded to study Biomedical Sciences at the Vrije Universiteit (VU) Amsterdam. During her bachelor internship at the molecular microbiology department of the VU Amsterdam, under supervision of Prof. dr. Wilbert Bitter, she got very excited to have a career in science. During her masters she specialized in infectious diseases and immunology and completed two internships. She studied the possibilities to improve detection of specific bacterial species for diagnostic purposes at the VU medical center under the supervision of Prof. dr. Paul Savelkoul and dr. Dries Budding. She also studied the effect of hepatitis B antigens on the activation of dendritic cells at the department of gastroenterology of the Erasmus MC under supervision of prof. dr. Andrea Woltman and dr. Nadine van Montfoort. Via Nadine she got invited to apply for a PhD position at the Rheumatology department of the LUMC. Hilde started her PhD in 2013 under the supervision of Prof. dr. René Toes, Prof. dr. Margreet Kloppenburg and Dr. Andreea Ioan-Facsinay and conducted experiments for the following 4.5 years and received her doctoral in 2022. Next to working with her beloved neutrophils, she developed an interest in the ethics and logistics of biobanking while working with patient materials during her PhD. Currently, Hilde works at the Erasmus MC as head of the Central Biobank facility.

List of publications

1. Hyaluronidase treatment of synovial fluid is required for accurate detection of inflammatory cells and soluble mediators. **Brouwers H**, von Hegedus JH, van der Linden E, Mahdad R, Kloppenburg M, Toes R, Giera M, Ioan-Facsinay A.Arthritis Res Ther. 2022 Jan 8;24(1):18. doi: 10.1186/s13075-021-02696-4.

2. Anti-Inflammatory and Proresolving Effects of the Omega-6 Polyunsaturated Fatty Acid Adrenic Acid. **Brouwers H**, Jónasdóttir HS, Kuipers ME, Kwekkeboom JC, Auger JL, Gonzalez-Torres M, López-Vicario C, Clària J, Freysdottir J, Hardardottir I, Garrido-Mesa J, Norling LV, Perretti M, Huizinga TWJ, Kloppenburg M, Toes REM, Binstadt B, Giera M, Ioan-Facsinay A. J Immunol. 2020 Nov 15;205(10):2840-2849. doi: 10.4049/jimmunol.1801653. Epub 2020 Oct 2.

3. Lipid metabolism of leukocytes in the unstimulated and activated states. Alarcon-Barrera JC, von Hegedus JH, **Brouwers H**, Steenvoorden E, Ioan-Facsinay A, Mayboroda OA, Ondo-Mendez A, Giera M. Anal Bioanal Chem. 2020 Apr;412(10):2353-2363. doi: 10.1007/s00216-020-02460-8. Epub 2020 Feb 14.

4. Effects of anticoagulants and storage conditions on clinical oxylipid levels in human plasma. Jonasdottir HS, **Brouwers H**, Toes REM, Ioan-Facsinay A, Giera M. Biochim Biophys Acta Mol Cell Biol Lipids. 2018 Dec;1863(12):1511-1522. doi: 10.1016/j.bbalip.2018.10.003. Epub 2018 Oct 9.

5. Targeted lipidomics reveals activation of resolution pathways in knee osteoarthritis in humans. Jónasdóttir HS*, **Brouwers H***, Kwekkeboom JC, van der Linden HMJ, Huizinga T, Kloppenburg M, Toes REM, Giera M, Ioan-Facsinay A.Osteoarthritis Cartilage. 2017 Jul;25(7):1150-1160. doi: 10.1016/j.joca.2017.01.018. Epub 2017 Feb 8. *contributed equally

6. Hepatitis B Virus Surface Antigen Activates Myeloid Dendritic Cells via a Soluble CD14-Dependent Mechanism. van Montfoort N, van der Aa E, van den Bosch A, **Brouwers H**, Vanwolleghem T, Janssen HLA, Javanbakht H, Buschow SI, Woltman AM. J Virol. 2016 Jun 24;90(14):6187-6199. doi: 10.1128/JVI.02903-15. Print 2016 Jul 15.

7. Lipid mediators of inflammation in rheumatoid arthritis and osteoarthritis. **Brouwers H**, von Hegedus J, Toes R, Kloppenburg M, Ioan-Facsinay A. Best Pract Res Clin Rheumatol. 2015 Dec;29(6):741-55. doi: 10.1016/j.berh.2016.02.003. Epub 2016 Mar 4.

8. An Advanced LC–MS/MS Platform for the Analysis of Specialized Pro-Resolving Lipid Mediators. Jonasdottir HS, Ioan-Facsinay A, Kwekkeboom JC, **Brouwers H**, Zuurmond AM, Toes REM, Deelder AM, Giera M. Chromatographia 2015 78:391–401. doi: 10.1007/s10337-014-2779-5

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