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

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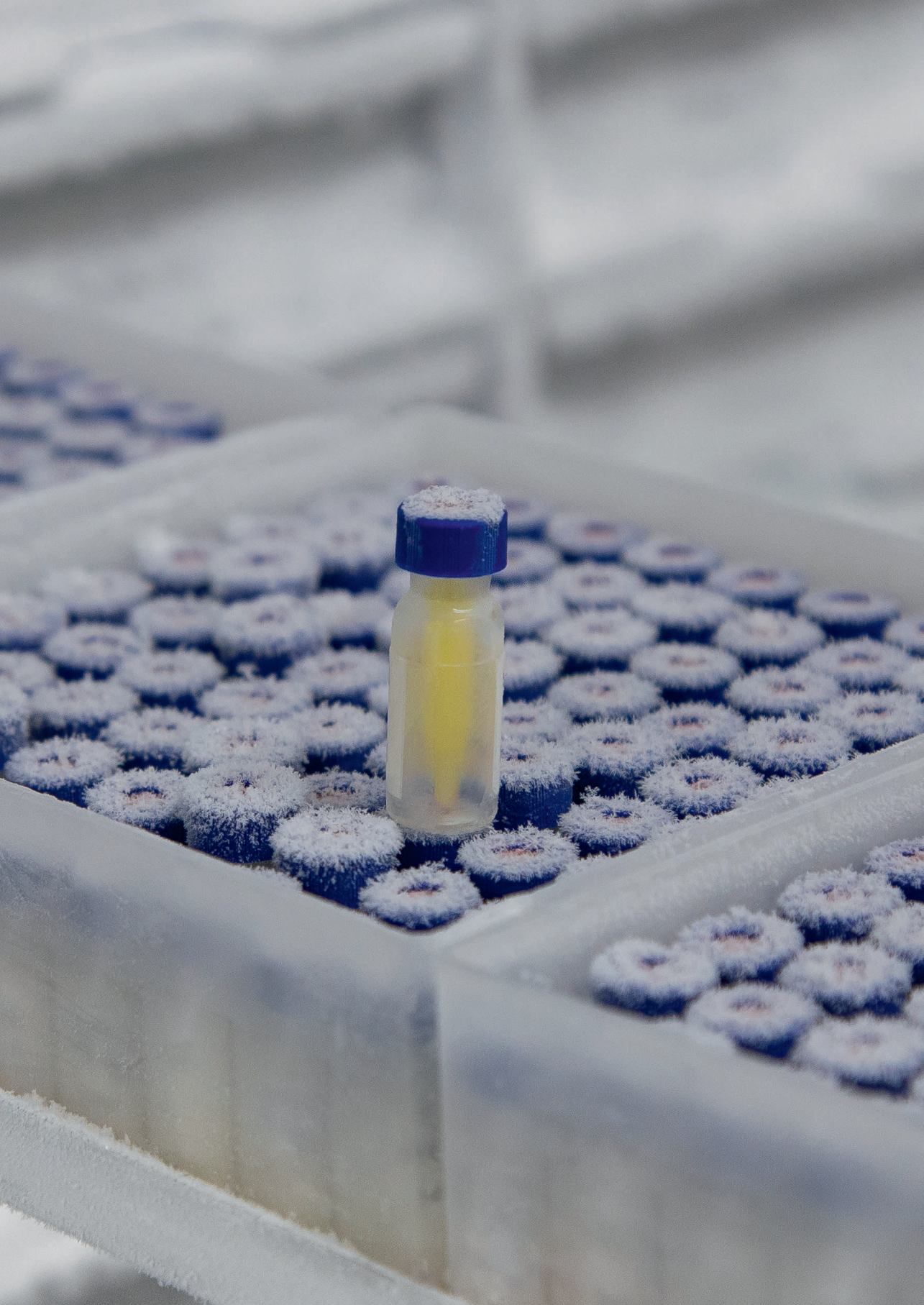

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General Discussion

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.

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