

Final report

Pro-resolving mediators in osteoarthritis: homeostatic signals in the joint organ?

Objectives and outcomes

We hypothesized that inflammation in osteoarthritis (OA) is sustained by a lack of proresolving molecules. Poly-unsaturated fatty acid metabolites called specialized pro-resolving mediators (SPM) (lipoxins, resolvins, protectins and maresins) were identified as factors generated within the inflammatory process supporting its resolution. We aimed to further understand their roles in OA using a systematic approach to document their presence in OA patients and their effects on key processes involved in joint development, homeostasis and disease. For this project we focussed on the following questions: **(1) Can we (further) document the presence of specific SPM in the joints of OA patients? (2) What are the effects of SPM on inflammation in joint tissues and cells? (3) What are their effects on the molecular phenotype of the articular chondrocytes including OA-associated hypertrophy?**

For question 1, the specific detection of the lipid mediators in tissues and synovial fluid was particularly challenging. One mL SF was treated with hyaluronidase, followed by centrifugation at 931 $\times g$ for 10 min. 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 $\times g$ for 15 min at 4 °C). The MeOH supernatant was removed, the protein pellet washed again with 1 mL MeOH and internal standard was added (LTB₄-d₄, 15(S)-HETE-d₈ and PGE₂-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. Targeted lipidomics analysis of the SF was carried out after SPE by Liquid-chromatography combined with mass spectrometry (LC-MS/MS) analysis. 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).

With the LC-MS/MS platform used in this study, we can detect 60 analytes, including SPMs, such as for example resolvin E2 (RvE2) in whole blood supplemented with EPA28 or RvD2 spiked into SF before hyaluronidase treatment. Of these analytes, 37 were detected in at least one of the SPE worked-up samples. 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). For 28 of the 37 detected analytes, the concentrations determined by the two methods correlated well. Further

analysis was restricted to these 28 analytes, which were determined upon SPE treatment in 24 OA and 10 RA samples.

Seven PUFAs were detected in SF of OA patients: the ω -6 FAs, AA, adrenic acid (AdA) and linoleic acid (LA), and the ω -3 FAs, EPA, DHA, docosapentaenoic acid (DPA ω -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 A₄), 17-HDHA (precursor of D series resolvins) and 18-HEPE (precursor of E series resolvins). LTB₄, 6-trans-LTB₄, and 20-OH-LTB₄ 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-LTB₄, and 20-OH-LTB₄, and the 15-LOX metabolite of AdA, 17-HDoTE, which were higher in RA than OA samples. **None of the SPMs that can be measured with our platform could be detected in any of the samples.**

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. Of these, four are generated via the 5-LOX pathway and three via the 15-LOX pathway. These metabolites included the SPM precursors 15-HETE and 17-HDHA. No differences were found in metabolites generated via the COX-driven pathway, or the activity of leukotriene A₄ hydrolase (LTA₄H), assessed indirectly by using the concentration ratio of LTB₄ to 5-HETE. **These data are interesting indicating that the 5-LOX and 15-LOX pathways are less activate in OA than in RA, while other pathways are similar.**

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). we detected PUFAs, the monohydroxylated precursors of the SPMs like 15-HETE and 17-HDHA, as well as pro-inflammatory LMs such as PGE₂ and thromboxane B₂ (TXB₂) in the soluble fraction of all patients. In the insoluble fraction, these analytes were also detectable in most patients. 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**, indicating that the complete resolution pathway is activated in OA and is detectable in the joint.

For question 2, we assessed 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. Calcium ionophore stimulation was used as a potent activator of cPLA₂ and subsequent bioactive LM synthesis, 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. The unstimulated synoviocytes contained detectable levels of AA, EPA and DHA, as well as AA 5-LOX derivatives 5-HETE and LTB₄, and 15-LOX derivative 15-HETE indicating presence of activated 5-LOX and 15-LOX in these cells. In contrast, these lipids were only detectable in a part of the patients in SFC. Remarkably, LTB₄ could not be detected in SFC samples, while it was detectable in synoviocytes of all patients. 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. Upon calcium ionophore stimulation, increased levels of AA, EPA, DHA, 5-HETE, 15-HETE, and LTB₄ were observed in synoviocytes of all patients. A similar trend was observed for SFC after calcium ionophore stimulation for AA, 5-HETE and LTB₄, although the data is likely underpowered to reach significance. LPS stimulation over 3 days had overall low effects and resulted in a significant, albeit small increase in EPA and 15-HETE in synoviocytes. 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 LM profile observed in SF of OA patients. In sharp contrast, no evidence for active production of the pro-resolving molecules could be reliably detected in primary human articular chondrocytes.**

Because macrophages are a major source of inflammation in the OA joint, we studied the effect of 17-HDHA, detected in SF of OA patients, on cytokine secretion by macrophages. LPS-induced secretion of TNF α was decreased in M1 macrophages (differentiated from monocytes of healthy volunteers in the presence of GM-CSF), while IL-10 secretion was increased by 17-HDHA in M2 (M-CSF differentiated monocyte-derived) macrophages. **These data indicate an anti-inflammatory function of 17-HDHA with potential effects in the OA joint.**

For question 3, we encountered major technological hurdles: commercially available pro-resolving lipids could not be demonstrated to have biological activity despite trying several approaches towards preservation and handling of the molecules.

Involvement of patient research partners

For this basic research project, patient input was, unfortunately limited. All patients of whom samples were used were informed about research and the importance of making progress in osteoarthritis. As is also the case for the osteoarthritis study group, OA patients are strongly underrepresented in the traditional patient organisations such as Reumanet in Belgium. Nevertheless, we have informed patient organisations about the work during the national conferences in which part of the data were presented.

Communication of results to community and patients

Inflammation is the hallmark feature of many rheumatic and musculoskeletal diseases. The importance of inflammation as a factor that contributes to the severity and symptoms of osteoarthritis

which was traditionally considered as degenerative joint disease. Our body disposes of a few mechanisms to control inflammation. Recently, a class of small lipid molecules was discovered that has strong negative effects on inflammation. A role or a lack of these molecules in inflammatory disease such as rheumatoid arthritis has been demonstrated and we hypothesized that such molecules may also be present in patients with osteoarthritis. Indeed, by optimizing complex analysis techniques we were able to demonstrate small amounts of precursor lipids in the joint fluid of OA patients. We also identified synovial cells as potential sources. In contrast, we could not demonstrate specific effects of these molecules on cartilage cells, most likely due to methodological limitations.