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Metabolic profiling reveals differences in concentrations of oxylipins and fatty acids secreted by the infrapatellar fat pad of end-stage osteoarthritis and normal donors

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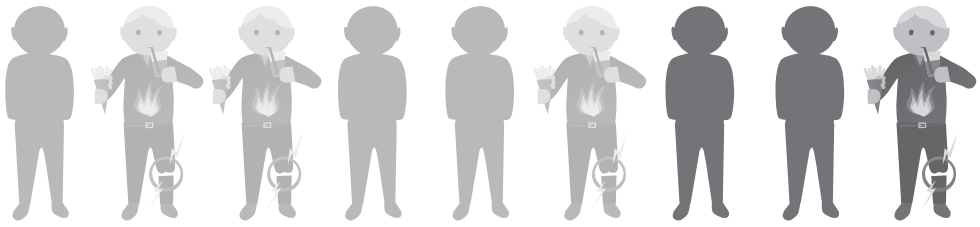

Abstract

Objective: The infrapatellar fat pad (IPFP) in the knee joint is hypothesized to contribute to osteoarthritis (OA) development possibly by influencing inflammatory processes. Oxylipins are essential mediators in the inflammatory process. We aimed to identify the secretion of fatty acids and oxylipins derived thereof by the IPFP.

Methods: IPFP explants of 13 OA (joint replacement surgery) and 10 normal (*post-mortem*) donors were cultured for 24 hours and supernatants were collected (FCM). Liquid chromatography-tandem mass spectrometry detected fatty acids and oxylipins in FCM samples. Univariate and multivariate (PLS-DA; Partial Least Squares Discriminant Analysis) analyses were performed followed by pathway analysis. To validate these outcomes a second set of OA FCM samples was measured (n=23).

Results: Twenty nine oxylipins and fatty acids could be detected in FCM. Univariate analysis showed no differences between normal and OA FCM, however PLS-DA revealed an oxylipin/fatty acid profile consisting of 14 mediators associated with OA (accuracy rate 72%). Most important contributors to the model were lipoxin A4 (decreased), thromboxane B2 (increased) and arachidonic acid (increased). The statistical model predicted 64% of the second set of OA FCM samples correctly. Pathway analysis indicated differences on individual mediators rather than complete pathways.

Conclusion: IPFP secretes multiple and different oxylipins and a subset thereof provides a distinctive profile for OA donors. The observed changes are likely regulated by the OA process rather than a consequence of basal metabolism changes as the increase in fatty acid levels was not necessarily associated with an increase in oxylipins derived from this fatty acid.

Osteoarthritis (OA) is a complex joint disease with multiple risk factors leading to cartilage degradation (1). For a long time, the emphasis in OA research has primarily been on degeneration of articular cartilage. Nowadays it is considered a disease of the whole joint in which multiple joint tissues, such as synovial tissue, ligaments, muscles and bone, are involved or affected by the process (2, 3).

An important risk factor for OA is obesity, which was until recently related to increased mechanical load in obese individuals leading to cartilage degradation. However, recent findings, such as the association between obesity and OA in non-weight bearing joints, suggest that there are other connections between obesity and OA (4, 5). Adipocytes and infiltrating immune cells in the adipose tissue actively secrete numerous cytokines and adipokines that in turn can influence metabolism and inflammatory responses in the body (6). These factors derived from adipose tissue may therefore contribute to the development of OA.

A special form of adipose tissue, named the infrapattelar fat pad (IPFP), is located intracapsularly and extrasynovially in the joint and is in close contact with synovial layers and articular cartilage. Its main role is to facilitate the distribution of synovial fluid and to absorb mechanical forces through the knee (7). Given the fact that adipose tissue is a highly active metabolic and endocrine organ, IPFP possibly has an important role in OA pathogenesis which only recently received attention. Its exact role in OA development still needs to be elucidated (8). Several cytokines are locally produced in the knee joint by the IPFP (9) and it has been found that IPFP derived from OA patients secretes higher levels of inflammatory mediators and has a different cell composition compared to subcutaneous fat (10). On the other hand, IPFP conditioned medium from end stage OA patients had anti-catabolic effects on cartilage explants in an *in vitro* culture experiment (11).

Other important signaling molecules implicated in the modulation of inflammatory responses are oxylipins. Oxylipins are formed from essential fatty acids via oxygenation and their biosynthesis is initiated via three major pathways, cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP). These lipid mediators are found in all tissues in the body and are involved in the control of many physiological processes (12, 13). The activity of the COX, LOX and CYP pathways and the binding of their products to corresponding receptors are important drug targets

for multiple inflammatory diseases such as rheumatoid arthritis, cardiovascular diseases and psoriasis (14). Pro-inflammatory oxylipins such as prostaglandin (PG)E2 and leukotrienes (LT)B4 are produced by oxidation of omega (ω)-6 fatty acids such as arachidonic acid (AA) by the COX enzyme system. Oxidation of (ω)-3 fatty acids (such as EPA and DHA) give rise to the lesser inflammatory mediators as PGE3 following COX oxidation, and LTB5 following LOX activity (15).

The role of PGs in OA has been explored extensively (16, 17) and non-steroidal anti-inflammatory drugs (NSAIDs) which reduce the production of PGE2 are commonly used in medical management of OA aiming to reduce pain. These drugs interfere, however, not only with PGE2 but also with other mediators that are generated by the same enzymatic pathways (18). The individual role of other mediators in OA has not received full attention, but may provide valuable targets for OA therapy (19, 20).

The main aim of this study was to investigate which fatty acids and oxygenated derivatives thereof (oxylipins) are secreted by the IPFP. Furthermore, we questioned whether an oxylipin and fatty acid secretion profile of the IPFP could distinguish between normal and OA donors using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis approach (21-23). The results were used to construct biological networks, providing visualization of the interactions between identified markers. This integrated inspection might provide new insight in the role of IPFP in the OA process.

Materials & Methods

Chemicals and reagents

Indomethacin, paraoxon and butylated hydroxytoluene (BHT) were from Sigma (Steinheim, Germany). Phenylmethylsulfonyl fluoride (PMSF) was from Fluka (Steinheim, Germany). 12-[(tricyclo[3.3.1.13,7]dec-1-ylamino) carbonyl]amino]-dodecanoic acid (AUDA) was purchased from Cayman (Ann Arbor, MI, USA). Milli-Q water (MQ) (Milli-Q Advantage unit, Millipore, Amsterdam, The Netherlands) was used in all analyses. Ultra Liquid Chromatography (ULC)-grade acetonitrile (ACN), formic acid (FA) and trifluoro acetic acid (TFA) were obtained from Biosolve (Valkenswaard, The Netherlands). LC-MS grade methanol was from Riedel-de-Häen (Steinheim, Germany). Isopropanol and ethanol were from JT Baker (Deventer, The Netherlands). All analytical and internal standards were purchased from Cayman. For oxylipins, stock solutions were prepared in ethanol, aliquoted and stored at -80°C until analysis. Hydrophilic-lipophilic-balanced reversed-phase sorbent for acids, bases and neutrals (HLB) solid phase extraction (SPE) columns (Oasis, 60 mg, 3 mL) were from Waters (Etten-Leur, The Netherlands).

Preparation of fat-conditioned medium of infrapatellar fat pad

IPFP was obtained from 13 OA patients undergoing joint replacement surgery (OA-I) or from *post-mortem* material of 10 non-OA donors with macroscopically no signs and no clinical history of OA (normal). IPFP were obtained according to legal and ethical requirements approved by the institutional review board of the University of Pennsylvania including anonymous informed written consent from the donor or nearest relative (Articular Engineering, Northbrook, USA). Due to the anonymous collection, no information was available about these donors excluding age, sex and body mass index (BMI). Immediately after collection the inner parts of the fat pads, where no synovial tissue is present, were cut in small pieces and cultured in Dulbecco's Modified Eagle medium (DMEM) + glutamax (Invitrogen, Paisley, UK), 1:100 Insulin-transferrin-sodium selenite supplement (ITS) (Roche Diagnostics, Indianapolis, USA), 1% penicillin and streptomycin (Pen Strep) (Biowitthaker Verviers, Belgium) and 0.1% lactalbumine (Sigma-Aldrich, St. Louis, USA) in a concentration of 50 mg tissue/ml

and incubated at 37°C. After 24 hours, the supernatant, i.e. fat-conditioned medium (FCM) was harvested, centrifuged at 300 g for 8 minutes and frozen at -80˚C in aliquots until analysis (Articular Engineering, Northbrook, USA). This incubation time was chosen arbitrarily since each mediator has its own optimum regarding release kinetics Furthermore, 23 FCM samples from end-stage OA patients, obtained and processed with a comparable protocol but at another laboratory, were included and are called OA-II in this manuscript (Erasmus MC, Rotterdam, the Netherlands, MEC-2008-181). These OA-II FCM samples were used to validate the outcomes of the study. Demographic data of all included donors can be found in table 1.

Table 1. Demographic data of the normal and osteoarthritis (OA-I and OA-II) donors included.

	Normal (n=10)	OA-I (n=13)	OA-II (n=23)
Age	62.1 (11.9)	62.5 (6.9)	64.5 (9.5)
Sex, M/F	6/4	8/5	7/16
BMI	24.4 (4.5)	32.0 (10.5)	29.9 (3.9)

Data are presented as mean (SD). Number of donors (n), Age in years, male (M), female (F), Body Mass Index (BMI).

Extraction of oxylipins and fatty acids from fat-conditioned medium

Samples were precipitated with methanol and 50 µl internal standards (Table S1) were added to 1 ml of the FCM samples and put on ice for 30 minutes. Negative controls, containing culture medium used in the processing of normal, OA-I and OA-II samples which was not incubated with IPFP, were also included. Samples were subsequently centrifuged (5' at 3000 x g and 4°C) and the supernatant was transferred to a glass tube. Just before loading on activated HLB columns, 4.75 mL MQ water containing 0.1% v/v FA was added to the methanol extract, diluting the extract to 20% methanol. After loading, the columns were washed with 2 mL 20% methanol in MQ water containing 0.1% FA, and the columns were allowed to dry for 15 minutes. The SPE columns were eluted with 2 mL methanol and the samples were captured in tubes already containing 20 µL of 10% glycerol and 500 µM butylhydroxytolueen (BHT) in ethanol. The tubes were placed in a water bath at 40°C and the methanol was evaporated under a gentle stream of nitrogen, after which the samples were reconstituted in 100 µL ethanol containing internal standard (CUDA) (for monitoring the system performance) and immediately used for LC-MS/MS analysis.

Liquid chromatography-mass spectroscopy (LC-MS)/MS analysis of oxylipins and fatty acids

All analyses were performed on an ultra-performance liquid chromatography (UPLC) coupled to a Xevo TQ-S mass spectrometer (Waters). Five µl extract was injected on an Acquity C18 BEH (ethylene bridged hybrid technology) UPLC column (2.1 x 100 mm, 1.7 µm) and was separated using gradient elution with a stable flow of 600 µl/min. The gradient started with 95% A (MQ water with 0.1% FA) and 5% B (ACN with 0.1% FA) followed by a linear increase to 70% A and 30% B which was achieved at 5.0 minutes. This was followed by a linear increase towards 50% B which was achieved at 11.25 minutes and maintained until 13.25 minutes. The system was subsequently switched to 100% B, which was achieved at 15.75 minutes and maintained until 16.75 minutes, after which the column was left to equilibrate at 5% B for approximately 3 minutes. The column was maintained at 50°C during analysis, and the samples were kept at 10°C. The MS was operating in selective reaction mode using electrospray ionization in negative ion mode, with a capillary voltage of 3.3 kV, a source temperature of 150°C and a desolvation temperature of 600°C. Cone voltage and collision energy were optimized for each compound individually (consult table S1 for parent and product m/z values). Peak identification and quantification were performed using MassLynx software version 4.1. Calibration curves were run in duplicate from which one regression equation was generated after internal correction.

Statistical analysis and interpretation

Partial least square discriminant analyses (PLS-DA) (24) was used to identify oxylipins and fatty acids that differed between 13 OA-I and 10 normal FCM samples. Levels lower than the lowest level of the detection limit were replaced by 0.5 times the lowest level of detection. In PLS-DA, a y-variable containing class membership information is correlated to a data matrix (X-block). OA-I donors and normal donors were assigned to class 0 and class 1 respectively. The PLS-DA model was validated using a 10-fold double cross validation approach (25). The 23 OA-II samples were used to validate the statistical model for OA FCM samples. All multivariate data analyses were performed using Matlab Version 7.11.0 (R2010b, the Mathworks,

Inc). Based on the outcomes of the PLS-DA, a pathway analysis was performed using Pathvisio in which the observations were visualized (www.pathvisio.org). A custom made pathway was created based on literature searches. Univariate analyses were performed with the parametric student t-test or non-parametric Mann-Witney test depending on their normal distribution as assessed with Levene's test by using SPSS version 20.. Graphs were made by GraphPad Prism version 17.0.

Results

Lipid content of fat-conditioned medium

Metabolic profiling of oxylipins and fatty acids in FCM samples of 13 OA-I donors and 10 normal donors was performed. This screening approach led to the identification of the lipid mediators presented in table 2. Out of 52 oxylipins and fatty acids that can be measured by this approach, 29 could be detected in the FCM samples. Additionally, 23 OA-II FCM samples, processed with the same protocol but at another laboratory, were measured and revealed to contain the same lipid mediators as the OA-I FCM samples, but at different concentrations (table S2).

Most fatty acids were detected (with exception of linoleic acid), i.e. arachidonic acid (AA) (ω-6), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (both ω-3), in the FCM samples of OA-I, OA-II and normal donors. No significant differences were detected between OA-I and normal donors when applying univariate statistical analysis, although AA showed a tendency of higher concentrations in OA-I FCM than in normal FCM ($p=0.10$) (Figure 1). DHA also showed a tendency towards higher concentrations in OA-I FCM than in normal FCM ($p=0.10$).

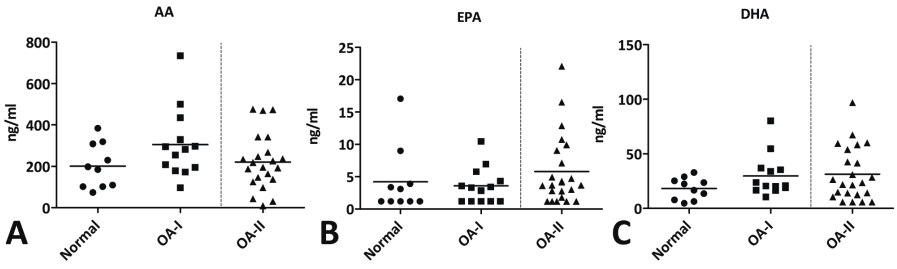

Figure 1. Concentrations of essential fatty acids detected in fat-conditioned medium (FCM) samples of normal (n=10) and osteoarthritis ((OA)-I, n=13) donors. Concentrations of independently isolated OA FCM samples are also presented (OA-II, n=23). Line indicates the mean. Arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

Using multivariate statistical analysis (PLS-DA), a set of 14 out of 29 quantified oxylipins and fatty acids was retained in a model which distinguished OA-I and from normal FCM samples with a median error rate of 28% after 10-fold double cross validation. The model provided a ranking (Table 3), in which lipoxin A4 was the most discriminative oxylipin between OA-I and normal FCM. The essential fatty acid AA was ranked on the third position and DHA was ranked as fifth. The individual concentrations of the 3 most discriminating oxylipins are illustrated in figure 2. Levels of lipoxin A4 were lower in OA-I than in normal FCM. Thromboxane B2 (TBXB2), an inactive metabolite of the biologically active TBXA2, was ranked on the second position and was higher in OA-I FCM than in normal FCM, whereas prostaglandin PGD3 (ranked fifth), a derivative from the ω-3 fatty acid EPA, was found to be lower in OA-I FCM. Furthermore, the precursor for resolvin D1, 17(S)HDoHE (derived from DHA), was higher in OA-I FCM. AA-derived PGF2α and D2 were higher in OA-I FCM, whereas PGB2 and F2β levels were lower. Finally, linoleic acid-derived 9-HODE and 13-HODE were found to be lower in OA-I FCM. None of the resolvins, maresins and leukotrienes could be detected.

Table 2. Oxylipins and essential fatty acids detectable in fat-conditioned medium (FCM) of 13 osteoarthritis (OA-I) and 10 normal donors.

	LLOD (ng/ml)	OA-I				Normal			
		Mean (ng/ml)	SD	CV (%)	N	Mean (ng/ml)	SD	CV (%)	N
Essential fatty acids									
EPA	2.34	3.6	2.8	78	5	4.2	5.1	121	5
AA	46.85	305.8	168.7	55	0	200.8	107.5	54	0
DHA	11.70	29.9	19.2	64	0	18.2	9.9	55	0
EPA-derived oxylipins (ω-3)									
PGE3	0.06	0.1	0.2	175	10	0.2	0.6	238	8
PGD3	0.10	0.2	0.4	187	10	0.5	1.0	188	5
DHA-derived oxylipins (ω-3)									
17(S)_HDoHE	0.10	0.2	0.5	213	9	0.1	0.1	168	9
AA-derived oxylipins									
13,14-dihydro-15-keto-PGD2	0.01	0.04	0.1	280	11	0.05	0.1	259	8
13,14-dihydro-15-keto-PGE2	0.02	0.8	1.7	212	2	0.8	2.1	271	2
13,14-dihydro-15-keto-PGF2α	0.08	2.4	4.5	190	1	1.5	2.5	169	0
15(S)-HETE	0.01	0.2	0.1	81	0	0.2	0.1	73	0
11(S)-HETE	0.01	0.1	0.1	85	0	0.1	0.2	116	0
12(S)-HETE	0.01	0.2	0.2	81	0	0.1	0.1	45	0
5(S)-HETE	0.02	0.1	0.1	105	0	0.1	0.1	69	0
TBXB2	0.05	2.1	2.1	98	0	0.9	1.2	134	1
PGB2	0.02	0.2	0.3	153	2	0.6	1.3	206	3
PGF2β	0.02	0.1	0.1	159	7	0.2	0.2	128	1
PGD2	0.01	0.8	1.03	131	0	1.0	1.3	133	0
PGE2	0.02	74.6	133.3	179	0	90.0	207.2	230	0
Lipoxin A4	0.01	0.04	0.04	85	3	0.1	0.2	128	1
8-iso-PGF2α	0.02	0.3	0.6	161	1	0.3	0.6	189	0
11b-PGF2α	0.02	1.8	2.6	145	1	0.9	2.2	244	3

PGF2α	0.02	5.0	6.8	136	0	5.0	8.7	173	0
11,12-DiHETrE	0.01	0.05	0.05	101	0	0.04	0.03	76	1
14,15-DiHETrE	0.01	0.04	0.03	80	1	0.05	0.03	64	1
8,9-EET	0.08	0.05	0.03	63	1	0.05	0.03	54	1
8,9-DiHETrE	0.02	0.05	0.07	145	4	0.03	0.03	103	5
12(S)-HHTrE	0.16	0.2	0.24	102	7	0.3	0.3	115	1
LA-derived oxylipines									
9(S)-HODE	0.02	2.5	1.0	39	0	4.5	3.7	84	0
13(S)-HODE	0.04	3.2	0.8	25	0	4.8	3.5	72	0

Standard deviation (SD), coefficient of variance (CV), lower level of detection (LLOD), number of samples below the LLOD (N). Eicosapentaenoic acid (EPA), arachidonic acid (AA), docosahexaenoic acid (DHA), prostaglandin (PG), hydroxydocosahexaenoic acid (HDoHE), hydroxyeicosatetraenoic acid (HETE), thromboxane (TBX), dihydroxyeicosatrienoic acid (diHETre), eicosatrienoic acid(EET), hydroxyheptadecatrienoic acid (HHTrE), linoleic acid (LA), hydroxyoctadecadienoic acid (HODE).
Resolvin D1, Resolvin D2, Maresin, Leukotriene B4, E4, D4, n-acetyl-leukotriene E4. TBXB3, 11,12 EET, 5,6 EET, 14,15 EET, 10(S)-17(S) diHDoHE, 6-keto-PGF1α, 2,3-dinor-8-iso-PGF2α, 12(S)- 12S-hydroxyeicosapentaenoic acid (HEPE), 5(S)-HEPE, 20(S)-HEPE, 15-deoxy-d-12,14-PGJ2, 5,6-DiHETre, 19,20-dihydroxydocosapentaenoic acid (HDoPE), 17-keto, 4(z),7(z),10(z),13(z),15(E)),19(z)-DHA, 12,13-di-hydroxyoctadecenoic acid (HOME) and 9,10-diHOME could not be detected in these samples.

Validation of the osteoarthritis fat-conditioned medium samples

To assess whether the collected OA-I FCM samples were representative for OA, we included independently isolated FCM samples from 23 OA donors (OA-II) in the study. Regarding individual levels, the 14 lipid mediators obtained from the PLS-DA model had different absolute mean values in the OA-I samples compared to the OA-II samples, although the majority was changed in the same direction compared to normal FCM (Figure 1 and 2, table S2). For TBXB2, 9(S)HODE and 17(S) HODE different patterns were seen in the OA FCM samples from different laboratories; with respect to normal FCM samples the TBXB2 levels were higher in OA-I while lower in OA-II samples. For 9(S)HODE and 17(S) HODE lower levels were found in OA-I than in normal FCM samples, in contrast to higher levels in OA-II samples. Additionally, the OA-II FCM samples were analyzed within the built statistical PLS-DA model of OA-I and normal samples. Eight out of 23 OA-II FCM samples were not classified as OA FCM sample by using this approach (error rate of 36%).

Table 3. Ranking in partial least square discriminant analysis (PLS-DA) of 13 osteoarthritis (OA-I) and 10 normal donor derived fat-conditioned medium samples.

Rank	Name	Regression coefficient
1	Lipoxin A4	-0.144
2	TBXB2	0.135
3	AA	0.110
4	PGD3	-0.095
5	DHA	0.093
6	17(S)_HDoHE	0.087
7	PGB2	-0.073
8	14,15-DiHETrE	-0.067
9	11b-PGF2α	0.059
10	15(S)-HETE	-0.057
11	13,14-dihydro-15-keto-PGF2α	0.053
12	PGF2β	-0.037
13	13(S)-HODE	-0.034
14	9(S)-HODE	-0.026

The contribution of a variable in the PLS-DA model is expressed as its regression with a positive score indicating an increase and a negative score indicating a decrease in OA-I compared to control FCM samples. A greater absolute value means more contribution of this oxylipin to the measured differences between OA-I and normal FCM samples. Thromboxane B2 (TBXB2), arachidonic acid (AA), prostaglandin (PG), docosahexaenoic acid (DHA), hydroxy docosahexaenoic acid (HDoHE), dihydroxy eicosatrienoic acid (diHETrE), hydroxyl eicosatetraenoic acid (HETE), hydroxyl octadecadienoic acid (HODE).

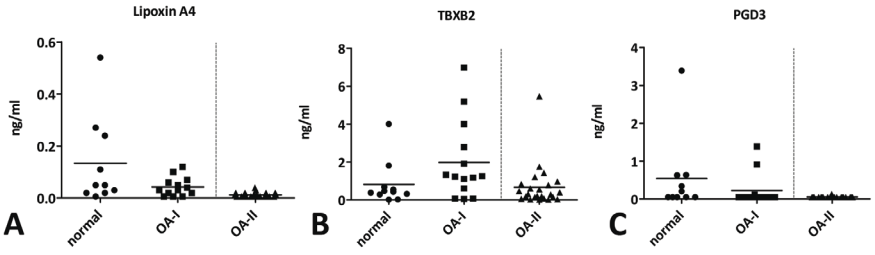

Figure 2. Concentrations of oxylipins which are high in the ranking in the contribution to the distinction between normal (n=10) and osteoarthritis ((OA)-I, n=13) donors derived fat-conditioned medium (FCM) samples after partial least square discriminant analysis (PLS-DA). Concentrations of independently isolated OA FCM samples are also presented (OA-II, n=23). Thromboxane B2 (TBXB2) and prostaglandine (PG)D3. Line indicates the mean.

Pathway analysis

To visualize the results from the quantified fatty acids and oxylipins in the OA-I FCM samples, all data were imported and analyzed using Pathvisio (Figure 3). In this overview, the quantified fatty acids and oxylipins are shown together with their main enzymes (LOX, COX, and CYP). Herein it is shown that higher concentrations of the fatty acids not necessarily lead to higher concentrations of its downstream products. AA was found in higher concentrations in OA-I FCM than in normal FCM, but for its downstream products both higher and lower concentrations were found. None of the main enzymes (COX, LOX or CYP) seemed to be consistently up or down regulated, although all LOX-derived oxylipins in the PLS-DA model showed lower concentrations in OA-I patients. However, 3 out of 7 quantified LOX-derived oxylipins did not contribute to the discrimination of OA-I from normal donors. Oxylipins involved in the synthesis of lipoxin A4 (lipoxin A4 and 15-HETE) and downstream derivatives of linoleic acid (9-HODE and 13-HODE) showed decreased concentrations in OA-I FCM.

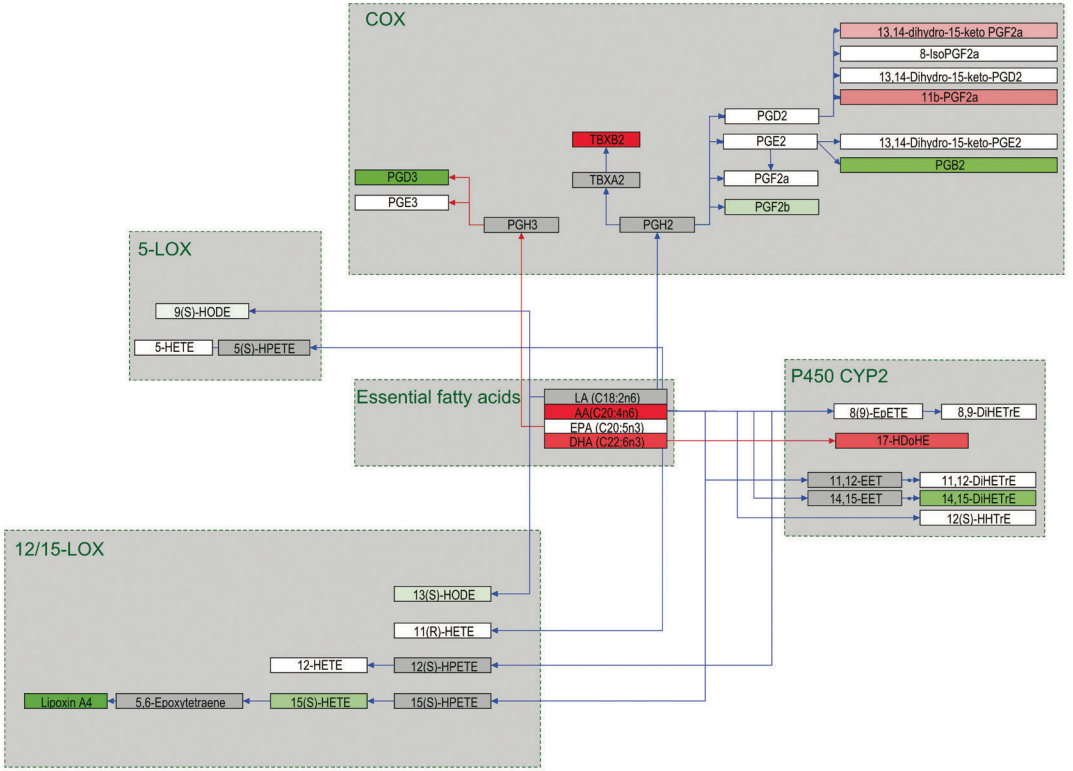

Figure 3. Overview of essential fatty acids and oxylipins differences in fat-conditioned medium (FCM) samples from osteoarthritis (OA-I) and normal donors as detected by partial least square discriminant analysis (PLS-DA). The following is indicated by the colors: grey, not measured or detected; white, quantified but not different between OA-I and normal FCM; red, increased concentrations in OA-I FCM; green, decreased concentrations in OA-I FCM. The more intense the color, the higher the ranking in the PLS-DA model.

Discussion

The IPFP located in the knee joint is considered as a tissue that may contribute to local inflammatory and destructive processes in OA (8). Important signaling molecules implicated in the modulation of inflammatory responses are oxylipins (14). In this study, we provide a comprehensive approach to identify oxylipins and fatty acids secreted by the IPFP in the knee joint. Using an LC-MS/MS approach, 29 out of the measurable 52 oxylipins and fatty acids could be detected in the FCM samples, indicating the presence of bioactive lipids in IPFP. None of these 29 lipid mediators differed significantly between OA-I and normal donors based on univariate statistics. By using multivariate analysis (PLS-DA), an oxylipin profile associated with OA-I was obtained with an accuracy rate of 72% after 10-fold double cross validation. The validation of the statistical model with independently isolated OA FCM samples (OA-II) revealed a comparable accuracy rate for OA samples (64%). These accuracy rates are acceptable given the relatively small included number of donors and the biological variety in these sample type. The measured changes visualized by pathway analysis demonstrated that the profile associated with OA FCM is likely to be specifically changed by the disease process rather than due to alterations in basal metabolism or generic enzymes as the increase of a fatty acid concentration was not necessarily associated with an increase in oxylipins derived from this fatty acid.

In addition to the profiling of essential fatty acids and oxylipins secreted by IPFP, we wondered whether the profile of IPFP from OA would differ from normal donors. A difference may reveal more insight in the role of IPFP in the OA disease process. Based on multivariate analysis lipoxin A4, which is derived from AA, was the most discriminative oxylipin between OA-I and normal FCM with lower levels in OA donors. Also 15-HETE, an intermediate in lipoxin A4 synthesis, was found in decreased concentrations in OA-I compared to normal FCM. Lipoxin A4 is known to have a role in the resolution of inflammation, is produced by cartilage explants (26) and is present in OA and rheumatoid arthritis (RA) synovial fluid (27). Lipoxin A4 inhibits interleukin (IL)-1β induced IL-6 and IL-8 release in fibroblast-like synoviocytes (FLS) and reduces matrix metalloproteinase (MMP)-3 release and increases tissue inhibitor of MMP (TIMP) release in IL-1β stimulated FLS (28, 29). Deletion of

12/15-lipoxygenase (12/15-LOX), a major enzyme involved in the generation of a subclass of oxylipins including lipoxin A4, in a mouse model for arthritis leads to uncontrolled inflammation and tissue damage (30). The lower concentrations of lipoxin A4 and 15-HETE in the OA-I compared to normal FCM samples contribute to the concept that lipoxin A4 may act as counter regulatory signal and has a potential to limit inflammation-induced tissue damage (31).

TBXB2 was ranked as the second oxylipin by PLS-DA that contributed to the distinction between OA-I and normal FCM samples. TBXB2 was elevated in OA-I compared to normal FCM samples. In FCM of the OA-II samples, however, this oxylipin was not different from normal FCM. This discrepancy may be explained by several reasons. Firstly, although the same protocol for the collection and processing of OA-I and OA-II FCM was applied, reliable detection of oxylipins may be disturbed by *ex vivo* degradation of lipid mediators due to rapid enzymatic conversion or chemical instability (32, 33). Therefore the generated absolute values and the differences between OA-I and OA-II samples should be interpreted with caution. Secondly, due to the anonymous collection of the FCM samples, the medical history of these patients is unknown. OA-I and OA-II FCM samples were collected in different countries and different protocols for the use of drugs prior to surgery may explain the discrepancy between OA-I and OA-II FCM samples. Especially, the use of pain killers such as NSAIDs or COX inhibitors, which interfere in pathways leading to the production of TBXB2, may explain these differences. It should as well be noted that the lack of information about the medical history of the donors also counts for the other observed differences between OA-I and normal donors and it cannot be excluded that this influences the detected levels. Most of the essential fatty acids were detected in the FCM samples. It has been postulated that the intake of dietary essential fatty acids can influence the development of OA (34). In a spontaneous OA guinea pig model an ω-3 rich diet was reported to improve histological and biochemical markers of OA (35). Unexpectedly, in our data DHA (ω-3) levels were higher in OA-I than normal derived FCM samples. EPA (ω-3) was not altered in OA-I compared to normal FCM samples. The higher secretion of the pro-inflammatory ω-6 fatty acid AA by OA IPFP was in line with our expectations. Notably, the different PGs derived from AA were either unchanged, increased and decreased in OA-I compared to normal FCM

samples. PGs exert diverse and complex modulatory roles during pathophysiologic conditions and are extensively investigated in relation to OA (16, 17, 20). PGE2, for example, is known to increase the productions of MMPs and pro-inflammatory soluble mediators which can lead to alterations in the cartilage, synovial membrane and bone. Remarkably, no differences between OA-I and normal FCM were observed for PGE2. Furthermore, PGD2 concentrations, which can augment the production of PGE2 (26), were not different between OA-I and normal FCM. The increase of the essential fatty acid AA but not necessarily their by-products supports the idea that the observed changes are specifically for the disease process and indicates the complexity of systemic equilibrium in these pathways.

Although essential fatty acids were detected, some apparent by-products from pathways instigated by these fatty acids were not measured in the FCM samples, such as the leukotrienes which are suggested to increase bone resorption and to induce IL-1 synthesis (20, 36). Resolvins, which play an important role in the resolution of inflammation, were also not identified using this approach (37). The absence of these oxylipins might be due to the detection limit of our analysis and more sensitive methods may reveal their presence. Furthermore, as mentioned previously, concentrations may be influenced by *ex vivo* degradation of lipid mediators due to rapid enzymatic conversion or chemical instability (32). For all detected oxylipins and fatty acids, having identical MS/MS transitions, counts that they were distinguished by retention time after optimization of the chromatographic conditions using commercially available standards, however it cannot be fully excluded that other isomers may be present in biological materials that co-elute with those tested.

There are few references to such a broad lipid mediator approach performed in tissues and fluids in the knee joint. Attur et al. analyzed the excretion of a selected panel of oxylipins and their related fatty acids by OA and normal cartilage explants. The main outcomes were the increased spontaneous secretion of PGE2 and leukotriene B4 by OA compared to normal cartilage explants (26). A study with equine synovial fluid reported a radical change of the oxylipin profile after experimental induction of transient acute synovitis (e.g. PGE2, PGD2, PGF2a, leukotriene B4 and TBXB2) (38). Our data contribute to the theory that during the OA process the essential fatty acids and oxylipin profiles in various joint tissues change. It remains the question, however,

whether these changes in profiles contribute to the process of cartilage degeneration or are a consequence thereof.

The extensive biological variance which was measured in the samples led to a multivariate statistical model with an error rate of 28%. This should be taken into account when interpreting the data. By the validation of the model with independently isolated OA FCM samples (OA-II) a comparable error rate for OA samples was obtained, which strengthens the observations measured in the OA-I FCM sample set. An expansion of the number of donors is needed to investigate whether the error rate can be reduced, thereby improving the reliability of the model and the validity that differences in oxylipin secretion are present between OA and normal IPFP.

This study demonstrates that OA and normal IPFP generate multiple and different oxylipins. OA patients can be distinguished from normal donors based on the secretion of lipid mediators involved in the oxylipin pathways by IPFP. Pathway analysis underlined the complexity of equilibrated mechanisms in the OA process as an increase in a fatty acid not necessarily led to an increase in a by-product of the instigated pathway. These data may provide a basis for development of therapeutics in OA.

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Supplementary information

Table S1. MS/MS parent and product ion m/z values

Compounds	Parent (m/z)	Product (m/z)
12-HHTrE	279.0	179.3
13-HODE	295.1	195.0
9-HODE	295.1	171.0
EPA	301.1	257.2
ARA	303.1	259.2
12,13-DiHOME	313.2	183.0
9,10-DiHOME	313.2	201.0
15-deoxy-d-12,14-PGJ_2	315.0	271.1
12-HEPE	317.2	179.0
5-HEPE	317.2	115.0
11,12-EET	319.1	167.0
12-HETE	319.1	179.2
5-HETE	319.1	203.1
14,15-EET	319.1	219.2
5,6-EET	319.1	191.3
11-HETE	319.1	167.0
8,9-EET	319.1	167.0
15-HETE	319.2	219.1
20-HETE	319.2	275.3
2,3-dinor-8-iso-$PGF_{2\alpha}$	325.1	237.2
DHA	327.1	283.1
9,10,13-TriHOME	329.2	171.1
9,12,13-TriHOME	329.2	211.1
PGB2	333.2	174.9
LTB4	335.1	194.8
14,15-DiHETrE	337.1	207.0
11,12-DiHETrE	337.1	166.9
5,6-DiHETrE	337.1	144.8
8,9-DiHETrE	337.1	127.0
17-keto-4(z),7(z),10(z),13(z),15(e),19(z) DHA	341.5	111.0
17-HDoHE	343.1	281.4
PGE3	349.0	269.1
PGD3	349.1	269.1
PGD2	351.1	271.1
Lipoxin A4	351.1	114.9
13,14-dihydro-15-keto-PGD2	351.1	175.0
13,14-dihydro-15-keto-PGE2	351.1	175.1
PGE2	351.1	271.2
13,14-dihydro-15-keto-PGF2	353.1	113.1

Compounds	Parent (m/z)	Product (m/z)
8-iso-PGF2α	353.1	193.0
11-PGF2α	353.1	193.0
PGF2α	353.1	193.0
PGF2α	353.2	193.1
10,17-DiHDoHE	359.1	152.9
Maresin	359.6	177.1
19,20-DiHDoPE	361.1	272.7
TBXB3	367.1	168.9
TBXB2	369.1	169.0
Resolvin D2	375.1	175.0
Resolvin D1	375.1	140.8
LTE4	438.0	351.0
n-acetyl LTE4	480.0	351.1
LTD4	495.1	142.9
Internal standards (quantification)		
13-HODE-d4	299.2	198.2
ARA-d8	311.2	267.2
20-HETE-d6	325.1	281.3
15-HETE-d8	327.2	226.1
14,15-EET-d11	330.2	268.3
PGB2-d4	337.1	178.9
LTB4-d4	339.1	197.1
8,9-DiHETrE-d11	348.2	127.0
PGE2-d4	355.1	275.1
13-14-dihydro-15-keto-PGF2α-d4	357.1	187.0
8-iso-PGF2α-d4	357.1	196.9
11β-PGF2α-d4	357.1	313.4
PGF2-d4	357.1	313.4
PGD2-d9	360.3	280.1
TBXB2-d4	373.1	173.0
LTD4-d5	500.0	142.9
Internal standard (system performance)		
CUDA	339.1	214.1

Table S2. Essential fatty acids and oxylipins concentrations in fat-conditioned medium samples of 10 normal, 13 osteoarthritis (OA)-I and 23 OA-II donors.

	Normal			OA-I			OA-II					
	Mean ng/ml	95 % CI		Mean ng/ml	95 % CI		Mean ng/ml	SD	CV (%)	N	95% CI	
Essential fatty acids												
EPA	4.2	1,049	7,398	3.6	2,052	5,087	6.8	4.6	68	5	4,923	8,690
AA	200.8	134,199	267,454	305.8	214,123	397,537	290.4	115.3	40	5	243,300	337,506
DHA	18.2	12,003	24,331	29.9	19,448	40,276	41.4	26.4	64	4	30,630	52,231
EPA-derived oxylipins (ω-3)												
PGE3	0.2	-0,110	0,572	0.1	0,007	0,266	0.1	0.1	148	19	0,027	0,109
PGD3	0.5	-0,091	1,182	0.2	-0,004	0,455	0.1	0.0	0	22	0,051	0,051
DHA-derived oxylipins (ω-3)												
17(S)_HDoHE	0.1	-0,003	0,170	0.2	-0,037	0,503	0.05	0.02	43	21	0,037	0,053
AA-derived oxylipins												
13,14-dihydro-15-keto-PGD2	0.05	-0,028	0,122	0.04	-0,020	0,096	0.02	0.03	152	12	0,007	0,031
13,14-dihydro-15-keto-PGE2	0.8	-0,529	2,081	0.8	-0,123	1,726	0.4	1.0	251	5	-0,010	0,782
13,14-dihydro-15-keto-PGF2α	1.5	-0,067	3,071	2.4	-0,077	4,863	1.1	2.7	253	8	-0,037	2,185
15(S)-HETE	0.2	0,112	0,296	0.2	0,090	0,232	0.1	0.05	73	6	0,044	0,081
11(S)-HETE	0.1	0,042	0,255	0.1	0,067	0,182	0.1	0.09	89	6	0,063	0,134
12(S)-HETE	0.1	0,102	0,182	0.2	0,114	0,291	0.1	0.1	121	3	0,056	0,165
5(S)-HETE	0.1	0,056	0,141	0.1	0,047	0,173	0.1	0.02	44	6	0,045	0,065
TBXB2	0.9	0,154	1,635	2.1	0,995	3,261	0.6	0.6	91	0	0,395	0,865
PGB2	0.6	-0,170	1,392	0.2	0,037	0,403	0.2	0.4	165	7	0,070	0,358
PGF2β	0.2	0,032	0,278	0.1	0,008	0,115	0.04	0.03	81	8	0,028	0,055
PGD2	1.0	0,169	1,741	0.8	0,225	1,346	0.2	0.3	103	4	0,142	0,350
PGE2	90.0	-38,358	218,440	74.6	2,168	147,083	33.6	71.6	213	1	4,293	62,807
Lipoxin A4	0.1	0,028	0,239	0.04	0,023	0,063	0.01	0.01	77	11	0,010	0,019
8-iso-PGF2α	0.3	-0,054	0,672	0.3	0,043	0,647	0.2	0.4	186	2	0,055	0,401
11b-PGF2α	0.9	-0,470	2,313	1.8	0,376	3,173	0.8	1.5	190	3	0,179	1,413

PGF2α	5.0	-0,373	10,381	5.0	1,308	8,683	3.3	5.3	160	2	1,146	5,484
11,12-DiHETrE	0.04	0,023	0,064	0.05	0,023	0,080	0.02	0.02	69	3	0,015	0,027
14,15-DiHETrE	0.05	0,028	0,066	0.04	0,024	0,060	0.01	0.01	95	9	0,008	0,018
8,9-EET	0.05	0,034	0,069	0.05	0,031	0,064	0.05	0.02	33	19	0,040	0,052
8,9-DiHETrE	0.03	0,009	0,041	0.05	0,010	0,080	0.02	0.02	92	9	0,015	0,033
12(S)-HHTrE	0.3	0,084	0,495	0.2	0,107	0,371	0.1	0.1	74	16	0,096	0,179
LA-derived oxylipins												
9(S)-HODE	4.5	2,660	6,949	2.5	2,730	3,608	11.6	4.3	37	2	9,844	13,352
13(S)-HODE	4.8	2,151	6,778	3.2	1,959	3,013	7.0	2.4	34	2	6,005	7,964

Standard deviation (SD), coefficient of variance (CV), number of samples below the lower level of detection (N) and 95 % confidence interval (CI). Eicosapentaenoic acid (EPA), arachidonic acid (AA), docosahexaenoic acid (DHA), prostaglandin (PG), hydroxydocosahexaenoic acid (HDoHE), hydroxyeicosatetraenoic acid (HETE), thromboxane (TBX), dihydroxyeicosatrienoic acid (diHETre), eicosatrienoic acid(EET), hydroxyheptadecatrienoic acid (HHTrE), hydroxyoctadecadienoic acid (HODE).
Resolvin D1, Resolvin D2, Maresin, Leukotriene B4, E4, D4, n-acetyl-leukotriene E4. TBXB3, 11,12 EET, 5,6 EET, 14,15 EET, 10(S)-17(S) diHDoHE, 6-keto-PGF1α, 2,3-dinor-8-iso-PGF2α, 12(S)- 12S-hydroxyeicosapentaenoic acid (HEPE), 5(S)-HEPE, 20(S)-HEPE, 15-deoxy-d-12,14-PGJ2, 5,6-DiHETre, 19,20-dihydroxydocosapentaenoic acid (HDoPE), 17-keto, 4(z),7(z),10(z),13(z),15(E)),19(z)-DHA, 12,13-dihydroxyoctadecenoic acid (HOME) and 9,10-diHOME could not be detected in these samples.