Osteoarthritis and Cartilage 32 (2024) 990–1000

Osteoarthritis and Cartilage

Serum levels of hydroxylated metabolites of arachidonic acid cross-sectionally and longitudinally predict knee pain progression: an observational cohort study

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ARTICLE INFO

Article history:
Received 3 November 2023
Accepted 11 April 2024

Keywords:
Osteoarthritis
Oxylipins
Arachidonic acid
Knee pain
Biomarkers

SUMMARY

Objective: To examine associations between serum oxylipins, which regulate tissue repair and pain signalling, and knee pain/radiographic osteoarthritis (OA) at baseline and knee pain at 3 year follow-up.
Method: Baseline, and 3 year follow-up, knee pain phenotypes were assessed from 154 participants in the Knee Pain in the Community (KPIC) cohort study. Serum and radiographic Kellgren and Lawrence (KL) and Nottingham line drawing atlas OA scores were collected at baseline. Oxylipin levels were quantified using liquid chromatography coupled with mass spectrometry. Associations were measured by linear regression and receiver operating characteristics (ROC).
Results: Serum levels of 8,9-epoxyeicosatrienoic acid (EET) (β(95% confidence intervals (CI)) = 1.809 (−0.71 to 2.91)), 14,15-dihydroxyeicosatrienoic acid (DHET) (β(95%CI) = 0.827 (0.34–1.31)), and 12-hydroxyeicosatetraenoic acid (HETE) (β(95%CI) = 4.090 (1.92–6.26)) and anandamide (β(95%CI) = 3.060 (1.35–4.77)) were cross-sectionally associated with current self-reported knee pain scores (numerical rating scale (NRS) item 3, average pain). Serum levels of 9- (β(95%CI) = 0.467 (0.18–0.75)) and 15-HETE (β(95%CI) = 0.759 (0.29–1.22)), 14-hydroxydocosahexaenoic acid (β(95%CI) = 0.483(0.24–0.73)), and the ratio of 8,9-EET:DHET (β(95%CI) = 0.510(0.19–0.82)) were cross-sectionally associated with KL scores. Baseline serum concentrations of 8,9-EET (β(95%CI) = 2.166 (0.89–3.44)), 5,6-DHET (β(95%CI) = 152.179 (69.39–234.97)), and 5-HETE (β(95%CI) = 1.724 (0.677–2.77) showed positive longitudinal associations with follow-up knee pain scores (NRS item 3, average pain). Combined serum 8,9-EET and 5-HETE concentration showed the strongest longitudinal association (β(95%CI) = 1.156 (0.54–1.77) with pain scores at 3 years, and ROC curves distinguished between participants with no pain and high pain scores at follow-up (area under curve (95%CI) = 0.71 (0.61–0.82)).
Conclusions: Serum levels of a combination of hydroxylated metabolites of arachidonic acid may have prognostic utility for knee pain, providing a potential novel approach to identify people who are more likely to have debilitating pain in the future.

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https://doi.org/10.1016/j.joca.2024.04.006

Introduction

Osteoarthritis (OA) is a major cause of chronic pain worldwide,[1] and is the commonest form of arthritis.[2] The knee is a common site for OA and associated pain often changes from being intermittent to constant as the disease progresses.[3] Diagnostic approaches that predict the likely course of individuals' pain progression may improve clinical decision-making and provide a rationale for the inclusion of individuals in future clinical trials of novel treatments. The complexity of chronic OA pain mechanisms substantially contributes to the challenges of effective treatment,[4] which may be aided by identifying prognostic molecules that predict future OA pain and pathology,[5–7] and could facilitate personalised treatments.[8]

Inflammatory signalling molecules, and related enzymatic cascades (cyclooxygenase-2 (COX2), lipoxygenase (LOX)) pathways, including prostaglandin E2 (PGE2), are elevated in multiple joint tissues in people with OA.[9–11] The oxylipins, a distinct class of bioactive lipids, which are derived from omega-3 and -6 polyunsaturated fatty acids (PUFAs) via COX, LOX and cytochrome P450 (CYP450) enzymatic pathways,[12] are key regulators of inflammatory signalling and are associated with progression/severity of OA and other inflammatory conditions[12–14] (Fig. 1). Joint inflammation is associated with knee pain[15] and cartilage pathology in people with OA.[16,17] The knee joint tissues produce a wide range of molecules including prostaglandins, cytokines, chemokines, and nerve growth factor, which may contribute mechanistically to this association.[18,19] Plasma levels of PGE2 and 15-hydroxyeicosatetraenoic acid (HETE) are increased in knee OA compared to non-OA controls, which may reflect low grade knee inflammation and may have diagnostic or prognostic value.[20]

The multiple oxylipins, which have known roles in the resolution of inflammation,[21] have attracted scant attention in OA pain and their potential value as prognostic biomarkers of pain progression. Two endogenous anti-inflammatory pathways, the specialised pro-resolution molecules (SPMs) and the soluble epoxide hydrolase (sEH) pathway, have been associated with OA pain. Serum levels of one SPM intermediate, 17-HDHA, are associated with heat pain thresholds in healthy volunteers and lower pain scores in people with OA.[22] Robust inhibitory effects of 17-HDHA on experimental OA pain behaviour[23] and inflammatory arthritis responses[24] have been reported. The sEH pathway regulates levels of the epoxyeicosatrienoic acid (EETs), which are derived from AA via the CYP450 pathway and have anti-inflammatory effects.[25] Members of this pathway are also associated with OA pain (serum)[26] and radiographic OA progression (synovial fluid).[27] Preclinical evidence supports the role of the sEH pathway in experimental models of OA pain.[26,28]

Our main aim was to identify baseline serum oxylipins (pro- and/or anti-inflammatory), or combinations, which distinguish participants with knee pain progression at 3 years from non-pain progressors, among participants with knee pain at baseline. As part of this analysis, we provide further cross-sectional evidence that sEH pathway derived oxylipins and pro-inflammatory molecules are associated with knee pain and radiographic OA.

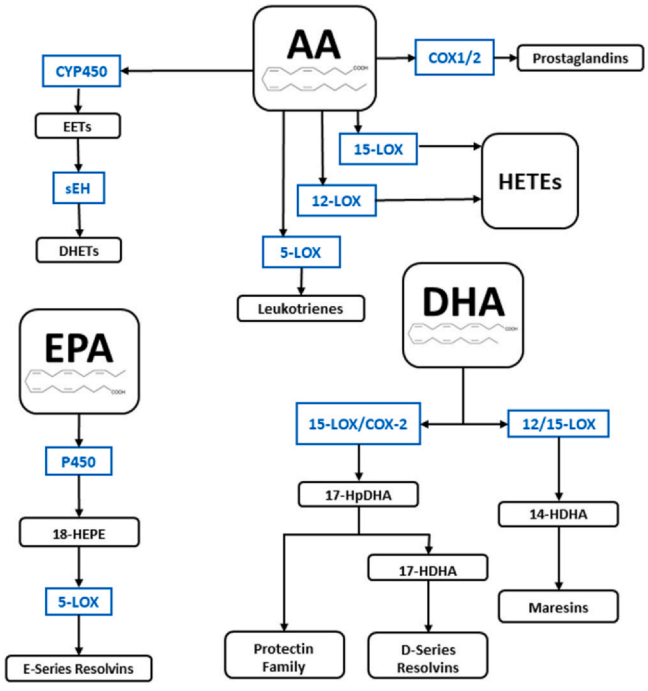

Fig. 1 Osteoarthritis and Cartilage

Simplified diagram showing the key biosynthetic pathways involved in the production of oxylipins measured in this study, items in black show lipids, and items in blue show enzymes. AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; DHETs, dihydroxyeicosatrienoic acids; HETEs, hydroxyeicosatetraenoic acids; HDHA, hydroxydocosahexaenoic acid; HpDHA, hydroperoxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid.

Methods

Participants

The Knee Pain in the Community (KPIC) cohort had 400 participants who were extensively phenotyped (pain questionnaires, quantitative sensory testing, x-ray, and ultrasound) and had blood collected at baseline. This cohort was recruited by stratification into 3 groups: recent onset knee pain (within 3 years, n = 200), established knee pain (> 3 years, n = 100) and no knee pain (n = 100). These participants (n = 400) were invited for a follow-up visit 3 years after their baseline visit.[29] One hundred and fifty-four participants had the required sample and data availability necessary for this cross-sectional and longitudinal study, participants with missing pain data at either baseline or follow-up were excluded from this study (Fig. 2).

Ethical approval was provided by Nottingham University Hospitals NHS Trust and the Nottingham Research Ethics Committee 1 (Ref:14/EM/0015), all participants provided written informed consent. Participants included in this study had a range of radiographic OA and knee pain severities (Table I).

Patient and public involvement statement

A patient and public involvement group reviewed a lay summary of our proposed study and provided feedback through a focus group. They agreed that being able to predict future pain intensity is desirable if it would help with the clinical management of OA pain progression.

Pain and pathology assessments

Participants were invited to complete self-reported pain and participate in further clinical assessments including pain pressure detection threshold (PPT), knee ultrasound, and radiographs. Detailed methods, including self-reported pain questions, are described in the Supplementary Information and the KPIC study protocol.[29]

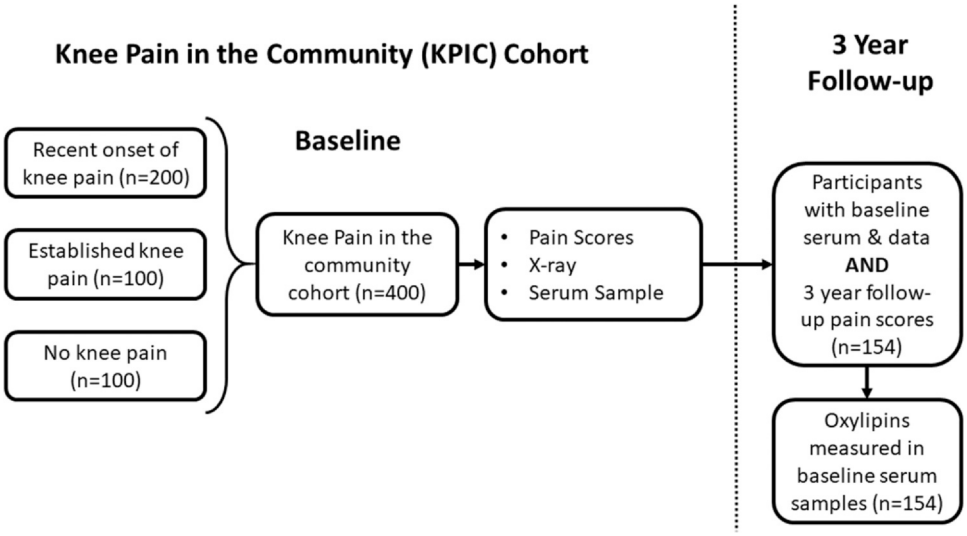

Fig. 2 Osteoarthritis and Cartilage

Flow diagram showing the selection of participants from the KPIC cohort included in this study. Inclusion criteria comprised of availability of a serum sample, x-ray, and pain assessments at baseline and pain assessments at 3 year follow-up (n = 154).

	All participants	Undefined joint damage-lower pain	OA-higher pain	Remaining participants
No of participants	154	56	45	53
Age	61 [53–68]	57 [52–67]	61 [55–66]	64 [57–70]
BMI (kg/m^2)	28 [25–32]	27 [24–31]	29 [26–33]	28 [25–33]
Sex (%F)	61.7%	62.5%	62.2%	61.2%
Average NRS	5 [3–7]	3 [2–4]	7 [6-8.5]	5 [3–6]
PDQ	6 [3–10]	4 [2–8]	11 [8–14]	5 [3–8]
KL grade	2 [0–3]	0 [0,1]	3 [2,3]	2 [1–3]
Knee effusion:				
Both knees	3.6 [1.5–5.3]	2.5 [0.9–4.3]	4.5 [3.4–7.5]	3.9 [1.3–5.3]
Worst knee	4.5 [2.5–7.1]	3.5 [1.7–5.3]	6.3 [4.1–10.1]	4.5 [2.1–7.2]
Synovial hypertrophy:				
Both knees	0.5 [0–2.6]	0.0 [0.0–1.4]	2.3 [0.0–4.9]	0.0 [0.0–2.9]
Worst knee	1.0 [0–5.0]	0.0 [0.0–2.5]	3.8 [0.0–7.1]	0.0 [0.0–4.1]
C-reactive protein (mg/L)	2.1 [1.0–3.9]	1.9 [1.4–3.7]	2.3 [1.2–7.9]	1.6 [0.7–3.5]
Comorbidities				
High cholesterol	35 [22.7%]	11 [19.6%]	11 [24.4%]	12 [22.6%]
Heart attack/angina	7 [4.5%]	2 [3.6%]	4 [8.9%]	1 [1.9%]
Hypertension	45 [29.2%]	14 [25.0%]	15 [33.3%]	16 [30.2%]
Diabetes	18 [11.6%]	5 [8.9%]	8 [17.8%]	5 [9.4%]
Stroke	0 [0.0%]	0 [0.0%]	0 [0.0%]	0 [0.0%]
IBS	19 [12.3%]	5 [8.9%]	8 [17.8%]	6 [11.3%]
Fibromyalgia	0 [0.0%]	0 [0.0%]	0 [0.0%]	0 [0.0%]
Cancer	16 [10.4%]	9 [16.1%]	2 [4.4%]	5 [9.4%]

IBS, irritable bowel syndrome.

Table I Osteoarthritis and Cartilage

Clinical characteristics at baseline of the full cohort (n = 154); undefined joint damage-lower pain group (defined as having KL ≤1 and average NRS score of ≤5); OA-higher pain group (defined as having KL ≥2 and average NRS score ≥6); and participants whose clinical features did not place them in early or advanced groups (remaining). Data are presented as median [interquartile range]. Knee effusion and synovial hypertrophy are presented for both knees (calculated as mean of both knees), and worst knee (highest score from either knee). BMI = body mass index, NRS = numerical rating scale (based on question 3 of the questionnaire), PDQ = pain detect questionnaire, KL = Kellgren-Lawrence radiographic grade.

Quantification of serum oxylipins by liquid chromatography with tandem mass spectrometry

Thirty-six oxylipins were quantified in serum samples and met the required analytical acceptance criteria using the method previously method,[30] which was updated to include a larger number of analytes and updated instrumentation. Detailed methods are described in the Supplementary Information.

Data analysis

Participant characteristics are presented as the median ± interquartile range as data were not normally distributed. Concentrations of oxylipins were log-transformed for regression analyses in order to achieve a normal distribution. For some analyses the ratio of precursor to product oxylipins levels was calculated. Data were analysed in Prism (Graphpad v.8) or R programming software (www.r-project.org). Regression analyses between oxylipin levels and pain scores were performed without adjustment for age, sex, BMI, or KL, and separate analyses were performed between these clinical descriptors and oxylipin levels. Analyses were adjusted for multiple tests using Bonferroni correction. To investigate the prognostic potential of baseline oxylipins, regression analyses with follow-up pain were not adjusted for baseline pain.

At baseline, participants were stratified based on KL grades and average NRS scores (item 3, average pain) of knee pain into an undefined joint damage with below median overall pain score group (KL ≤ 1 and NRS ≤ 5 n = 56) or an established structural OA with above median overall pain score group (KL ≥ 2 and NRS ≥ 6 n = 45). These two groups were selected to focus on the two extremes of this condition. These cut-offs were based on the established classification for OA on the KL scale[31] and the median value for average NRS score at baseline. Univariate analysis compared levels of oxylipins between these two groups was undertaken using Mann-Whitney U test.

At follow-up, participants were stratified into three groups based on NRS score (item 3, average pain): no pain (NRS = 0 (n = 51)); lower pain (NRS ≤ 5 (n = 57)); higher pain (NRS ≥ 6 (n = 46)). Univariate analysis compared the levels of oxylipins at baseline between these groups was undertaken using Kruskal-Wallis Test. Receiver operating characteristic (ROC) curves were performed between the 'no pain' and 'higher pain' groups to assess the ability for baseline oxylipins to distinguish between these two groups at follow-up. Area under curve (AUC) data from ROC analysis were compared to assess potential differences between the oxylipins performance in distinguishing between the two groups.

Results

Participant characteristics

Participants had an average age of 61 years and 62% were female (Table I). Seventy-three of 154 (47.4%) had a KL grade below 2 and 81/154 (52.6%) had a KL grade of 2 or above in their worst knee. Sixty-two (40.3%) had an average NRS score (item 3, average pain) of 5 or less, and 92 (59.7%) had an average NRS score of 6 or more. The median levels of C-reactive protein (CRP) were 2.1 mg/L (1.0–3.9 interquartile range). The most common comorbidities were hypertension (29.2%) and high cholesterol (22.7%). Two subgroups were analysed based on KL score and average NRS score: undefined joint damage-lower pain (KL ≤ 1 and NRS ≤ 5, n = 56) or OA-higher pain (KL ≥ 2 and NRS ≥ 6, n = 45). Participants in each subgroup had similar age, sex, and BMI (Table I). The participants who were not allocated to either group had either a KL≥ 2 and NRS ≤ 5 (n = 36) or had KL ≤ 1 and NRS ≥ 6 (n = 17) (Table I).

Cross-sectional associations between serum levels of oxylipins with knee pain and radiographic OA at baseline

A total of 34 oxylipins met the required analytical criteria and serum levels were quantified in the full cohort (n = 154) at baseline (Table II). Linear regression analysis, Bonferroni corrected for multiple tests, revealed associations of levels of DHA (β(95% confidence intervals (CI)) = 13.359 (5.51–21.21)) and thromboxane B2 (β(95%CI) = −1.976 (−3.09 to −0.86) with age (Table III, Supplementary Table I), and negative associations of EPA (β(95%CI) = −7.572 (−11.46 to −3.86)) and DHA (β(95%CI) = −9.646 (−14.79 to −4.49)) with BMI (Table III, Supplementary Table I). In addition, levels of 5,6-DHET were lower in females (β(95%CI) = −0.450 (0.23–0.67)) (Table III, Supplementary Table I). None of the lipids measured in the study were significantly associated with CRP levels.

Linear regressions were performed to identify potential associations of oxylipin levels with current pain and with radiographic OA at baseline. Levels of 8,9-EET (β(95%CI) = 1.809(−0.71 to 2.91)), 14,15-DHET (β(95%CI) = 0.827(0.34–1.31)), 12-HpETE (β(95%CI) = 4.090 (1.92–6.26)), and anandamide (AEA) (β(95%CI) = 3.060(1.35–4.77)) were cross-sectionally associated with the NRS score for 'how would you rate your pain at the present time?' (Table IV). Levels of AEA had a positive cross-sectional association with pain detect questionnaire (PDQ) scores (β(95%CI) = 5.875 (2.43–9.31)) (Table IV, Supplementary Table II). The ratio of 8,9-EET:DHET levels (β(95%CI) = 0.510 (0.19–0.82)), and levels of 9-HETE (β(95%CI) = 0.467 (0.18–0.75)) and 15-HETE (β(95%CI) = 0.759 (0.29–1.22)) and 14-HDHA (β(95%CI) = 0.483 (0.24–0.73)) had positive cross-sectional associations with KL score (Table III). Levels of 12-HpETE (β(95%CI) = 4.727 (1.89–7.57)) were positively associated with osteophyte scores (Supplementary Table I). There were no significant associations between levels of any of the oxylipins measured with knee effusion, knee hypertrophy, total joint, or joint space narrowing scores (Supplementary Table I).

To further explore the associations between oxylipins and OA pain and pathology identified in the full cohort (n = 154), a univariate analysis compared levels of these oxylipins between participants with established OA and higher levels of knee pain and those with an undefined diagnosis and lower levels of knee pain. Of the oxylipins identified by the linear regression in all participants, levels of 8,9-EET, 12-HpETE, and AEA were significantly higher in the OA-higher pain group compared to undefined joint damage-lower pain group (Table II, Figure 3A-C).

Levels of 14,15-DHET were not significantly different between the two groups (Figure 3D). These univariate analyses also revealed that levels of the SPM precursors 17-HDHA and 14-HDHA and various pro-inflammatory molecules (5-, 9-, 11-, 12-, and 20-HETE) were higher in the OA-higher pain group compared to undefined joint damage-lower pain group (Table II, Supplementary Figure 2). Consistent with a previous study,[20] levels of 15-HETE were significantly higher in the OA-higher pain group compared to undefined joint damage-lower pain group (Supplementary Figure 3A), however there were no significant differences in the levels of PGE2 between groups in our study (Supplementary Figure 3B). Linear regression analyses between oxylipin levels and measures of pain in the sub-cohort (participants with established OA and higher levels of knee pain and those with an undefined diagnosis and lower knee pain) were performed. In addition to the associations identified in the full cohort, levels of 5,6-, (β(95%CI) = 1.340 (0.63–2.05)) and 11,12-EET (β(95%CI) = 1.310 (0.63–1.99)); 5,6-, (β(95%CI) = 1.505 (0.79–2.22)) and 11,12-ratio (β(95%CI) = 1.282 (0.60–1.96)); 9-, (β(95%CI) = 1.435 (0.68–2.19)) 12-, (β(95%CI) = 1.323 (0.66–1.99)) and 20-HETE (β(95%CI) = 1.388 (0.71–2.06)); 14-HDHA (β(95%CI) = 1.309 (0.69–1.93)), each had a positive cross-sectional association with at least one of the four NRS item scores (n = 101, Table IV, Supplementary

Lipid	Full cohort (n = 154) Mean (nM) ± SD	Undefined joint damage-lower pain (n = 56) Mean (nM) ± SD	OA-higher pain (n = 45) Mean (nM) ± SD	Remaining (n = 53) Mean (nM) ± SD
5,6-EET	24.18 ± 42.25	11.37 ± 17.78	40.11 ± 56.17	27.20 ± 43.45
5,6-DHET	0.58 ± 0.41	0.56 ± 0.36	0.61 ± 0.46	0.60 ± 0.40
5,6-Ratio	44.64 ± 93.90	32.98 ± 108.44	59.16 ± 69.10	44.80 ± 74.90
8,9-EET	0.77 ± 1.04	0.49 ± 0.49	1.11 ± 1.38	0.76 ± 0.96
8,9-DHET	0.35 ± 0.19	0.35 ± 0.18	0.35 ± 0.20	0.34 ± 0.27
8,9-Ratio	45.98 ± 429.75	1.63 ± 1.65	101.18 ± 639.54	134.55 ± 638.16
11,12-EET	125.11 ± 220.71	59.67 ± 94.27	206.54 ± 293.79	142.62 ± 226.11
11,12-DHET	0.84 ± 0.47	0.85 ± 0.45	0.82 ± 0.49	0.88 ± 0.49
11,12-Ratio	178.69 ± 337.10	88.05 ± 159.01	291.48 ± 447.93	240.63 ± 432.88
14,15-EET	0.30 ± 0.17	0.29 ± 0.18	0.31 ± 0.15	0.25 ± 0.13
14,15-DHET	0.53 ± 0.53	0.54 ± 0.63	0.52 ± 0.38	0.54 ± 0.39
14,15-Ratio	28.28 ± 77.14	43.21 ± 91.37	9.70 ± 48.41	4.26 ± 15.91
12-HpETE	3.55 ± 2.09	2.94 ± 1.14	4.32 ± 2.68	3.27 ± 1.98
TBXB2	12.46 ± 21.23	8.88 ± 11.86	16.93 ± 28.30	13.39 ± 24.70
PGE2	0.46 ± 0.59	0.38 ± 0.41	0.55 ± 0.74	0.63 ± 1.14
LTB4	0.50 ± 0.97	0.40 ± 0.71	0.61 ± 1.20	0.65 ± 0.94
20-HETE	43.19 ± 78.08	19.78 ± 31.37	72.31 ± 104.54	47.50 ± 74.00
18-HEPE	0.63 ± 0.61	0.56 ± 0.54	0.71 ± 0.68	0.68 ± 0.76
16-HETE	0.50 ± 0.33	0.53 ± 0.39	0.46 ± 0.22	0.53 ± 0.34
15-HETE	5.47 ± 7.27	3.75 ± 4.29	7.61 ± 9.36	5.84 ± 6.70
13-oxoODE	13.92 ± 8.43	14.90 ± 9.26	12.71 ± 7.08	17.48 ± 8.84
11-HETE	2.44 ± 2.40	1.98 ± 1.54	3.01 ± 3.07	2.53 ± 2.27
9-oxoODE	7.04 ± 4.53	7.73 ± 4.93	6.18 ± 3.82	7.48 ± 3.67
9-HODE	8.28 ± 5.79	8.40 ± 5.72	8.13 ± 5.87	14.59 ± 31.60
9-HETE	2.23 ± 4.08	1.00 ± 1.51	3.76 ± 5.50	2.41 ± 3.92
12-HETE	117.40 ± 206.99	54.28 ± 86.16	195.94 ± 275.31	133.35 ± 208.80
5-HETE	1.18 ± 1.37	0.88 ± 0.78	1.56 ± 1.79	0.85 ± 0.71
17-HDHA	1.24 ± 1.66	0.78 ± 0.75	1.80 ± 2.22	1.67 ± 2.61
14-HDHA	23.93 ± 52.53	8.66 ± 16.86	42.93 ± 72.03	28.05 ± 51.90
AEA	0.73 ± 0.40	0.62 ± 0.38	0.86 ± 0.38	0.73 ± 0.40
AA	13879.96 ± 4958.74	12661.60 ± 4411.58	15396.14 ± 5179.13	13409.35 ± 4829.68
LA	21896.43 ± 9355.66	22250.42 ± 10148.67	21455.91 ± 8242.00	23235.95 ± 9011.40
EPA	752.97 ± 407.61	747.47 ± 388.32	759.81 ± 430.31	670.66 ± 383.45
DHA	16327.41 ± 7033.10	16083.18 ± 6408.57	16631.35 ± 7729.47	15453.54 ± 6471.32

AEA, anandamide.

Table II

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Average concentration (nM) ± standard deviation of oxylipins quantified in serum in full cohort (n = 154); undefined joint damage-lower pain group (KL ≤ 1 and NRS ≤ 5); OA-higher pain group (KL ≥ 2 and NRS ≥ 6); and participants whose clinical features did not place them in defined groups (remaining).

	Age		Sex		BMI		KL	
Lipid	Beta (SE)	P Value	Beta (SE)	P Value	Beta (SE)	P Value	Beta (SE)	P Value
5,6-DHET	ns		-0.450 (0.110)	0.00002	ns		ns	
8,9-EET:DHET ratio	ns		ns		ns		0.510 (0.160)	0.0014
TBXB2	−1.976 (0.569)	0.0005	ns		ns		ns	
15-HETE	ns		ns		ns		0.759 (0.236)	0.0013
9-HETE	ns		ns		ns		0.467 (0.145)	0.0013
14-HDHA	ns		ns		ns		0.483 (0.125)	0.0001
EPA	ns		ns		−7.572 (1.984)	0.0001	ns	
DHA	13.359 (4.007)	0.0009	ns		−9.646 (2.626)	0.0002	ns	

SE, standard error.

Table III

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Significant associations (linear regression analysis) between baseline serum levels of oxylipins with age, sex, BMI, and KL score in the full cohort of participants (n = 154). P values were adjusted for multiple comparisons using Bonferroni correction. Full dataset and all statistical values provided in Supplementary Table I. BMI, body mass index; KL, Kellgren-Lawrence; SE, standard error.

Table III). Levels of AEA (β(95%CI) = 9.146 (4.73–13.56)) and 14,15-DHET (β(95%CI) = 2.019 (0.88–3.16)) were cross-sectionally associated with higher PDQ scores in this subset of participants, whereas the ratio of 14,15-EET:DHET (β(95%CI) = −1.700 (−2.67 to −0.73)) showed a negative cross-sectional association associated with PDQ scores (Table IV).

Baseline serum levels of pro- and anti-inflammatory oxylipins predict pain scores at 3 years

We next investigated whether baseline levels of oxylipins were longitudinally associated with future pain scores 3 years later

Lipid	Present Time (Q1)		Worst Pain (Q2)		Average Pain (Q3)		Mean of Q1-3		PDQ	
	Beta (SE)	P value	Beta (SE)	P value	Beta (SE)	P value	Beta (SE)	P value	Beta (SE)	P value
Baseline: full cohort (cross-sectional analysis)										
8,9-EET	1.809 (0.561)	0.0013	ns		ns		ns		ns	
14,15-DHET	0.827 (0.248)	0.0009	ns		ns		ns		ns	
12-HpETE	4.090 (1.109)	0.0002	ns		ns		ns		ns	
AEA	3.060 (0.870)	0.0004	ns		ns		ns		5.875 (1.756)	0.0008
Baseline: undefined joint damage-lower pain/OA-higher pain subgroups (cross-sectional analysis)										
5,6-EET	ns		1.340 (0.360)	0.0002			1.249 (0.335)	0.0002	ns	
5,6-EET:DHET ratio	ns		1.505 (0.367)	4.17E-05	1.210 (0.364)	0.0009	1.249 (0.348)	0.0003	ns	
8,9-EET	2.410 (0.725)	0.0009	ns		2.132 (0.623)	0.0011	2.171 (0.628)	0.0006	ns	
11,12-EET	ns		1.310 (0.345)	0.0001	ns		1.178 (0.323)	0.0003	ns	
11,12-EET:DHET ratio	ns		1.282 (0.346)	0.0002	ns		1.105 (0.325)	0.0007	ns	
14,15-DHET	1.006 (0.289)	0.0005	ns		ns		ns		2.019 (0.580)	0.0005
14,15-EET:DHET ratio	ns		ns		ns		ns		-1.700 (0.497)	0.0006
12-HpETE	5.465 (1.515)	0.0003	4.718 (1.431)	0.0010	4.835 (1.316)	0.0002	5.030 (1.308)	0.0001	ns	
20-HETE	ns		1.388 (0.344)	5.56E-05	1.104 (0.335)	0.0010	1.262 (0.322)	8.78E-05	ns	
9-HETE	ns		1.435 (0.387)	0.0002	1.260 (0.377)	0.0008	1.340 (0.361)	0.0002	ns	
12-HETE	ns		1.323 (0.339)	9.54E-05	1.048 (0.329)	0.0014	1.193 (0.317)	0.0002	ns	
14-HDHA	1.368 (0.370)	0.0002	1.245 (0.348)	0.0003	1.285 (0.333)	0.0001	1.309 (0.317)	3.71E-05	ns	
AEA	4.491 (1.124)	6.43E-05	3.586 (1.074)	0.0008	4.356 (0.018)	1.88E-05	4.067 (0.971)	2.82E-05	9.146 (2.252)	4.89E-5
3 year follow-up: full cohort (longitudinal analysis)										
8,9-EET	ns		ns		2.166 (0.650)	0.0009	1.949 (0.601)	0.0012	ns	
5-HETE	ns		2.030 (0.602)	0.0008	ns		1.724 (0.534)	0.0010	ns	
8,9-EET + 5-HETE	0.904 (0.292)	0.0019	1.344 (0.359)	0.0002	1.230 (0.338)	0.0003	1.156 (0.312)	0.0002	ns	
8,9-EET + 5-HETE + 5,6-DHET	0.623 (0.249)	0.0132	0.926 (0.307)	0.0029	0.879 (0.288)	0.0027	0.809 (0.266)	0.0028	ns	

AEA, anandamide; SE, standard error.

Table IV Osteoarthritis and Cartilage

Significant associations (linear regression analysis), after adjusting for multiple comparisons, between baseline levels of oxylipins and baseline NRS scores for the full cohort (n = 154), baseline NRS scores in the undefined joint damage-Lower pain and OA-higher pain groups (n = 101), and 3 year NRS scores in the full cohort (n = 154). The NRS scores were calculated from the responses to the following questions: Q1: How would you rate your most painful knee pain on a 0–10 scale at the present time, that is right now, where 0 is 'no pain' and 10 is 'pain as bad as could be'?; Q2: In the past month, how intense was your 'worst knee pain' rated on a 0–10 scale, where 0 is 'no pain' and 10 is 'pain as bad as could be'?; Q3: In the past month, on average, how intense was the pain in your most painful knee rated on a 0–10 scale, where 0 is 'no pain' and 10 is 'pain as bad as could be'? Mean: average score of questions 1–3. PDQ: PainDETECT questionnaire score. P values were adjusted for multiple tests using Bonferroni correction. All statistical values are provided in Supplementary 2–4.

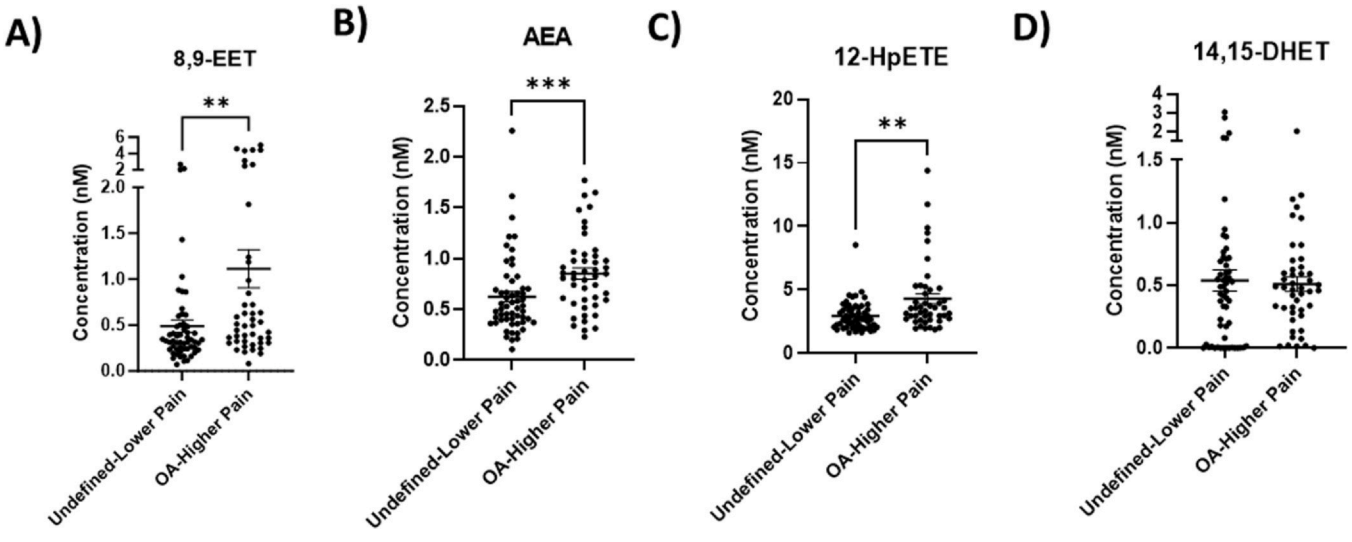

Fig. 3 Osteoarthritis and Cartilage

Serum concentration of (A) 8,9-EET; (B) AEA; (C) 12-HpETE; and (D) 14,15-DHET in baseline samples based on the undefined joint damage-lower pain and OA-higher pain groups. Significance was assessed using Mann-Whitney test. *P = < 0.05, **P = < 0.01, ***P = < 0.001. AEA, anandamide.

(n = 154, Supplementary Table IV). Linear regression analysis revealed that baseline levels of pro-inflammatory 5-HETE (β(95%CI) = 1.724 (0.677–2.77) and anti-inflammatory 8,9-EET (β(95%CI) = 2.166 (0.89–3.44)) each had positive longitudinal association with self-reported pain scores at 3 years (Table IV). Baseline levels of 5,6-DHET (β(95%CI) = 152.179 (69.39–234.97)) were associated with higher PPT (tibialis anterior) scores at 3 years (β(SE) = 152.179 (42.24); P = 0.0003, Supplementary Table IV). We then explored whether a combined measurement of 5-HETE, 8,9-DHET and/or 5, 6-DHET may have improved utility in predicting pain at 3 years. In this analysis, combining baseline levels of 5-HETE and 8,9-EET further strengthened the associations with pain score at 3 years follow-up (β(95%CI) = 1.156 (0.54–1.77)), however the addition of 5,6-DHET weakened this association (β(95%CI) = 0.809 (0.29–1.33), Table IV). When compared to age, sex, BMI, and KL score individually, the combination of 5-HETE + 8,9-EET showed significantly higher R^2 values for the prediction model (Supplementary Figure 4A). These analyses also demonstrated that a combination of lipids, age, sex, and BMI gave the highest R^2 value when comparing prediction models (Supplementary Figure 4A).

We then undertook a univariate analysis to explore whether the relationships between baseline levels of oxylipins with pain at 3 years were substantiated by sub-cohort analysis (Supplementary Table V). In addition, we investigated whether ROC curves for these baseline oxylipins were able to distinguish between people with no pain and high pain 3 years later. Baseline levels of 5-HETE were higher in both the lower and higher pain group, compared to the no pain group (Figure 4A) and ROC analysis significantly distinguished between the no pain group and high pain group (AUC(95%CI) = 0.68 (0.57–0.78); P = 0.0019) (Figure 4F). Levels of 8,9-EET were higher in the higher pain group compared to the no pain group (Figure 4B) and ROC analysis was significantly able to distinguish between the no and high pain groups (AUC(95%CI) = 0.70(0.59–0.80); P = 0.0007) (Figure 4G). Levels of 5,6-DHET showed no significant differences between groups (Figure 4C), and ROC analysis did not distinguish the no pain group from the high pain group (AUC (95%CI) = 0.57 (0.46–0.69); P = 0.207) (Figure 4H). Analysis of the combined concentrations of 5-HETE and 8,9-EET for the two groups strengthened the statistical power of this analysis (AUC (95%CI) = 0.71 (0.61–0.82); P = 0.0002) (Figure 4D,I) and gave a sensitivity value of 0.67 (0.52 to 0.80) and a specificity value of 0.75 (0.62 to 0.86). By contrast, the inclusion of concentrations of 5,6-DHET into this analysis did not strengthen the significance of this finding (AUC (95%CI) = 0.68 (0.58–0.78); P = 0.0017) (Figure 4E,J). Of the other oxylipins measured, baseline levels of DHA were significantly higher in the high pain group compared to the low pain group, and levels of 14,15-EET and 16-HETE were significantly higher in the low pain group compared to the no pain group (Supplementary Figure 5).

Discussion

Here we report that serum concentrations of multiple oxylipins are cross-sectionally associated with current self-reported pain scores and radiographic knee OA in a community cohort of participants. Importantly from a prognostic perspective, baseline concentrations of 8,9-EET, 5-HETE, and 5,6-DHET showed a positive longitudinal association with pain measures at the 3 year follow-up. Our data suggest that the measurement of a combination of specific hydroxylated metabolites of AA in serum may have utility as a prognostic marker for pain, providing a possible novel approach to identify people with knee pain who are more likely to have moderate to severe pain after 3 years.

Serum pro- and anti-inflammatory oxylipins associated with current knee pain

We quantified multiple pro-inflammatory oxylipins, however only 12-HpETE was cross-sectionally associated with current pain at baseline. Given that 12-HpETE, which is derived from AA via the 12-LOX pathway, is reported to be an activator of TRPV1, a key receptor

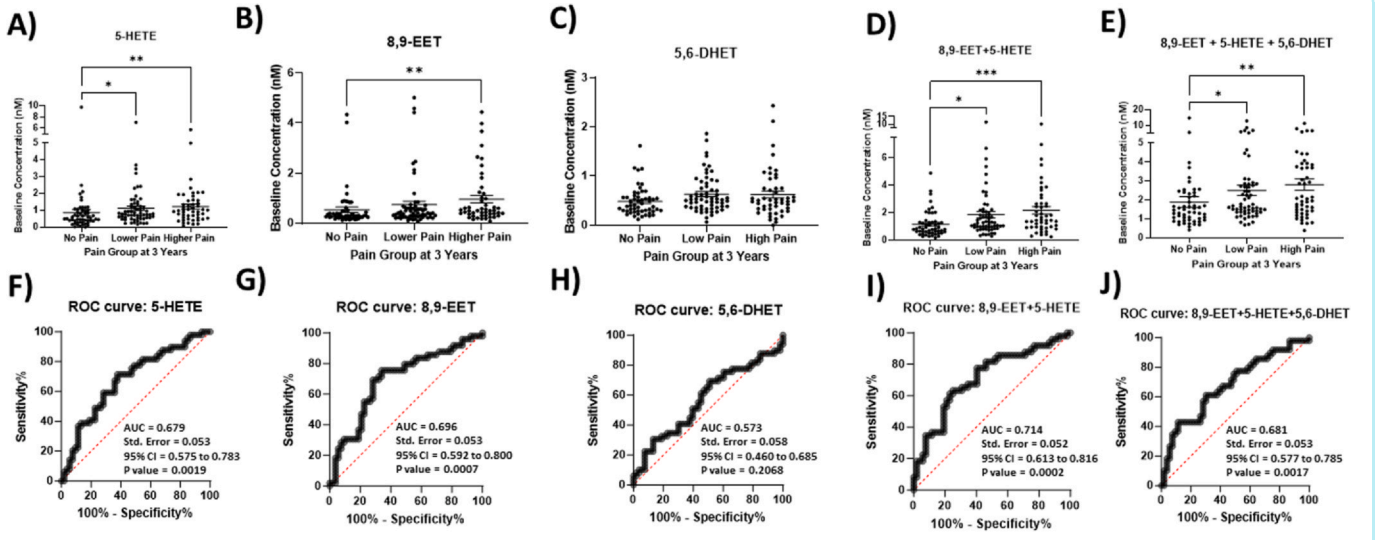

Fig. 4

Osteoarthritis and Cartilage

Baseline serum levels of (A) 5-HETE, (B) 8,9-EET, (C) 5,6-DHET; (D) 5-HETE + 8,9-EET; and (E) 5-HETE + 8,9-EET + 5,6-DHET in groups based on 3 year follow-up pain scores from Q3 (average pain). No pain = score of 0; lower pain = score 1–5; and higher pain = score 6–10. Significance was assessed using Kruskal-Wallis test with multiple comparisons. *P = < 0.05, **P = < 0.01. ROC analysis of the ability of baseline concentrations of 5-HETE (F), 8,9-EET (G), 5,6-DHET (H) and combinations of these oxylipins (I and J) to distinguish between the no pain group and high pain group at 3 year follow up. AUC, SE, 95%CI, and P values are presented for each curve. SE, standard error.

in pain signalling[32] this association with current OA pain has a potential mechanistic basis. Furthermore, we have previously reported a significant cross-sectional association between plasma 12-HpETE levels and synovitis scores in an animal model of OA.[33]

A number of other oxylipins were also cross-sectionally associated with current pain at baseline. 8,9-EET and 14,15-DHET, part of the anti-inflammatory sEH pathway, were negatively associated with current knee pain, suggesting a role in counteracting ongoing pain.[26] To explore further the potential relationships between knee OA and pain with serum levels of EETs and DHETS, participants were selected by radiographic OA score and knee pain score. Univariate analysis revealed higher levels of 5,6-, 8,9-, and 11,12-EET and their respective EET:DHET ratios in the OA-higher pain group compared to the undefined joint damage-lower pain group. These findings are consistent with a previous study in which we reported cross-sectional associations between 5,6-, 8,9-, and 11,12-EET and NRS score.[26] Collectively these new, and previous,[26,34] datasets support our emerging understanding of the role of the sEH pathway in modulating OA pain mechanisms.[26] Inhibitors of sEH are currently under development for clinical studies and are already being tested in for neuropathic pain (ClinicalTrials.gov identifier: NCT04228302), and therefore could be a potential treatment for chronic knee pain.

Our analytical method included precursors of some of the SPMs (17- and 14-HDHA, and 18-HEPE), as well as some of the SPMs. Consistent with our previous OA studies we did not detect the SPMs in our serum samples.[26,27] Levels of the SPM precursors, 14- and 17-HDHA and 18-HEPE, were detectable and quantified, but were not associated with current knee pain at baseline in our full cohort. However, univariate analysis revealed significantly higher levels of 14-HDHA and 17-HDHA in the OA-higher pain group compared to undefined joint damage-lower pain group, consistent with our previous work on 17-HDHA.[22] 14-HDHA is an intermediary molecule in the DHA pathway and is a precursor to the maresins, which are mainly produced by macrophages and act to drive M2 macrophage activity, including the release of anti-inflammatory cytokines and the resolution of inflammation.[35] Serum levels of the inhibitory endocannabinoid AEA were also associated positively with current knee pain at baseline in the full cohort analysis. AEA has well described anti-inflammatory and analgesic effects both in humans and experimental pain models.[36,37] Previously we reported the presence of AEA in synovial fluid from participants with OA and RA, however control groups were not available at that time.[38] Studies using experimental models of pain support a role of the endocannabinoids, including AEA, in regulating nociceptive responses in models of chronic pain.[39] Levels of AEA have been reported to be elevated in the spinal cord in the monosodium iodoacetate model of OA pain[40] and pharmacological treatments which prevent the degradation of AEA attenuate pain behaviour in models of OA pain.[40–42]

Pro- and anti-inflammatory oxylipins at baseline predict OA knee pain score at 3 years

Regression analysis performed between baseline levels of oxylipins and pain scores 3 years later revealed positive longitudinal associations between 8,9-EET and 5-HETE with two items of the self-reported knee pain scores, and between 5,6-DHET and higher PPT scores. Analysis of the combined levels of 8,9-EET and 5-HETE strengthened the existing longitudinal associations with two items of the NRS scores, and led to associations with a further two items, improving on the prognostic utility of serum levels of these individual molecules. By contrast, the addition of 5,6-DHET into the model reduced the strength of the associations. Building on this linear regression, univariate analysis compared baseline lipid levels between groups stratified by pain at 3 years. Consistent with the regression analysis in the full cohort, participants with higher pain at 3 year had significantly higher levels of 8,9-EET and 5-HETE, but not 5,6-DHET. These data were further supported by ROC analysis which revealed that both 8,9-EET and 5-HETE can distinguish participants with no pain from high pain at 3 year follow-up. Additional statistical comparison of the ROC datasets confirmed the utility of the combination of serum 8,9-EET and 5-HETE concentrations over 5,6-DHET in distinguishing between these two groups. The combined model of 8,9-EET and 5-HETE using ROC analysis showed good sensitivity and specificity values. Collectively, data from the linear regression, univariate, and ROC analyses suggest that of the molecules quantified, a combined measurement of serum 8,9-EET and 5-HETE has the highest potential to predict future OA knee pain so as to inform treatment strategies. Further studies are required to determine whether combining these molecules with other factors (not measured in this study) may further improve prediction value.

Serum levels of 8,9-EET were both cross-sectionally and longitudinally associated with knee pain. Current understanding of the biological effects of 8,9-EET are quite mixed, local in vivo administration of 8,9-EET induced a short-lived mechanical hyperalgesia in mice supporting a pro-nociceptive role.[43] Lower concentrations of 8,9-EET inhibited the activation of NLRP3 inflammasome in murine macrophages,[44] however, higher concentrations of 8,9-EET sensitised and directly activated TRPA1 expressing nociceptive neurons.[43,45] The serum concentrations of 8,9-EET quantified in the present study were around 1000-fold lower (in the nanomolar range) compared to concentrations used in the above studies and are in the same range as those we previously reported in synovial fluid in people with radiographic OA.[27] Thus, it is feasible that the concentrations of 8,9-EET at their site of action in knee joint (macrophages or nociceptors) are closer to those reported to have anti-inflammatory activity in in vitro assays. Relevant to OA pathology and pain, 8,9-EET is a substrate for COX2, which generates the proangiogenic oxylipin 8,9-epoxy-11-hydroxy-eicosatrienoic acid (ct-8,9-E-11-HET).[46] Given the complex role of angiogenesis in structural damage and pain in OA,[47] the role(s) of 8,9-EET and its metabolites in driving the underlying pathology and pain in OA requires further mechanistic interrogation.

Pro- and anti-inflammatory oxylipins are cross-sectionally associated with radiographic knee OA

As well as knee pain, joint pathology was also associated with multiple serum oxylipins. Of the oxylipins associated with KL score, 15-HETE was the only lipid associated with KL score, but not pain. A previous larger study however reported higher plasma levels of 15-HETE in symptomatic knee OA patients (n = 300) compared to non-OA controls (n = 100).[20] Differences between these studies may reflect the group sizes, and/or the inclusion of a non-OA control group. In our study, none of the oxylipins measured were associated with ultrasound measurements of synovial effusion or hypertrophy, which are indicative of localised inflammation in the knee joint. This may suggest a disconnect between serum levels of oxylipins and those in the joint.

The ratio of levels of 8,9-EET:DHET showed a positive cross-sectional association with KL score, consistent with our earlier report that levels of 8,9-DHET were associated with radiographic knee progression over 3.3 years.[27] 8,9-DHET may have utility as a biomarker for OA joint damage in the absence of pain. Experimental studies also support a role of this pathway in joint pathology damage.[33] Given the reported anti-inflammatory role of the EETs,[25] upregulation/increased activity of CYP450 could be an endogenous response to joint damage and associated inflammation. Levels of the SPM precursor molecule 14-HDHA and the pro-inflammatory molecules 9- and 15-HETE were also cross-sectionally associated with KL score in the full cohort at baseline. 14-HDHA and 15-HETE are both produced from their respective PUFA substrates (DHA and AA) via LOX enzymes (12/15-LOX),[48] and both are precursors to the

specialised pro-resolving mediators maresins (14-HDHA) and lipoxins (15-HETE). These data are consistent with experimental studies reporting that expression of 12/15-LOX in cartilage is increased during progression of OA-like pathology in mice, and that 12/15-LOX knockout mice had more severe cartilage degeneration models of OA.[49] In addition, deletion of 12/15-LOX led to uncontrolled inflammation and tissue damage in inflammatory models of arthritis.[50] Collectively, evidence suggests that LOX pathways play a protective role in the joint, potentially slowly disease progression but is insufficient to halt the structural changes driving the disease completely. Contingent upon further validation and replication studies, the oxylipins cross-sectionally associated with KL score may have clinical utility as markers of disease severity, which may help inform patient care and clinical studies without the reliance upon radiographic information.

Limitations section

This study investigated associations of serum oxylipin levels in samples from the KPIC cohort which has been used already for other exploratory analyses.[26,51] The outcomes of these exploratory analyses support hypotheses that can be prospectively tested in future purpose designed studies to establish cross-sectional and longitudinal construct validity of these lipid markers. Further studies should also investigate whether oxylipin levels are able to improve existing prognostic models based on other variables (e.g. comorbidities, etc). Although our study is focused on knee pain and pain progression, and radiographic changes of OA occur slowly, the absence of follow-up KL scores is a limitation. Another limitation is we focused solely on tibio-femoral changes and did not take account of potential patello-femoral changes. Our sub-group analysis which focused on the extreme groups, may have resulted in some bias as we excluded participants who either have asymptomatic radiographic OA or who may have non-OA related knee pain. Our study criteria required participants with serum samples and follow-up pain scores, which may have biased our cohort to participants who are more likely to engage with research and/or are proactive in managing their OA and pain, which may not be representative of the wider population of people with OA. Although follow-up pain scores at 3 years were collected, a lack of pain measures between these two time points means we have no measure of the regularity and severity of knee pain over this period. The generalisability of our data to the broader socioeconomic and ethnic diversity of people living with OA pain is limited due to the poor representation of these groups in our cohort.

The rationale for the analysis of serum oxylipin levels, rather than synovial fluid, was to support the potential future translation of these findings into a clinically useful test. Nevertheless, the findings of this study cannot infer a causative role of the oxylipins in the development of knee pain and joint pathology. Previously we have reported concordance of the associations between serum and synovial fluid levels of a number of DHETs and radiographic OA and OA progression,[27] however pain measures were not available for this previous study. The method used to measure the profile of oxylipins requires high resolution analytical facilities not amenable to routine diagnostics. However, the identification of a small number of oxylipins that have predictive pain value paves the way for the development of simpler alternative methods of detection that may have higher clinical utility in the future.

Conclusions

Cross-sectional analyses reveal potential lipid biomarkers for knee pathology which, with further validation, could provide a measure of diseases severity. Improved prediction of future pain will aid identification of individuals at risk of having the greatest OA pain burden, and may provide a rationale for earlier and or specific treatment interventions. Previously, the prognostic value of combining imaging and inflammatory blood gene expression biomarkers for the prediction of radiographic knee OA[52] and plasma lipids and leukocyte biomarkers for symptomatic OA[20] has been reported. With replication and validation in other OA and chronic pain cohorts, our longitudinal data support the prognostic utility of serum 8,9-EET and 5-HETE for future OA pain. Evaluation of the usefulness of combining these oxylipins with other wet and/or dry biomarkers may further improve the predictive validity of these molecules as biomarkers of future pain.

Ethical Approval Information

Ethical approval was provided by Nottingham University Hospitals NHS Trust and the Nottingham Research Ethics Committee 1 (Ref 14/EM/0015), all participants provided written informed consent.

Author Contributions

MD, WZ, AMV, & DAW, GF, and AS established the Knee Pain in the Community (KPIC) cohort of participants from which this study analysed samples from. GF, AS, and RFB collected and curated the clinical data associated with participants in this cohort. VC, AMV, DAB, DAW, and JT conceived the study detailed in the current manuscript. JT and RRJ conducted lipidomic and statistical analysis. MD, WZ, AMV, DAW, DAB, DHK, PG, and VC supervised data collection and analysis. All authors contributed to the preparation of the manuscript and approved the final version.

Conflicts of interest

The authors declare no financial arrangements that may represent a possible conflict of interest.

Funding

This research was funded by Versus Arthritis (Grants: 20777 and 21960) and the NIHR Nottingham Biomedical Research Centre. AMV was supported by the NIHR Nottingham BRC, MRC grants MR/Y010175/1 and MR/W026813/1 and by Versus Arthritis (grant 23139). The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

Data availability

Data can be released to bona fide researchers using the normal procedures overseen by the University of Nottingham and the Nottingham NIHR BRC and its ethical guidelines. Please contact the corresponding author to receive the application form.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.joca.2024.04.006.

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