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OXFORD

Original Article

Imbalance Between Omega-6- and Omega-3-Derived Bioactive Lipids in Arthritis in Older Adults

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Abstract

Elderly-onset rheumatoid arthritis (EORA) and polymyalgia rheumatica (PMR) are common rheumatic diseases in older adults. Oxylipins are bioactive lipids derived from omega-6 (n-6) and omega-3 (n-3) polyunsaturated fatty acids (PUFAs) that serve as activators or suppressors of systemic inflammation. We hypothesized that arthritis symptoms in older adults were related to oxylipin-related perturbations. Arthritis in older adults (ARTIEL) is an observational prospective cohort with 64 patients older than 60 years of age with newly diagnosed arthritis. Patients' blood samples at baseline and 3 months posttreatment were compared with 18 controls. A thorough clinical examination was conducted. Serum oxylipins were determined by mass spectrometry. Data processing and statistical analysis were performed in R. Forty-four patients were diagnosed with EORA and 20 with PMR. At diagnosis, EORA patients had a mean DAS28CRP (Disease Activity Score 28 using C-reactive protein) of 5.77 (*SD* 1.02). One hundred percent of PMR patients reported shoulder pain and 90% reported pelvic pain. Several n-6- and n-3-derived oxylipin species were significantly different between controls and arthritis patients. The ratio of n-3/n-6 PUFA was significantly downregulated in EORA but not in PMR patients as compared to controls. The top two candidates as biomarkers for differentiating PMR from EORA were 4-HDoHE, a hydroxydocosahexaenoic acid, and 8,15-dihydroxy-eicosatrienoic acid (8,15-diHETE). The levels of n-3-derived anti-inflammatory species increased in EORA after treatment. These results suggest that certain oxylipins may be key effectors in arthrtis in older adults and that the imbalance between n-6- and n-3-derived oxylipins might be related to pathobiology in this population.

Keywords: Oxylipins, Arthritis, Omega-3, Omega-6 polyunsaturated fatty acids

Elderly-onset rheumatoid arthritis (EORA) is defined as a de novo illness that usually develops after 65 years of age and has different characteristics compared to young-onset RA (YORA): more equal sex distribution, more frequent acute onset with constitutional symptoms (fever, weight loss, and asthenia), large joints more frequently involved, and a larger percentage of EORA subjects are negative for both rheumatoid factor (RF) and anti-cyclic citrullinated peptide (CCP) antibodies (1). This may lead to significant diagnostic difficulties at first presentation, as there are many similarities between seronegative EORA and other rheumatologic diseases, including polymyalgia rheumatica (PMR) (2,3). PMR is characterized by pain and stiffness in the shoulder and the hip girdle and elevated inflammatory markers, that occur in people older than 50 years of age (4). While a lot of research has focused on YORA, which is rapidly diag-

nosed and is generally treated adequately, EORA and PMR have received less attention. Thus, mechanistic studies aimed at identifying key pathobiological factors to assess the biological systems with a putative role in EORA and PMR are few in number, and mechanisms driving or maintaining arthritis in older adults are relatively less well known.

Oxylipins and related bioactive lipid mediators derived from polyunsaturated fatty acids (PUFAs) constitute a major bioactive lipid network, which is among the most complex and challenging pathways to map in a physiological context. PUFAs can be classified into n-3 fatty acids and n-6 fatty acids (5). Arachidonic acid (AA) is synthesized from the n-6 essential fatty acid linoleic acid (LA) which comes from diet (vegetable oils, meats, and eggs) and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are synthesized from the n-3 essential fatty acid α-linolenic acid (ALA), also from diet (green leafy vegetables, flax and chia seeds, canola, walnut, and soybean oils) with the participation of the same enzymes: Δ6 desaturase, the limiting step of the pathway, Δ5-desaturases and elongases (Supplementary Figure 1A). Synthesis of n-6 and n-3 PUFA-derived oxylipins is performed by a set of highly conserved enzymes: cyclooxygenases (COXs), lipoxygenases (LOXs), and cytochrome P450 enzymes (6–8). AA, the predominant n-6 PUFA, is the precursor of proinflammatory oxylipins, such as prostaglandins, thromboxanes, and leukotrienes, as well as anti-inflammatory lipoxins. EPA and DHA are the precursors of oxylipins that have a critical role in the resolution phase of inflammation, named specialized pro-resolving mediators (SPMs; resolvins, maresins, protectins) and antagonize the proinflammatory effects of n-6 fatty acids (Supplementary Figure 1B).

Oxylipins control many physiological and pathological processes, often in opposing directions. Each of these oxylipins serves a specific role as either activators or suppressors of systemic inflammation and collectively exert complex controls in regulating human physiology (6–8). Among the proinflammatory oxylipins, prostaglandin E2 (PGE2) is considered a key mediator of various aspects of inflammation, including swelling, fever, and inflammatory pain (9). Other oxylipins are involved in the resolution phase of inflammation, such as the cyclopentenone 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) and lipoxin A4 (LXA4), and have instead been shown to limit inflammatory processes. Moreover, an altered ratio of omega-6 to omega-3 PUFAs is involved in diseases including cancer, cardiovascular diseases, and inflammatory disease (10). In arthritis, phospholipase A2, the enzyme that releases fatty acids from membranes, was found to be overexpressed in synovial fluid from RA patients. COX2 is upregulated in synovial fibroblasts, mononuclear cells, and endothelial cells in the sublining and is also induced in vitro in RA cultured synovial fibroblasts stimulated with proinflammatory cytokines. PGE2 was also found to be increased in RA patients' synovial fluid. High levels of 5-LOX and leukotriene B4 were also found in RA patients' synovial fluid, serum, and synovium (11). However, while some information is available regarding the role of PG and leukotrienes in arthritis, very few studies have addressed the role of other oxylipins in this field.

Aging is accompanied by an increase in the circulating levels of saturated fatty acids and a decrease of the unsaturated ones (12). A previous publication found increased levels of proinflammatory oxylipins in healthy older adults compared to younger individuals (13), suggesting they could be one of the underlying mechanisms of the low-grade inflammation described in aging population (14,15). It could also offer an explanation for the higher prevalence of inflammatory conditions such as arthritis, diabetes, and obesity, with increasing age (16,17). We hypothesized that oxylipin-related perturbations may be related to arthritis in older adults, and that by defining this oxylipin profile, we might be able to define elements of inflammation pathobiology in this population. Here, we compared the oxylipin profile in older adults that developed arthritis with age-matched controls. We also compared the oxylipin profile of EORA patients with PMR patients.

Patients and Methods

Patient Selection and Assessment

This is an observational longitudinal prospective study (ARTIEL—Arthritis in Older Adults), which enrolled older adults with new-onset arthritis. The study was approved by the University Hospital Germans Trias i Pujol Institutional Review Board, and included patients older than 60 years with clinically newly diagnosed peripheral and/or rhizomelic arthritis. Patients with infections, neoplasias, dementia, and immunodeficiencies, or who had received glucocorticoids or any disease-modifying anti-rheumatic drugs (DMARDs) in the last 6 months were excluded. Patients were identified by a primary care physician and then referred to a rheumatologist who prescribed treatment according to the standard of care.

Clinical assessment included presence/absence of pelvic and shoulder pain, stiffness, edema, fatigue and loss of appetite, global pain using a Visual Analogue Scale (VAS: 0–10), evaluation of the number of tender (TJC) and swollen joints (SJC) (out of 28), functional status as assessed by Health Assessment Questionnaire (HAQ), and assessment of global disease severity by patients, and a global assessment of disease by physicians, using a VAS ranging from 0 to 10. Composite measures of peripheral arthritis were calculated using the above measures: Disease Activity Score using a 28-joint count and C-reactive protein (DAS28CRP), Clinical Disease Activity Index (CDAI), and Simple Disease Activity Index (SDAI). Blood samples were collected at baseline (first consult in rheumatology clinics) and 3 months posttreatment, processed immediately, and sera aliquots were stored at −80°C until analysis. Blood samples from 18 controls in the same age range were also collected.

Out of 64 patients that fulfilled the inclusion criteria, 44 were diagnosed with EORA according to the ACR/EULAR 2010 criteria (18) and 20 patients were diagnosed with PMR (2012 EULAR/ACR criteria (19)). Forty-three patients with EORA and 19 patients with PMR were also evaluated at 3 months posttreatment.

Lipid Extraction and LC-MS Measure of Oxylipin

All sera samples at baseline were stored at −80°C, thawed once, and immediately used for free fatty acid and oxylipin isolation as described (20). Briefly, 50 µL of sera was spiked with a cocktail of 26 deuterated internal standards that also included some selected PUFAs (individually purchased from Cayman Chemicals, Ann Arbor, MI) and brought to a volume of 1 mL with 10% methanol. The samples were then purified by solid phase extraction on Strata-X columns (Phenomenex, Torrance, CA), using an activation procedure consisting of consecutive washes with 3 mL of 100% methanol followed by 3 mL of water. The oxylipins were then eluted with 1 mL of 100% methanol, and the eluent was dried under vacuum, dissolved in 50 µL of buffer A (consisting of water–acetonitrile–acetic acid, 60:40:0.02 [v/v/v]), and immediately used for analysis.

Oxylipins in sera were analyzed and quantified by LC/MS/MS as previously described (20,21). Briefly, oxylipins were separated by reverse-phase chromatography using a 1.7 µm 2.1 × 100 mm BEH

Shield Column (Waters, Milford, MA) and an Acquity UPLC system (Waters). The column was equilibrated with buffer A, and 10 µL of sample was injected via the autosampler. Samples were eluted with a step gradient starting with 100% buffer A for 1 minute, then to 50% buffer B (consisting of 50% acetonitrile, 50% isopropanol, and 0.02% acetic acid) over a period of 3 minutes, and then to 100% buffer B over a period of 1 minute. The LC was interfaced with an IonDrive Turbo V ion source, and mass spectral analysis was performed on a triple quadrupole AB SCIEX 6500 QTrap mass spectrometer (AB SCIEX, Framingham, MA). Oxylipins were measured using electrospray ionization in negative ion mode and multiple reaction monitoring (MRM) using the most abundant and specific precursor ion/product ion transitions to build an acquisition method capable of detecting 158 analytes and 26 internal standards. The ionspray voltage was set at −4,500 V at a temperature of 550°C. Collisional activation of the oxylipin precursor ions was achieved with nitrogen as the collision gas with the declustering potential, entrance potential, and collision energy optimized for each metabolite. Oxylipins were identified by matching their MRM signal and chromatographic retention time with those of pure identical standards.

Oxylipins and free fatty acids were quantitated by the stable isotope dilution method. Briefly, identical amounts of deuterated internal standards were added to each sample and to all the primary standards used to generate standard curves. To calculate the amount of oxylipins and free fatty acids in a sample, ratios of peak areas between endogenous metabolite and matching deuterated internal standards were calculated. Ratios were converted to absolute amounts by linear regression analysis of standard curves generated under identical conditions. Oxylipin levels are expressed in picomol/milliliter (pmol/mL). To account for batch effects, quality control samples were run in each batch; the average coefficient of variance for the quantified oxylipins was 6% (*SD* 0.01)

The desaturase enzymes Δ6 and Δ5 are considered the limiting step in the conversion of ALA to EPA and DHA, as well as LA to dihomo-γ-linolenic acid (DGLA) and AA (Supplementary Figure 1A). Activity of desaturase enzymes can be inferred from product to precursor ratios (22). DGLA/LA, calculated as the sum of DGLA-derived oxylipins divided by the sum of LA-derived oxylipins, was used to estimate the Δ6 desaturase activity. Sum of EPA-derived oxylipins/sum of ALA-derived oxylipins and sum of DHA-derived oxylipins/sum of ALA-derived oxylipin ratios were used to estimate the activity of the Δ6 and Δ5 desaturases, since no oxylipins derived from eicosatrienoic acid were detected. The sum of AA-derived oxylipins/sum of DGLA-derived oxylipins ratio was used to estimate the activity of the Δ5 desaturase.

Data Analysis

The data were processed using R, version 3.5.1 (www.r-project.org). Continuous variables were expressed as mean ± *SD* and the categorical variables as percentage. Chi-squared test was used to compare categorical variables. Comparisons of oxylipins at baseline between the control group and the arthritis population, between EORA and PMR patients, as well as between baseline and 3 months posttreatment, were adjusted for confounders (age, sex, body mass index [BMI], non-steroidal anti-inflammatory drugs [NSAIDs] use, and DMARDs), by including as covariates in linear regression models. The Benjamini–Hochberg method was used to adjust for multiple comparisons. A partial least squares discriminant analysis (PLS-DA) was utilized to evaluate whether oxylipins could differentiate between arthritis and control subjects. In an effort to identify a minimal number of oxylipins that could distinguish between clinical phenotypes of EORA and PMR, a sparse PLS-DA was subsequently performed with the number of principal components restricted to two and the number of variables/oxylipins in each component restricted to two. MetaboAnalystR was utilized to perform the PLS-DA. The variables for the PLS-DA were normalized to the median, log transformed, and scaled using range scaling. Heatmaps were performed using the gplots package (heatmap.2 function) after data scaling and hierarchical clustering with euclidean distance metric. To identify the best combination of oxylipins that discriminates between EORA an PMR, stepwise discriminant analysis was employed, with the use of multivariate cross-validation and "leave-one-out" classification. Discriminant analyses were performed to determine coefficients for linear combinations of variables that assigned cluster membership to individual cases with the SPSS software version 25.0. The change in oxylipins from baseline to 3 months was assessed by subtracting the baseline concentration from the concentration at 3 months. The comparison in changes between EORA and PMR was also performed adjusting for confounders as stated above.

Ethics Approval and Consent to Participate

Patients were enrolled following written informed consent. Ethical approval was granted by the Institutional Review Board (IRB) at Hospital Universitari Germans Trias i Pujol (PI-13-001).

Results

Cohort Demographics and Disease Characteristics

Characteristics of control and patient population are summarized in Table 1. Eighteen controls (average age: 75.38, *SD* 6.04) and 64 patients (average age: 74.97, *SD* 7.03) were analyzed. Of these patients, 44 were diagnosed with RA and 20 with PMR. Sixteen EORA patients were seropositive (RF and/or CCP positive: EORA+), while 28 were seronegative (RF and CCP were negative: EORA−). EORA− patients were younger compared to EORA+ patients (Table 1).

At baseline, EORA patients had a mean DAS28CRP of 5.77 (*SD* 1.02) and a mean HAQ of 1.7 (*SD* 0.8). One hundred percent of PMR patients reported scapular pain and 90% reported pelvic pain. As expected, arthritic subjects, that is, those with EORA and PMR, presented with higher erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and platelet count, and lower hemoglobin (Hb) than controls with similar comorbidities (Table 1). Within the arthritic population, PMR patients presented with more pelvic pain and less peripheral arthritis than RA patients (Table 1). We did not observe any significant difference in ESR, CRP, and platelet counts between PMR and EORA patients. Comorbidities including diabetes mellitus (DM), high blood pressure (HBP), and dyslipidemia (DL) were also similar in both arthritic populations and controls. About 29.2% of the patients were on daily NSAIDs. At baseline (first visit in rheumatology clinics), none of them were on steroids, or on any synthetic or biological DMARDs.

Oxylipin Profiling and Clustering in Control Population

Eighty-five oxylipins, which are derived from AA, eicosapentaenoic acid (EPA), LA, DGLA, ALA, and docosahexaenoic acid (DHA), were identified by reverse-phase LC/MS in our cohort (Figure 1A; Supplementary Table 1). Forty of the detected oxylipins are in general considered proinflammatory and 45 anti-inflammatory species. Figure 1A shows the pro- (in red) and anti-inflammatory

Table 1. Baseline Characteristics of the Patients Included in the Analysis

				EORA (44)					
Characteristic	Controls (18)	Patients (64)	*p*	Total	EORA – (28)	EORA+ (16)	*p* EORA– vs EORA+	PMR (20)	*p* EORA vs PMR
Female (%)	61.11	59.38	1	47.73	42.85	56.25	.58	85	**.014**
Age (years), mean *SD*	75.38 (6.04)	74.97 (7.03)	.82	74.34 (7.76)	76.93 (6.98)	69.81 (7.11)	**.002**	76.4 (4.99)	.2
BMI (kg/m^2), mean *SD*	28.83 (5.56)	27.93 (4.61)	.49	28.23 (4.77)	28.52 (4.63)	27.73 (5.12)	.6	27.27 (4.32)	.44
DM (%)	16.67	35.94	.24	40.91	53.57	18.75	.052	25	.31
HBP (%)	61.11	71.88	.78	65.91	71.43	56.25	.48	85	.38
Dyslipidemia (%)	50	65.62	.58	68.18	67.86	68.75	1	60	.82
Hemoglobin (mg/dL)	13.72 (1.48)	12.43 (1.5)	**<.001**	12.68 (1.57)	12.53 (1.48)	12.94 (1.73)	.41	11.88 (1.2)	**.04**
Platelets (×10^9/L)	193.71 (32.13)	264.85 (78.04)	**.004**	261.85 (79.94)	274.42 (60.85)	251.87 (102.27)	.43	266.27 (78.06)	.83
ESR (mm/h)	17.92 (17.02)	55.59 (25.46)	**<.001**	54.59 (27.26)	53.29 (26.64)	56.88 (29.04)	.67	57.8 (21.5)	.64
CRP (mg/dL)	4.1 (6.78)	38.65 (48.84)	**<.001**	41.88 (55.76)	42.98 (51.58)	39.96 (64.16)	.86	31.56 (28.27)	.43
Fatigue (%)				68.18	71.43	62.5	.84	70	1
Loss of appetite (%)				45.45	53.57	31.25	.26	35	.6
Stiffness (%)				95.45	96.43	93.75	1	95	1
Edema (%)				45.45	57.14	25	.08	0	**.0008**
Patient general health score (1–100)				75 (18.08)	74.11 (17.27)	76.56 (20)	.66	81.25 (17.46)	.25
Physician general health score (1–100)				77.27 (16.89)	76.43 (16.82)	78.75 (17.46)	0.66	—	—
Shoulder pain (%)				77.27	78.57	75	1	100	.34
Pelvic pain (%)				45.45	46.43	43.75	1	90	**.002**
Tender joints count (0–28)				10.16 (6.03)	8.96 (5.9)	12.25 (6)	.08	2.7 (0.98)	**<.001**
Swollen joints count (0–28)				11.75 (5.62)	11.89 (6.06)	11.5 (5)	.82	0.25 (0.64)	**<.001**
HAQ				1.7 (0.8)	1.78 (0.79)	1.54 (1)	.34	1.5 (0.57)	.34
CDAI				37.14 (11.69)	35.91 (11.28)	39.28 (12.45)	.36	—	—
SDAI				41.32 (13.88)	40.21 (12.29)	43.27 (16.55)	.48	—	—
DAS28CRP				5.77 (1.02)	5.68 (0.96)	5.92 (1)	.46	—	—
NSAIDs (%)	0	29.2%		25	39	25	.52	34	.66

Notes: Demographics, comorbidities, and inflammatory parameters in controls and patients. Continuous variables are presented as means (*SD*) and categorical variables as percentage. BMI = body mass index; CDAI = Clinical Disease Activity Index; CRP = C-reactive protein; DAS28CRP = Disease Activity Score taking into account 28 joints and CRP; DM = diabetes mellitus; EORA = elderly-onset rheumatoid arthritis; ESR = erythrocyte sedimentation rate; HAQ = Health Assessment Questionnaire; HBP = high blood pressure; NSAIDs = non-steroidal anti-inflammatory drugs; PMR = polymyalgia rheumatica; SDAI = Simplified Disease Activity Index. The bold *p* values are considered statistically significant (for a level of significance $p < 0.05$).

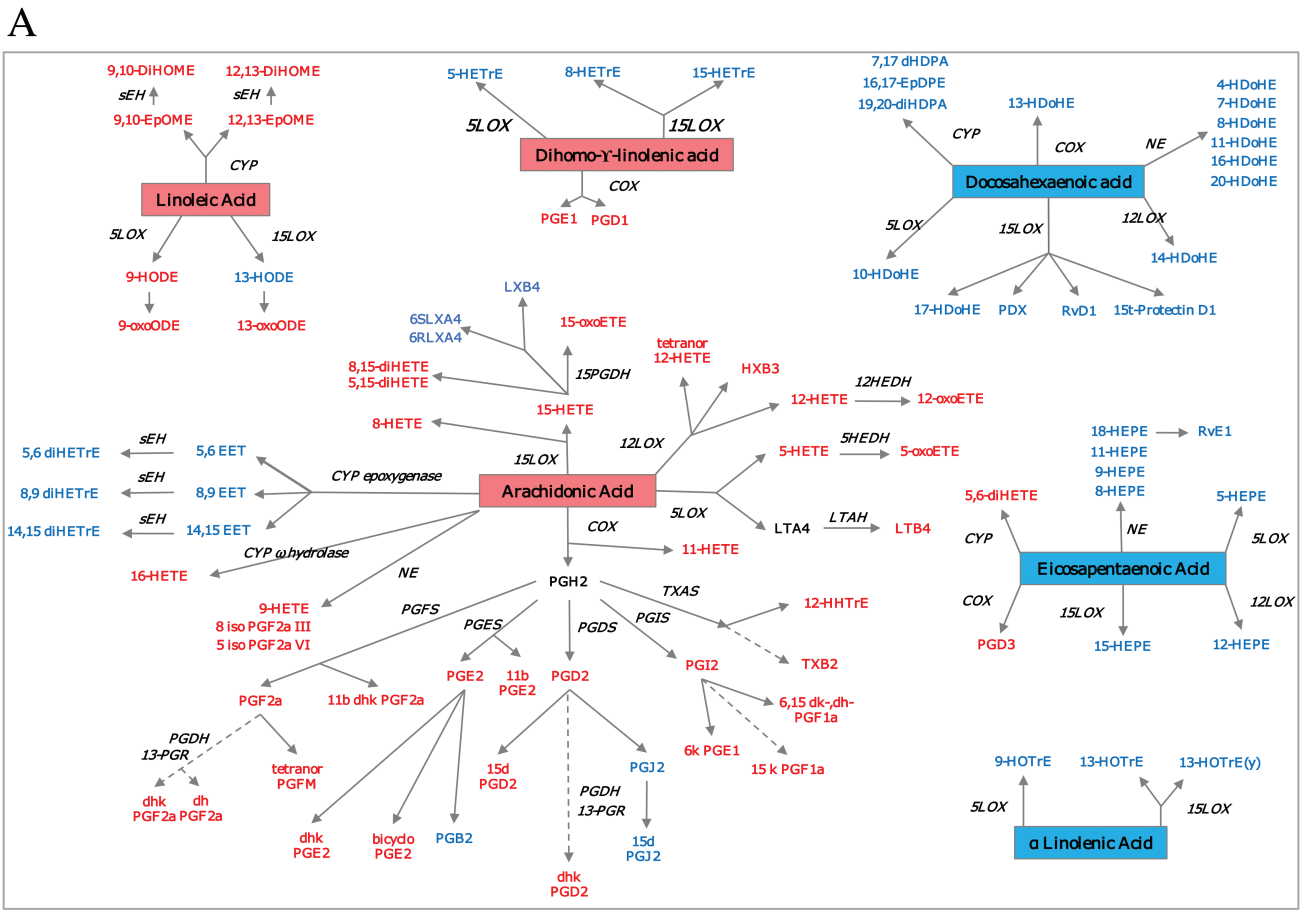


Figure 1. Oxylipin profiling in control population. (**A**) Oxylipins detected in our study by precursor and pathway are shown. Proinflammatory oxylipins are marked in red, while anti-inflammatory ones are marked in blue. The precursor n-3 polyunsaturated fatty acids (PUFAs) are marked in red, while the n-6 PUFAs are marked in blue, and the colors correspond to the barplot in Figure 1C. (**B**) Percentages of the different n-6 and n-3 PUFA-derived oxylipin mass in serum in the control population. (**C**) Total n-6 and n-3 PUFA mass in serum in control population. AA = arachidonic acid; aLA = alpha linolenic acid; COX = cyclooxygenase; CYP = cytochrome P450; DGLA = dihomo-gamma linolenic acid; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; HEDH = hydroxyeicosanoid dehydrogenase; LA = linoleic acid; LOX = lipoxygenase; LTAH = leukotriene A4 hydrolase; MDB = membrane dipeptidase; NE = non-enzymatic; PGDH = hydroxyprostaglandin dehydrogenase; PGDS = prostaglandin D synthase; PGES = prostaglandin E synthase; PGFS = prostaglandin F synthase; PGIS = prostaglandin I synthase; sEH = soluble epoxide hydrolase; TXAS = thromboxane A2 synthase; 13-PGR = 15-ketoprostaglandinΔ_{13} reductase.

(in blue) oxylipins and the different enzymatic pathways of the oxylipins detected in our samples. Among the detected oxylipins, 56 were derived from n-6 PUFAs, out of which 43 were derived from AA, 8 from LA, and 5 from DGLA. Out of the 29 n-3 PUFA-derived oxylipins, 3 were derived from ALA, 10 from EPA, and 16 from DHA (Figure 1A). Approximately, 80% of oxylipin mass in serum was derived from n-6 PUFA, particularly from AA (31%–63%), LA (18%–56%), and DGLA (1%–3%). n-3 PUFA-derived species were from DHA (8%–24%), EPA (1%–15%), and ALA (0.1%–0.5%; Figure 1B and C).

We then analyzed oxylipin clustering in the control population. We observed that anti-inflammatory oxylipins were grouped in two clusters, one comprised of DHA and EPA derivatives, and the other comprised of LA and ALA species (Supplementary Figure 2). Of interest, the first cluster negatively correlated with the second cluster. AA-derived species were distributed in two clusters, one comprised of most of the COX proinflammatory-derived oxylipin species, and the second comprised of most of the LOX proinflammatory-derived oxylipins.

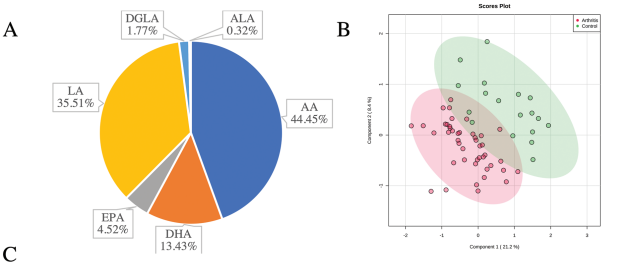
Oxylipin Profiling Differentiates Between Arthritic Patients and Controls

Similar oxylipin species were identified in the arthritic population. Approximately, 80% of oxylipin mass in serum was also derived from n-6 PUFA, particularly from AA (24%–60%), LA (19%–52%), and DGLA (0.7%–5%), and n-3 PUFAs-derived species were from DHA (7%–22%), EPA (1%–10%), and ALA (0.1%–1.4%) (Figure 2A). LA-derived oxylipins tended to be higher (p = .12), while EPA (p = .12) and DHA (p = .17) derived oxylipins tended to be lower in the arthritic patients compared to controls. Regression coefficients show positive relationships between identified oxylipin species in the arthritic population (Supplementary Figure 3). DHA-, EPA-, and ALA-derived anti-inflammatory oxylipins were also grouped in two different clusters as in the control group, yet, they positively correlated in the arthritic patients.

We then analyzed whether oxylipins could differentiate between arthritis and control subjects. Figure 2B shows that 21.2% of the variance can be explained by the first component and an additional 8.4% of the variance can be explained by the second component. This variance is sufficiently distinct to differentiate between control individuals and those with arthritis. In fact, several species were significantly different between control and patients after adjusting for age, sex, BMI, and NSAIDs (Figure 2C and D and Supplementary Table 2). A sparse PLS-DA (Supplementary Figure 3D) shows that tetranor-PGFM, 15-HEPE, 6R-LXA4, and LXB4 were the oxylipins that better distinguished between controls and arthritis patients. Of interest, most of the n-3 EPA and some DHA-derived oxylipin species were lower in patients compared to the control population (Figure 2D). Conversely, the n-3/n-6 PUFA ratio was lower in arthritis patients compared to control (p = .01; Figure 2E).

Baseline Oxylipin Profiling Differentiates Between EORA and PMR Patients

We further analyzed whether oxylipins were different between EORA and PMR. Oxylipin clustering shown in Supplementary Figures 4 and 5 differed in the EORA group compared to PMR patients. While oxylipin clustering in PMR population was similar to controls (Supplementary Figure 5), oxylipins clustered differently in EORA. The sparse PLS-DA between controls and EORA and controls and PMR was also different (Supplementary Figures 4D and 5D). While



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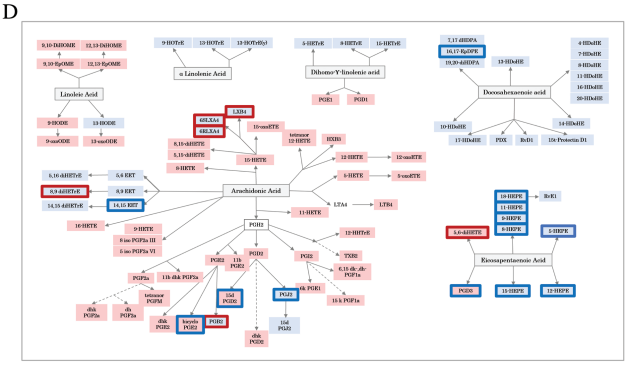
Oxylipin	Controls Relative abundance	Patients Relative Abundance	p	q BH
PGD3	1.07 (0.79)	0.78 (0.4)	0.02	0.15
bicyclo PGE2	0.08 (0.02)	0.05 (0.02)	<0.01	<0.01
11-HEPE	5.39 (3.02)	3.66 (2.4)	0.01	0.11
PGB2	5.16 (1.3)	5.74 (1.74)	0.03	0.17
PGJ2	0.23 (0.1)	0.18 (0.1)	0.18	0.45
15d PGD2	6.89 (2.53)	5.31 (1.73)	0.02	0.15
9-HEPE	1.91 (1.07)	1.28 (0.85)	0.01	0.11
5,6-diHETE	1.51 (0.52)	1.69 (0.51)	0.05	0.24
5-HEPE	18.56 (11.68)	13.68 (8.68)	0.03	0.17
6R-LXA4	0.91 (0.27)	1.11 (0.26)	0.02	0.15
6S-LXA4	0.38 (0.12)	0.47 (0.24)	0.06	0.24
LXB4	0.9 (0.17)	1.18 (0.31)	<0.01	<0.01
15-HEPE	2.17 (1.41)	1.19 (0.8)	<0.01	<0.01
8-HEPE	0.78 (0.47)	0.51 (0.34)	0.01	0.11
12-HEPE	5.02 (2.89)	3.46 (2.34)	0.03	0.17
18-HEPE	7.82 (4.83)	4.95 (3.22)	<0.01	<0.01
14,15-EET	1.45 (0.31)	1.14 (0.31)	0.02	0.15
16(17) EpDPE	0.48 (0.22)	0.39 (0.2)	0.05	0.24
8,9-diHETrE	0.03 (0.01)	0.04 (0.02)	0.99	0.99



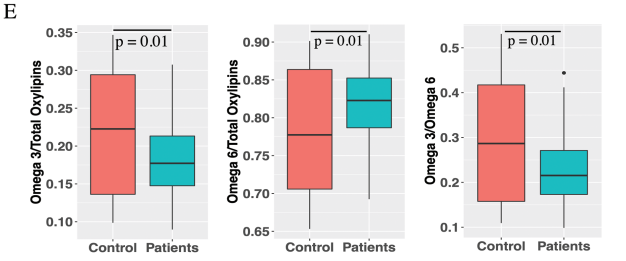


Figure 2. Oxylipins in patients compared to controls. (**A**) Percentages of n-6 and n-3 polyunsaturated fatty acid (PUFA)-derived oxylipin mass in serum in the arthritic population. (**B**) Partial least squares discriminant analysis (PLS-DA) between patients with arthritis and controls. The plot depicts the amount of variance between patients with arthritis and controls in the first two dimensions. Attributable variance is labeled on the axes. (**C**) The mean and *SD* of the relative abundance of the significantly different oxylipins between the two groups is presented with the correspondent *p*-values and *q*-values. (**D**) Upregulated (red squares) and downregulated (blue squares) proinflammatory (red background) and anti-inflammatory (blue background) oxylipins in patients compared to controls. (**E**) Total n-6 and n-3 PUFA mass, and n-3/n-6 ratio in control population compared to arthritis population. AA = arachidonic acid; aLA = alpha linolenic acid; DGLA = dihomo-gamma linolenic acid; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; LA = linoleic acid. Expansion for the abbreviations in C and D can be found in Supplementary Table 1.

tetranor-PGFM, 6R-LXA4, and LXB4 were the oxylipins that better distinguished controls and EORA, tetranor-PGFM, bicyclo-PGE2, PGB2, and LTB4 were the oxylipins that better distinguished controls and PMR, suggesting a different pathobiology in this disease. When comparing EORA+ and EORA– patients, five metabolites

were found to be different (PGD1, 62.04 ± 31.44 in EORA seropositive vs 44.51 ± 17.49 in EORA seronegative, p = .02; dhk PGD2, 86.64 ± 48.32 in EORA+ vs 59.34 ± 27.10 in EORA–, p = .03; 11b dhk PGF2a, 4.25 ± 2.20 in EORA+ vs 2.74 ± 1.42 in EORA–, p = .02; 9,10 EpOME, 8.98 ± 3.99 in EORA+ vs 7.51 ± 2.50 in EORA–, p = .02; 9,10 diHOME, 11.96 ± 20.23 in EORA+ vs 4.85 ± 2.24 in EORA–, p = .01).

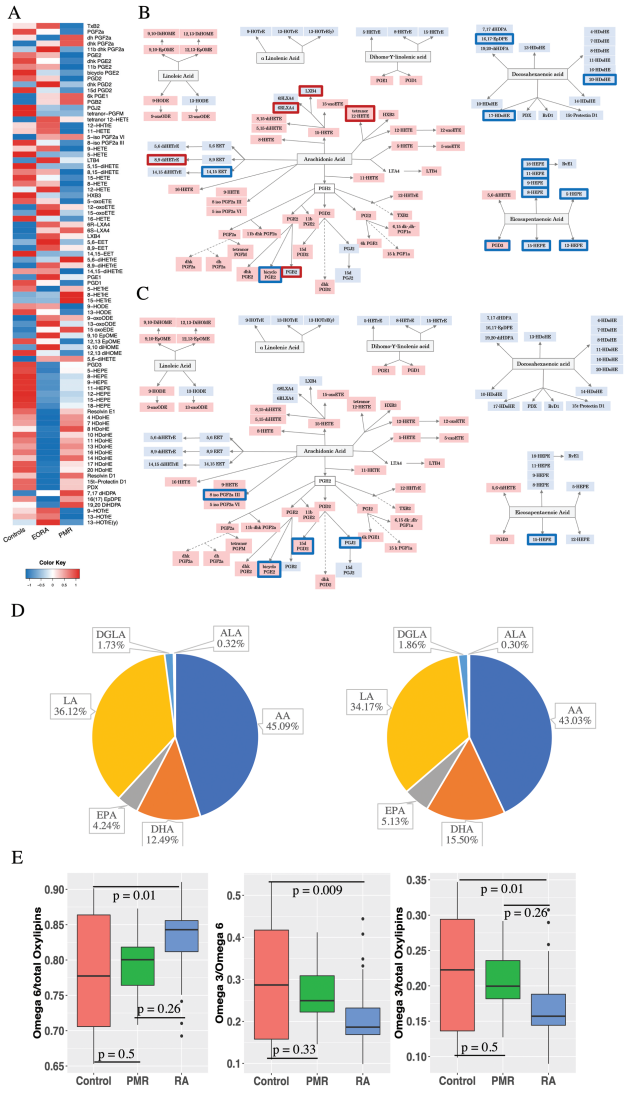


Figure 3. Oxylipins in elderly-onset rheumatoid arthritis (EORA) patients compared to polymyalgia rheumatica (PMR) patients. (**A**) Heatmap of relative abundance of oxylipins in EORA and PMR patients compared to controls. The relative abundances are scaled by row. (**B**) Upregulated (red squares) and downregulated (blue squares) proinflammatory (red background) and anti-inflammatory (blue background) oxylipins in EORA patients compared to controls. (**C**) Upregulated (red squares) and downregulated (blue squares) proinflammatory (red background) and anti-inflammatory (blue background) oxylipins in PMR patients compared to controls. (**D**) Percentages of n-6 and n-3 polyunsaturated fatty acid (PUFA)-derived oxylipin mass in serum in the EORA population. (**E**) Percentages of n-6 and n-3 PUFA-derived oxylipin mass in serum in the PMR population. (**F**) Total n-6 and n-3 PUFA mass, and n-3/n-6 ratio in EORA and PMR compared to control population. AA = arachidonic acid; aLA = alpha linolenic acid; DGLA = dihomo-gamma linolenic acid; DHA = docosahexaenoic acid; EPA - eicosapentaenoic acid; LA = linoleic acid. Expansion for the abbreviations in A, B and C can be found in Supplementary Table 1.

Figure 3A shows the heatmap of the relative abundance of oxylipins in EORA and PMR patients compared to controls. Several oxylipin species were significantly different between EORA, PMR patients, and the controls (Figure 3A and Supplementary Table 3). n-3 DHA- and EPA-derived oxylipins were significantly downregulated in EORA patients but not in PMR patients, while LA-derived oxylipins tended to be higher in both EORA and PMR patients compared to controls (Figure 3B and C). Conversely, the n-6 PUFA and n-3 PUFA mass, and the n-3/n-6 PUFA ratio was significantly different in EORA patients compared to controls, but not in the PMR group compared to controls (Figure 3D–F).

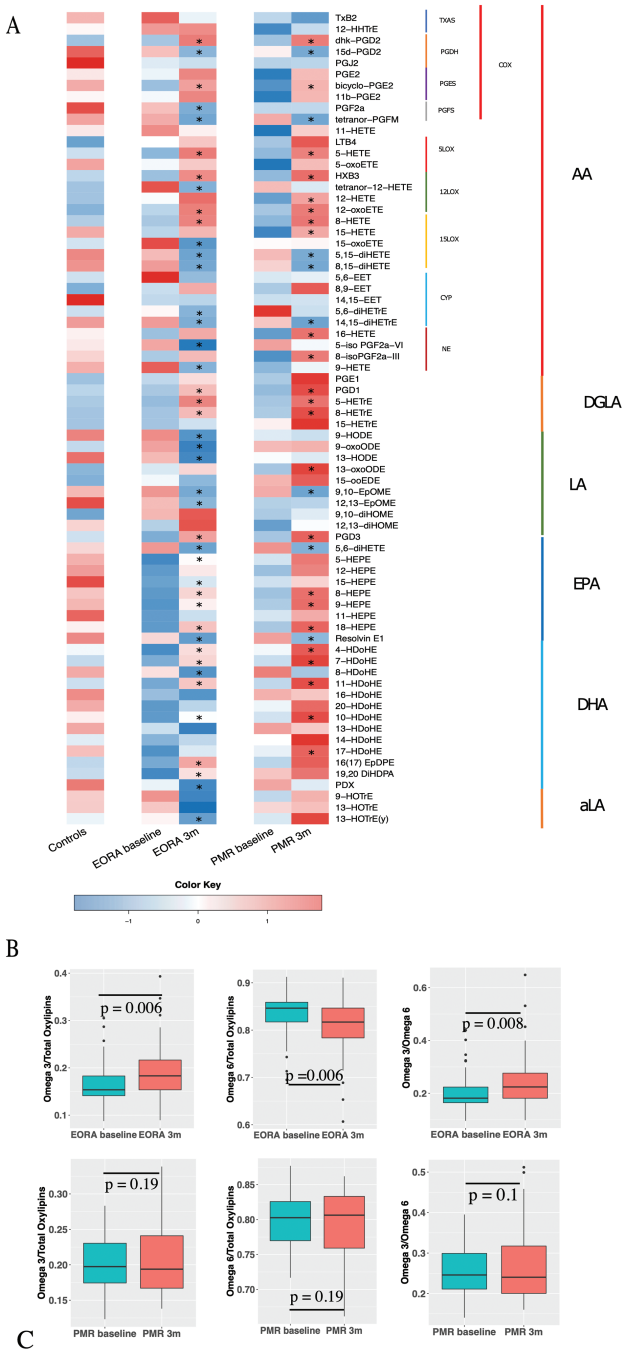
We then explored whether the oxylipins differentially expressed between controls and patients or between EORA and PMR were derived preferentially via either COX, LOX, or CYP pathways. As in Figures 2D and 3B and C, we did not observe a clear preference for COX-, LOX-, or CYP-derived oxylipins in arthritis patients. To determine whether the changes observed in EORA and PMR were related to changes in activity of the desaturase enzymes, the upstream step, we estimated their activity by studying the ratio between PUFA-derived oxylipins. The EPA/ALA ratio tended to be lower in EORA (17.27, p = .1) and PMR (18.21, p = .25) patients than in controls (25.72). The ratio of DHA/ALA (EORA 49.01, PMR 55.6, controls 64.5) tended to be lower in EORA than PMR patients and controls. DGLA/LA and AA/DGLA ratios were not different between the three studied groups (Supplementary Table 4).

We finally explored whether oxylipins in serum could discriminate between EORA and PMR. The stepwise discriminant analysis is presented in Supplementary Figure 6A. Two oxylipins, namely 4-HDoHE and 8,15-di dihydroxy-eicosatrienoic acid, were sufficient to correctly classify 70.3% of these patients. The canonical correlation of 0.821 and Wilks' lambda of 0.829 were found when these two variables were used, with high significance (p = .003; Supplementary Figure 6A). The top two candidates as biomarkers for differentiating PMR from RA, 4-HDoHE and 8,15-diHETE, had an area under the receiver operating characteristic curve (AUROC) of 0.76 (Supplementary Figure 6B). Yet, the disease phenotypes were unable to be fully distinguished using all the oxylipins as per a PLS-DA (Supplementary Figure 6C).

Ratio n-3/n-6 PUFA Increased in EORA Patients at 3 Months Posttreatment

At 3 months posttreatment, 39 out of the 44 patients diagnosed with EORA were on glucocorticoids (GCs, prednisolone) at an average dose of 5.51 mg/d (*SD* 3.27) (average dose after diagnosis was 8.83 mg/d, *SD* 3.21), and 32 received DMARD medication (methotrexate, leflunomide, or hydroxychloroquine). Thirty-three patients had a good and 8 patients a moderate response to treatment, according to the EULAR response criteria (23). Supplementary Table 5 shows the distribution of GCs and DMARDs across the response groups. At 3 months, all patients diagnosed with PMR were on GC at an average dose of 8 mg/d (*SD* 4.21) (average dose after diagnosis was 9.3 mg/d, *SD* 2.4). All PMR patients responded to treatment. Prednisolone dose was significantly higher (p = .01) in PMR patients compared to EORA patients at 3 months.

Seventy-two oxylipins were detected at 3 months posttreatment. Figure 4A shows a heatmap based on the concentrations of the oxylipins at baseline and 3 months posttreatment in EORA and PMR patients. The first column represents oxylipin concentrations in controls, which were quantified only at baseline. We compared the oxylipins at baseline and 3 months posttreatment. The significant



C

PUFA	Controls	EORA baseline	EORA 3m	p value	PMR baseline	PMR 3m	p value
AA (%)	44.67	44.317	49.033	0.07	43.049	45.413	0.683
DGLA (%)	1.76	1.828	3.04	< 0.001	1.99	3.089	0.019
LA (%)	32.34	37.189	28.608	0.001	35.083	30.242	0.063
EPA (%)	6.24	4.386	5.55	0.029	5.101	5.976	0.110
DHA (%)	14.68	11.964	13.536	0.005	14.486	15.002	0.337
aLA (%)	0.31	0.316	0.233	0.035	0.29	0.278	0.886

Figure 4. Oxylipins in controls compared to patients at baseline and 3 months. (**A**) Heatmap of relative abundance of oxylipins in elderly-onset rheumatoid arthritis (EORA) and polymyalgia rheumatica (PMR) patients at baseline and 3 months compared to controls. The relative abundances are scaled by row. The * represents a statistically significant difference (at a level of $p < 0.05$) between EORA at baseline and 3 months, and PMR at baseline versus 3 months post-treatment, respectively. (**B**) Total n-6 and n-3 polyunsaturated fatty acid (PUFA) mass, and n-3/n-6 ratio in EORA and PMR patients at baseline and 3 months compared to control population. (**C**) Percentage of the oxylipins for each PUFA precursor in the controls in the first column. The next columns show the percentage of oxylipins for each PUFA precursor in EORA and PMR patients at baseline and at 3 months posttreatment, along with the *p*-values. AA = arachidonic acid; aLA = alpha linolenic acid; DGLA = dihomo-gamma linolenic acid; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; LA = linoleic acid; PUFA = polyunsaturated fatty acid. Expansion for the abbreviations in A can be found in Supplementary Table 1.

changes are marked with asterisks. The n-3 EPA- and some of the DHA-derived anti-inflammatory oxylipins that were downregulated in EORA patients at baseline significantly increased in EORA compared to PMR patients (Figure 4A and Supplementary Table 6). Some of the EPA- and DHA- derived anti-inflammatory oxylipins also increased in PMR, although the total n-3 mass in PMR patients was similar between baseline and 3 months (Figure 4B and C and Supplementary Table 7). Both pro- and anti-inflammatory DGLA-derived oxylipins increased posttreatment in both disease groups. However, ALA-derived oxylipins decreased only in EORA ($p = .035$). Supplementary Table 8 shows the changes of oxylipins derived from each PUFA before and after treatment in both diseases. Interestingly, the desaturase activity changed significantly posttreatment in both EORA and PMR patients. The EPA/ALA and DHA/ALA, that tended to be lower in EORA compared to PMR and controls, increased in EORA patients (EPA/ALA: from 17.27 [11.21] at baseline to 27.9 [20.2], $p = .007$; DHA/ALA: from 49.01 [21.15] at baseline to 66.91 [30.31], $p < .001$). In Supplementary Table 9, it can also be observed that the ratios DHA/ALA and DGLA/LA significantly increased in both diseases posttreatment.

Discussion

Oxylipins comprise distinct classes of bioactive molecules with functions that are critical for joint disease (24). Evidence that the COX pathway might be involved in the pathogenesis of RA dates back to the 1970s, when elevated PG levels were reported in synovial fluid from patients with RA (25). Since then, studies in animals and patients have established a pivotal and complex role of PG in RA. High levels of phospholipase A2 (PLA_2), that catalyzes the release of AA and the production of its metabolites, such as PGs and leukotriene, are found in synovial tissue, inducing proliferative changes in synovial structures (26). However, few studies have addressed the role of other oxylipins in this field and none in the older adult population. The need for a more detailed understanding of how upstream oxylipin pathways influence disease risk is especially relevant to arthritis in older adults. Therefore, we conducted an extensive oxylipin profiling to comprehensively establish the association of circulating inflammatory oxylipins with arthritis in this population.

We hypothesized that oxylipin-related perturbations will be related to arthritic symptoms, and that by defining this oxylipin profile, we could define elements of inflammation pathobiology in this population. In this sense, new families of lipid mediators important in the resolution of inflammation have been discovered and are being investigated (27). The n-3 PUFA-derived oxylipins such as resolvins, protectins, and maresins have been identified in the resolving exudates of acute inflammation. EPA-derived E-series (RvE) and DHA-derived or D-series (RvD) resolvins display potent pro-resolving and immunoregulatory actions that include blocking the production of proinflammatory mediators. In addition to the D-series resolvins, DHA is also a precursor of other pro-resolving docosanoids named protectins (PDs) and maresins. These pro-resolving lipid mediators,

together with the above-mentioned resolvins, and the anti-inflammatory lipoxins, have been grouped together as SPMs (27). These are interesting compounds that constitute a novel topic of research, not only due to their bioactive role in the "return to homeostasis" process, but also in elucidating the physiological functions of other n-3 PUFAs (28).

Recent findings highlight that the omega-6 fatty acid AA appears increased, and omega-3 EPA and DHA decreased in most cancer tissues compared to normal ones. There is also compelling evidence that omega-3 PUFAs, particularly EPA and DHA, and an adequate balance of omega-6/omega-3 PUFAs play a determinant role in most physiological and biochemical processes occurring in cells and organisms, having great significance in decreasing the risk of many diseases or even resolving their inherent inflammation condition (28). In our study, we found a decreased n-3/n-6 PUFA-derived oxylipin ratio in patients compared to controls, with a stronger decrease in EORA compared to PMR patients. This profound decrease of n-3 PUFAs in EORA is not related to the degree of inflammation, as there were no differences in inflammatory biomarkers including CRP, ESR, or platelets count between PMR and EORA patients.

The DHA-derived downregulated oxylipins are the hydroxydocosahexaenoic acids (HDoHE—considered to have anti-inflammatory properties), among them 17-HDoHE, the precursor of resolvins, and 4-HDoHE, the precursor of maresins. The EPA-derived oxylipins that are downregulated are the hydroxy-eicosapentanoic acids (HEPE, also anti-inflammatory), 18-HEPE being another precursor of the resolvins. Total free DHA and EPA are also decreased in patients compared to controls, with a more prominent decrease in EORA compared to PMR patients, although without reaching a statistically significant difference. Interestingly, this was already observed in a previous study published by Rodríguez-Carrio and coworkers (29) who found decreased levels of palmitic, palmitoleic, oleic, arachidonic, total free EPA, and DHA in a Spanish cohort of YORA patients compared to healthy controls. Of interest, other anti-inflammatory oxylipins including lipoxins A4 and B4 that are derived from AA were upregulated in EORA, what would be expected to try to resolve a systemic inflammatory response.

We also explored whether the desaturase enzymes were inhibited in patients with arthritis. Δ5-desaturase and Δ6 desaturase exhibit affinity to metabolize n-3 over n-6 PUFA, provided that they exist in a ratio of 1:1–4. But, dietary intake of ALA is usually low compared to up to 30-fold higher intake of LA, which increases these enzymes' preference to metabolize n-6 PUFA, thus the conversion of ALA is poor in humans and only a small proportion is converted to EPA and DHA (30,31). Previous studies performed in RA patients suggest an increased activity of Δ6 desaturase, since they observed that treatment with TNF inhibitors decreased the activity of this enzyme (32) and higher levels of EPA were associated with a greater decrease of DAS28 in response to treatment with TNF inhibitors. In our study, the EPA/ALA ratio, but not the DGLA/LA and AA/DGLA ratios, surrogates for the activity of Δ5 and Δ6 desaturase, was lower in arthritis patients compared to controls (although it did not reach a statistical significance, likely due to a small sample size), and got back to control values after treatment. These data suggest a decreased activity of the desaturases only in the n-3 PUFA ALA pathway during inflammation. Steroids (dexamethasone, hydrocortisone, and triamcinolone) were shown to reduce the activity of the Δ5 and Δ6 desaturases (33) and are likely responsible of some of the changes observed at 3 months. In addition, we did not observe a clear preference for COX- or LOX-derived oxylipins in arthritis patients. However, these enzymes are active in the tissue and no studies have been performed to evaluate the relationship between tissue and circulating metabolites, hence, it makes the interpretation of our results difficult.

A larger percentage of EORA subjects are negative for both RF and anti-CCP antibodies (34–36). This may lead to significant diagnostic difficulties at first presentation, as there are many similarities between seronegative EORA and other rheumatologic diseases, including PMR. Moreover, an explosive onset of shoulder arthritis, resembling PMR, is observed in patients with early EORA (35). Conversely, peripheral arthritis has also been described in PMR patients (37). Imaging techniques such as FDG-PET/CT and ultrasound have shown some findings for differentiating PMR from EORA. In patients with PMR, abnormal FDG accumulation was observed at the entheses, suggesting the presence of enthesitis in addition to bursitis and synovitis (38). Ultrasound imaging showed that subdeltoid bursitis and biceps tendon sheath effusion were more frequent in patients with EORA, with a predominate symmetry and signs for massive inflammation (39). However, there is still an overlap of clinical and imaging features that would explain our oxylipin profiling results in these diseases. Although the n-3/n-6 ratio and some oxylipin levels were different in both diseases, the oxylipin profiling did not fully separate between RA and PMR. The stepwise discriminant analysis identified the combination of 4-HDoHE and 8,15-diHETE as sufficient to correctly classify 70% of the patients. 4-HDoHE is a metabolite of DHA via 5-LOX with anti-inflammatory properties and 8,15-diHETE is an AA-derived oxylipin via 5/15LOX. There is scarce data on both of these oxylipins. 4-HDoHE was shown to directly inhibit endothelial cell proliferation and angiogenesis via peroxisome proliferator-activated receptor γ (40), while 8,15-diHETE inhibits AA-induced autocrine neutrophil stimulation and LTB4-induced neutrophil chemotaxis, which also suggests an anti-inflammatory role (41). Since the biological relevance of these oxylipins in arthritis is unknown, more studies are required before deciding the adequacy of these metabolites as biomarkers of differential diagnosis between EORA and PMR.

Forty-one patients with EORA and 19 patients with PMR responded to treatment, which did not allow us to evaluate whether or not changes in the oxylipin profile were related to therapeutic response or could potentially predict response. However, we did observe that the ratio n-3/n-6 PUFA increased in EORA patients (Figure 4). In addition, most of these patients were receiving a low-dose prednisone. Although GCs have very well-known anti-inflammatory properties, there is no data about the effect of GC on systemic oxylipins, which could partially be responsible for the upregulation of most of the previously downregulated n-3 PUFA-derieved oxylipins. Although the effect of GC on COX enzymes is well known (42), little is published on its effect on LOX enzymes. Of note, a study showed that oral GC increased jejunal uptake of cholesterol and ileal uptake of lauric, palmitic, linoleic, and linolenic acid (43). Interestingly, the ALA-derived oxylipins 9-HOTrE, 13-HOTrE, and 13-HOTRE(γ) were still downregulated in EORA at 3 months.

Although these findings are certainly promising, this study is not without limitations. One of the limitations is the number of patients included in the study, but recruitment of patients with new-onset arthritis and DMARD naive is challenging. Confirmation of our results with a larger sample size from prospective cohorts of patients with new-onset inflammatory arthritis is necessary to strengthen our conclusions. Comparison with a YORA and other arthritides would help to determine if the described oxylipin changes are specific to rheumatoid arthritis/EORA/PMR or secondary to systemic inflammation. Yet, we believe that this work can improve our limited understanding of the role of oxylipins beyond AA metabolites and

leukotrienes in inflammatory arthritis and may lay the groundwork for a more targeted investigation of novel oxylipin- and lipidomics-based studies in arthritis.

Whether this abnormal n-3/n-6 ratio in serum reflects the tissue PUFA composition requires further studies. Oxylipin profiling of synovial or periarticular tissue could help to get more information about local and systemic oxylipin production or consumption. In addition, it is also unknown if altered patients' n-3/n-6 ratio at baseline could increase the risk of arthritis in older adults population. Research shows that the health-promoting effects of n-3 PUFAs are due to their competition with AA for the enzymatic metabolism, decreasing the formation of n-6-series lipid mediators that are predominately pro-angiogenic and proinflammatory and increasing n-3-series bioactive lipids with less detrimental and possibly beneficial effects (44–46). Further studies are needed to determine either a protective or therapeutic role of the n-3 PUFA diet in this population. Moreover, more studies are needed to evaluate the possible changes in the oxylipin profile related to corticosteroids and other treatments. In conclusion, we believe that this work can improve our limited understanding of the role of oxylipins beyond AA metabolites and leukotrienes in arthritis in older adults and may lay the groundwork for a more targeted investigation of novel oxylipin- and lipidomics-based studies in EORA and PMR.

Supplementary Material

Supplementary data are available at *The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* online.

Availability of Data and Materials

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

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Conflict of Interest

None reported.

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