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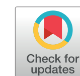
Targeted phospholipidomic analysis of synovial fluid as a tool for osteoarthritis deep phenotyping



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ABSTRACT

Objective: The aim of this study was to carry out a targeted phospholipidomic analysis on synovial fluid (SF) from patients with different grades of osteoarthritis (OA) and controls, in order to search for specific phospholipid profiles that may be useful for the deep phenotyping of this disease.

Design: Multiple reaction monitoring-mass spectrometry (MRM/MS) was applied to explore the potential phospholipidomic differences in the SF of knee OA patients (n = 15) (subclassified into early- and late-stage OA) and non-OA controls (n = 4). Multivariate statistical analyses conducted by partial least squares discriminant analysis (PLS-DA) and hierarchical clustering analysis (HCA) were performed to identify significantly altered phospholipids in OA, characterize phospholipidomic profiles associated with the radiographic stage of the disease and describe potential endotypes at early stages.

Results: Significant discrimination of phospholipid profiles between non-OA controls and the early- and late-stage OA groups were found by PLS-DA and HCA. Compared to SF from non-OA controls, OA patients showed higher levels of most quantified phospholipid species, including phosphatidylcholines (PC), phosphatidylserines and phosphatidylinositols. Furthermore, several PC species showed significant differences in abundance between the two OA subgroups and were negatively correlated with cartilage damage. Finally, two distinct endotypes of early-stage OA were identified based on the phospholipidomic profile of SF.

Conclusions: Our data provides a novel insight into the phospholipid profiles of OA synovial fluid, revealing specific alterations associated with the radiographic stage of the disease. This targeted phospholipidomic profiling also facilitated the characterization of two different OA endotypes at early stages of the disease.

1. Introduction

Osteoarthritis (OA) is the most common joint disorder. It is frequently associated with signs and symptoms of inflammation, including joint pain, swelling and stiffness, which lead to significant functional impairment and disability. OA has an increasing socioeconomic impact due to the ageing population [1,2]. It is a multifactorial disease that affects the whole joint tissues, including articular cartilage, subchondral bone, synovium, meniscus, periarticular ligaments, and adipose tissue, such as the infrapatellar fat pad in the knee [3]. OA has been recently defined as a syndrome comprised of multiple phenotypes rather than a single disease, in which several (or single) disease characteristics generate differences between patients with distinct clinical or structural OA outcomes [4].

The phenotypic heterogeneity of OA represents a huge impediment to improve understanding of disease pathogenesis, facilitate early diagnosis and detect the efficacy of current therapeutic interventions [5,6]. Thus, it

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is essential to delineate distinct clinical and pathogenic phenotypes or subgroups, mainly in the early stages of the disease, in order to correctly stratify OA patients. This would allow developing targeted treatments and prevention strategies for specific OA subpopulations. Accordingly, the definition of OA phenotypes is under active investigation. Substantial efforts have been made recently to classify subtypes of OA patients based on clinical, demographic, and anthropometric characteristics [7–9]. However, most patients may not fit easily into the proposed OA subsets defined by these methods due to the overlapping features among the subgroups and the complexity of the influencing risk factors [10,11]. Consequently, it is necessary to apply innovative approaches that can provide applicable classifications of OA subpopulations which may potentially improve the clinical management of OA patients [12].

Metabolomics is a reliable and promising tool for distinguish different subtypes of OA patients. This method provides the closest molecular portrait of phenotype as a result of environment and genetics, which is not predictable using other *omics* [13]. To date, its application to OA research has led to the identification of several metabolic perturbations underlying disease and novel metabolic markers for OA. Some of these recent metabolomic profiling approaches have revealed changes associated to pathways such as amino acid metabolism [14,15], and phospholipid metabolism involving the conversion of phosphatidylcholine (PC) to lysophosphatidylcholine (LPC) [16,17].

Synovial fluid (SF) is an ultrafiltrate of plasma, responsible for supplying nutrients and removing waste products from articular cartilage. In the context of OA, sampling the SF could be more indicative of the local metabolic state, since it is in direct contact with other affected tissues, and consequently may provide a better insight into the underlying mechanisms of disease [18]. Therefore, SF is a promising biofluid for metabolic studies focused on OA phenotyping.

Phospholipid metabolism is a metabolic pathway perturbed in OA and is likely contributing to disease pathogenesis [19]. To further explore the usefulness of its monitoring for patient stratification, in this work we have applied a targeted metabolomics approach to search and quantify potential alterations on the composition of phospholipids in human SF obtained from early- and late-stage OA patients and non-OA controls. Compared with untargeted metabolomics approaches, the multiple reaction monitoring (MRM) mode has been implemented in this study, providing better sensitivity and quantitative accuracy for lipid analysis. By defining targeted phospholipidomic profiles, in this study we also aimed to identify the association of levels of particular phospholipids with the radiographic severity of the disease from early-to late-stage, and classify early-stage OA patients into distinct subgroups based on their SF phospholipid profiles.

2. Method

2.1. Patients and synovial fluid sample collection

Human SF samples (n = 19) were obtained from the knee joints of patients with OA (n = 15) and non-OA donors (n = 4). OA SF samples were collected by direct aspiration from OA patients undergoing knee arthroscopy or total knee replacement surgery in the Rheumatology Department at Hospital Universitario de A Coruña, following the institutional regulations and procedures for sample collection. As selection criteria, we included in the study patients above 40 years, belonging to both genders and diagnosed with OA following the American College of Rheumatology criteria [20]. The severity of knee OA of each patient was assessed by the Kellgren-Lawrence (KL) grading system, based on radiography, osteophyte formation, joint space narrowing, sclerosis and joint deformity. Accordingly, SF samples were subclassified into early- (KL grades I and II, n = 7) and late-stage OA (KL grades III and IV, n = 8). SF from patients undergoing arthroscopy by traumatic event and without any type of secondary arthritis to any rheumatic disease or inflammatory joint effusion were also included in this study (non-OA control donors). The SF samples were stored at − 80 °C. Patients with history of malignancy, underlying severe conditions or diagnosed with other joint diseases were excluded from the study. Informed consent was obtained from all participants before surgery. The study was approved by the local Ethics Committee (Galicia, Spain).

2.2. Sample processing

A 250 μL aliquot of each SF was treated with 1 μg/mL of hyaluronidase (Sigma-Aldrich, St. Louis, (MO), USA) at 37 °C for 1 h to reduce viscosity and then centrifuged for 15 min at 3.500 rpm to remove insoluble material and debris. The supernatant was collected and stored at −80 °C until analysis. SF samples aliquots (100 μL) were treated according to previous publications [21,22]. Briefly, the samples were dissolved in 990 μL 17:1 (v/v) chloroform/methanol and centrifuged at 13, 000 rpm for 5 min. The supernatants containing non-polar lipids were removed and the pellets were suspended in 990 μL of 2:1 (v/v) chloroform/methanol to extract the polar lipids. A second centrifugation for 10 min at 13,000 rpm allowed the recovery of supernatants that were dried under vacuum and stored at −20 °C until the subsequent mass spectrometric analysis.

2.3. Targeted phospholipidomic analysis

Lipid levels in SF samples were determined using a targeted approach based on mass spectrometry in multiple reaction monitoring (MRM/MS) ion mode. Three technical replicates were carried out for each analysed sample. A standard mixture, containing heavy D-α-phosphatidylcholine, dipalmitoyl (d80, 98%), d6-L-α-phosphatidylethanolamine dioleoyl (Avanti Polar Lipids) was added to each human SF aliquot. The samples were analysed by using an AB Sciex QTrap 4000 mass spectrometer, coupled with an ExpressHT™-Ultra HPLC system (Eksigent). An aliquot of 5 μL of the extract was injected and separated on a Halo C18 1.0mm × 50 mm, 2.7 μm column using a 40 μL/min flow rate and a 12 min gradient (0 min 70% A, 2 min 50% A, 9 min 5% A, 11 min 50% A, 12 min 70% A). The column temperature was set to 40 °C, while the autosampler was cooled at 4 °C to avoid sample evaporation. Solvent A composition: acetonitrile/methanol/water 97:2:1 v/v/v acidified by acetic acid 0.2% and 5 mM ammonium acetate; solvent B composition: methanol acidified by acetic acid 0.2% and 5 mM ammonium acetate. The MRM/MS analyses were carried out in positive mode (ESI^+) and in negative mode (ESI^-). For each phospholipid specie, precursor ions, product ions and optimal collision energies were optimized for each MRM transition. The MS data were normalized according to the internal standard MS signal. The mass spectrometry procedure details of MRM/MS method used to detect PC (positive ion mode) and PA, PE, PI, PG, PS (negative ion mode) are listed in Supplementary Tables 1 and 2, respectively. Lipid concentrations were calculated in pg/μl.

2.4. Statistical analyses

Phospholipids present in at least 90% of patients were included in the dataset. The samples were divided into two groups: the early-stage OA group (eOA, KL grades I and II) vs. the late-stage OA group (lOA, KL grades III and IV), to investigate the alterations of phospholipids in relation with the structural severity of knee OA. Statistical analysis was performed using Metaboanalyst (https://www.metaboanalyst.ca/). Data were log transformed using the base-2 logarithm (log 2) to correct for non-normal distributions and standardized (mean centered divided by standard deviation). Standardized data were used for all analyses. Differences in lipid concentration between multiple groups (control donors, eOA and lOA) were determined by analysis of variance (ANOVA) F-test with Benjamini and Hochberg false discovery rate (FDR) at 5% level for correction of multiple comparisons using Prism v.7 (Graph Pad, San Diego, California, USA). To examine specific differences between sample groups, pairwise comparisons control vs. eOA; control vs. lOA; and eOA vs lOA were analysed by Student's t-tests with Welch's correction for

standard deviation. Sex ratios in the study groups were tested with the Chi-square test. The Pearson's correlation coefficient analysis was performed for all lipid species quantified in SF against the joint KL-score, in order to find potential associations between the severity of the disease and the absolute concentration of the lipid species. For this analysis, significance was declared when $r \geq 0.7$. A p-value < 0.05 was considered statistically significant for all analyses and statistical tests were two-sided. Phospholipids with a p-value (FDR corrected) less than 0.05 were considered statistically significant in these analyses.

Multivariate statistical analyses were also performed to evaluate changes in the phospholipid datasets. Unsupervised hierarchical clustering analysis (HCA) based on Euclidean distance and average cluster algorithm and principal component analysis (PCA) were initially carried out to separate SF samples into groups of similar abundance patterns, followed by supervised partial least squares discriminant analysis (PLS-DA). PLS-DA scores each phospholipid in each component showing how significant that phospholipid was in contributing to the sample separation. The robustness of the PLS-DA model was evaluated by cross validation test (Q_2: predictive ability). Those phospholipids with variable importance in projection (VIP) from the PLS-DA higher than 1.5 were considered biologically relevant in these analyses. Data are presented in the figure legends or text as mean ± standard deviation.

3. Results

3.1. Patient characteristics

The demographic characteristics of the study population are summarized in Table 1. There were no significant differences in age, gender proportions, body mass index, triglycerides and glucose levels among the study groups. The eOA group had higher levels of total cholesterol compared with the non OA-control group.

3.2. Phospholipidomic profiling of OA SF by MRM/MS targeted analysis

A total of 15 SF samples of OA with KL grading scales ranging from I to IV (7 eOA patients, 8 lOA patients) and 4 age-matched non-OA controls were analysed by MRM-MS for the targeted analysis of 84 phospholipid species. Among these, 40 were successfully determined in at least 90% of the patients and included in the data analysis. This includes 13 phosphatidylcholines (PC), 4 phosphatidic acids (PA), 5 phosphatidylethanolamines (PE), 3 phosphatidylglycerols (PG), 3 phosphatidylserines (PS), and 12 phosphatidylinositols (PI). Twenty-three phospholipid species were found significantly different between non-OA controls, eOA, and lOA SF samples after carrying out an ANOVA test (Table 2). These included several PI and PS that were higher in eOA and lOA compared to non-OA controls, such as PS 32:0, PI 26:0, PI 32:1, PI 34:1, PI 34:2 and PI 36:2 (Fig. 1). Moreover, the SF levels of several PC were notably higher in eOA samples compared to non-OA controls and lOA. On the other hand, the SF levels of certain species including PA 30:0 and PG 34:2 were significantly lower in eOA and lOA samples compared to non-OA controls.

Table 1
Descriptive statistics of the demographic characteristics of the study population.

	Non-OA controls (n = 4)	eOA (n = 7)	lOA (n = 8)	p-value
Age (years)	73 ± 15	71 ± 7	73 ± 12	0.998
Sex (female/male)	1/3	6/1	4/4	0.193
BMI (kg/m^2)	23.0 ± 2.5	32.5 ± 6.9	29.0 ± 4.0	0.058
Total cholesterol (mg/dl)	113.8 ± 52.8	217.1 ± 62.3[a]	191.8 ± 30.2	0.014
Triglycerides (mg/dl)	116.3 ± 43.8	115.9 ± 65.6	100.9 ± 13.4	0.683
Glucose (mg/dl)	94.0 ± 15.2	91.4 ± 4.7	90.7 ± 7.8	0.880
KL-score	0	I-II	III-IV	

Data are presented as mean ± SD, unless indicated.
eOA: early-stage osteoarthritis; lOA: late-stage osteoarthritis; BMI, body mass index; KL-score: Kellgren-Lawrence grading scale; SD, standard deviation.
[a] Significant difference from non-OA control (p = 0.023).

Multivariate analysis was performed in parallel to univariate analysis to identify phospholipids with the greatest discriminative capabilities for separating sample groups. PCA analysis exhibited certain separation among control, early- and late-stage OA patients based on absolute concentrations of all lipid classes quantified in SF (Fig. 2A). Phospholipids in each group were then divided according to the MS mode (positive or negative) and also analysed by PCA. As shown in Fig. 2B, non-OA control and OA samples were correctly clustered particularly by phospholipids quantified in negative ion mode. A PLS-DA was then performed to improve discrimination between the early- and late-stage OA groups, where $Q2 = 0.52$ showing good prediction of the model ($Q2>0.4$). The score plot of PLS-DA completely distinguished non-OA (control) from OA sample groups (DF1), and mostly separated with a minimum overlap early- and late-stage OA subgroups based on their phospholipidomic profiles (Fig. 2C). From the loading values of PLS-DA, PI 36:2, PI 26:0, PI 34:1 and PS 32:0 showed the greatest separation power to classify non OA-control and OA SF samples and constitute important candidates as potential biomarkers (all with VIP values > 1.5; Supplementary Figure 1). Using HCA, all SF samples were firstly classified based on their phospholipid signatures into two main clusters, corresponding to non-OA control and OA groups. The OA cluster could then be further stratified into two subgroups corresponding to eOA and lOA samples (Fig. 2D).

To examine specific differences between non OA-controls and OA cohorts, pairwise comparisons were carried out: non-OA controls vs. eOA; non-OA controls vs. lOA (Supplementary Tables 3 and 4). Sixteen phospholipid species were significantly different between non-OA controls and eOA SF, being 9 higher in eOA. On the other hand, other 16 phospholipids were also found significantly different between non-OA controls and lOA (62.5% of phospholipids showed similar alterations regardless of the radiographic stage of the disease). Taken together, these data indicate that the evaluated phospholipidome was significantly different between non-OA control, eOA and late OA.

3.3. Phospholipidomic changes in SF associated to the radiographic severity of OA

Changes of phospholipid concentrations between the eOA and lOA SF samples were also evaluated. Of the 12 PC whose levels in SF were significantly different between the eOA and lOA groups, all were higher in the SF of eOA patients than in the lOA according to Student's t-tests (Table 3). The fold change of the 12 PC ranged from 1.30 to 2.58. By examining the PLS-DA scores ($Q2 = 0.46$), the main significant contributors to the difference between eOA and lOA were PC 28:1, PC 30:0, PC 30:2, PC 32:2, PC 34:2 and PC 36:2. All of them had VIP scores >1.5 (Supplementary Figure 2). Moreover, the concentrations of most PC were positively correlated among each other. The highest significant correlation was found between PC 30:0 and PC 32:2 ($r = 0.98$) (Supplementary Figure 3 and Supplementary Table 5).

Additionally, PC were associated with the radiographic severity of OA, according to a Pearson correlation test. In fact, all quantified PC were negatively and significantly correlated with cartilage damage, except for PC 36:1 (Supplementary Table 6). D-α-PC dipalmitoyl ($r = -0.777$, $p = 0.001$), PC 30:0 ($r = -0.794$, $p = 0.001$), PC 32:1 ($r = -0.758$, $p = 0.002$), PC 32:2 ($r = -0.850$, $p = 0.0001$), PC 34:1 ($r = -0.747$, $p = 0.002$) and PC 34:2 ($r = -0.781$, $p = 0.001$) showed the strongest significant negative correlations between joint KL-score and SF levels (Fig. 3). No correlations were found between lipids quantified in negative ion mode and cartilage damage (Supplementary Table 6).

3.4. Identification of eOA endotypes by phospholipid profiling of SF

OA subgroups were further analysed by unsupervised HCA in order to

Table 2
Identification in SF of 23 phospholipids significantly modulated between sample groups, using a targeted phospholipidomic analysis.

N	Phospholipids	*m/z*	MS mode	Non-OA controls (n = 4)	eOA (n = 7)	lOA (n = 8)	*p*-value
1	D-α-PC dipalmitoyl	734.60	POS	3793.00 ± 2196.00	4420.00 ± 1451.00	2033.00 ± 743.80[b]	0.0162
2	PC 28:1	676.50	POS	378.70 ± 140.40	829.40 ± 389.50	322.10 ± 126.70[b]	0.0053
3	PC 30:0	706.50	POS	807.00 ± 538.20	1034.00 ± 385.50	454.40 ± 147.40[b]	0.0178
4	PC 32:1	732.60	POS	3524.00 ± 2149.00	4342.00 ± 1465.00	2057.00 ± 741.40[b]	0.0231
5	PC 32:2	730.50	POS	1537.00 ± 1336.00	2490.00 ± 683.00[a]	1098.00 ± 333.00[b]	0.0007
6	PC 34:1	760.60	POS	215.20 ± 51.09	667.30 ± 285.40[a]	311.80 ± 80.76[b]	0.0004
7	PC 34:2	758.60	POS	12985.00 ± 5144.00	16386.00 ± 3784.00	9784.00 ± 3116.00[b]	0.0152
8	PC 36:0	790.60	POS	9586.00 ± 4083.00	18282.00 ± 5341.00[a]	12515.00 ± 3860.00	0.0173
9	PCaa 24:0	622.44	POS	9675.00 ± 3690.00	16992.00 ± 4246.00[a]	11444.00 ± 3658.00[b]	0.0211
10	PA 30:0	619.40	NEG	290.90 ± 40.75	213.50 ± 26.32[a]	228.40 ± 32.35[a]	0.0070
11	PE 30:0	622.50	NEG	111.10 ± 4.02	144.20 ± 15.05[a]	139.30 ± 26.06[a]	0.0259
12	PE 36:1	744.50	NEG	905.04 ± 533.40	1672.00 ± 328.70[a]	1634.00 ± 311.60[a]	0.0059
13	PE 36:2	742.50	NEG	325.50 ± 65.38	162.60 ± 30.85[a]	179.10 ± 11.08[a]	<0.0001
14	PG 34:2	745.50	NEG	218.10 ± 86.04	74.76 ± 16.90[a]	87.17 ± 24.50[a]	<0.0001
15	PS 32:1	732.50	NEG	98.56 ± 6.82	197.00 ± 59.97[a]	163.00 ± 37.54[a]	0.0059
16	PS 32:0	734.50	NEG	497.20 ± 111.00	1070.00 ± 174.00[a]	1079.00 ± 156.00[a]	<0.0001
17	PI 26:0	725.50	NEG	344.20 ± 26.65	455.60 ± 13.88[a]	455.90 ± 12.41[a]	<0.0001
18	PI 30:0	781.60	NEG	797.00 ± 39.04	649.40 ± 48.82[a]	686.30 ± 63.13[a]	0.0038
19	PI 32:1	807.60	NEG	596.10 ± 45.55	900.50 ± 102.60[a]	923.60 ± 178.30[a]	0.0005
20	PI 34:1	835.60	NEG	1366.00 ± 73.98	1711.00 ± 115.50[a]	1725.00 ± 96.94[a]	<0.0001
21	PI 36:2	861.70	NEG	362.60 ± 63.57	648.20 ± 74.20[a]	708.00 ± 52.91[a]	<0.0001
22	PI 36:1	863.70	NEG	452.70 ± 148.80	669.10 ± 79.20[a]	675.20 ± 95.91[a]	0.0032
23	PI 36:0	865.70	NEG	186.50 ± 37.84	319.80 ± 56.48[a]	326.30 ± 63.73[a]	0.0005

Data are presented as mean ± SD, unless indicated. ANOVA F-test (FDR corrected <0.05) was used.
eOA: early-stage osteoarthritis; lOA: late-stage osteoarthritis; POS: positive ion mode; NEG: negative ion mode; PC: phosphatidylcholine; PA: phosphatidic acid; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; PS: phosphatidylserine; PI: phosphatidylinoisitol.
[a] Significant difference from control SF.
[b] Significant difference from eOA SF.

describe potential phospholipid-based endotypes. In eOA, HCA revealed two clusters of patients based on their phospholipid patterns in SF. These clusters, endotypes 1(E1) and 2 (E2), contained three and four individuals, respectively (Fig. 4A and B). PCA analysis was also applied to evaluate the separation between both potential endotypes, revealing a complete separation between E1 and E2. The first two components are associated with 53.6% of the variation between subsets (Fig. 4C). Moreover, PLS-DA ($Q_2 = 0.65$) further supported the identification of these two endotypes within eOA SF samples, by the clear discrimination between E1 and E2 participants (Fig. 4D). Additionally, the levels of ten phospholipid species were different between endotypes E1 and E2, including PS 32:0, PG 34:2 and 8 PC. We next analysed whether clinical data could be linked to the SF concentration of these ten significantly modulated phospholipids between E1 and E2 described above within early-stage OA. This would allow a deeper characterization of these two endotypes. Accordingly, we carried out a Pearson's test to evaluate the potential correlations between altered phospholipids and these clinical confounders in each endotype (see Supplementary Table 7). This analysis did not show significant correlations for any lipid specie with total cholesterol, triglycerides and BMI. However, it revealed that D-α-PC dipalmitoyl levels were negatively correlated with glucose in E2 ($r = -0.971$, p-value = 0.028). On the other hand, glucose was positively associated with changes in the concentration of PC 30:2 ($r = 0.998$, p-value = 0.041) in E1. Furthermore, two PC such as PC 28:1 ($r = -0.999$, p-value = 0.019) and PC 36:0 ($r = -0.998$, p-value = 0.030) were positively correlated with PCR values in E1. No significant associations were found between SF phospholipid levels and PCR in E2 patients. Finally, PCaa 24:0 was also positively correlated with ESR values in E2 participants ($r = -0.968$, p-value = 0.031).

4. Discussion

In this study, forty different phospholipids have been quantified in human SF using a targeted MRM/MS-based approach. By these means, specific phospholipidomic profiles have been determined for non-OA (control), eOA and lOA SF. Globally, the OA subgroups exhibited higher levels of several PI and PS species compared to controls. Conforming to this observation, our group recently reported that these species are increased in OA synovial membranes in comparison with control tissues, and also that PS are associated with areas characterized by a higher inflammation and vascularization [23]. Alterations in the phosphatidylinositol phosphate metabolism pathway have been also associated with OA SF [24], supporting our findings. Additionally, our data demonstrates that the levels of several PG were significantly lower in both early and late-stage OA SF. In line with these results, other lipidomic studies performed on SF have reported low concentrations of PG in samples from eOA and lOA compared to healthy donors [25]. Lastly, PE species that we found increased in OA subgroups were also elevated in human OA FLS treated with IL-β1 [26]. All these observations indicate that the variations in the phospholipidome reported herein might contribute to pathogenic processes underlying OA and could be useful markers for its diagnosis. Moreover, although further validation is required, this is the first study describing phosphatidylinositol species as potential SF markers of OA.

Focusing particularly on the OA group, our results pointed out that eOA patients certainly exhibited a quite different SF lipid profile compared with lOA individuals. In fact, most quantified PC species were significantly altered between eOA and lOA groups, presenting higher levels in the SF of eOA patients. Previous untargeted lipidomic analyses of OA SF had found alterations of phospholipid species between eOA and lOA, including PC species [27]. These studies reported elevated levels of certain PC species in eOA in comparison with lOA, which is consistent with our data [25]. Increased total lysophosphatidylcholines (LPC) to PC ratio in serum has been recently associated with knee OA risk and described as a metabolic marker for predicting advanced knee OA [16]. This elevated ratio indicates that the conversion of PC to LPC is highly activated in OA and may explain our findings regarding the lower levels of PC species detected in patients with lOA compared to eOA. In addition to this, we have found that the levels of PC species inversely correlate with the radiographic severity of OA ($p < 0.05$). A specific PC conversion (LPC 18:2/PC 44:3) in serum has been also associated with the cartilage loss in knee OA [17], supporting our findings. In contrast, plasma levels of the PC (38:7) were found significantly increased in a DMM model of OA and positively correlated with cartilage damage [28]. This

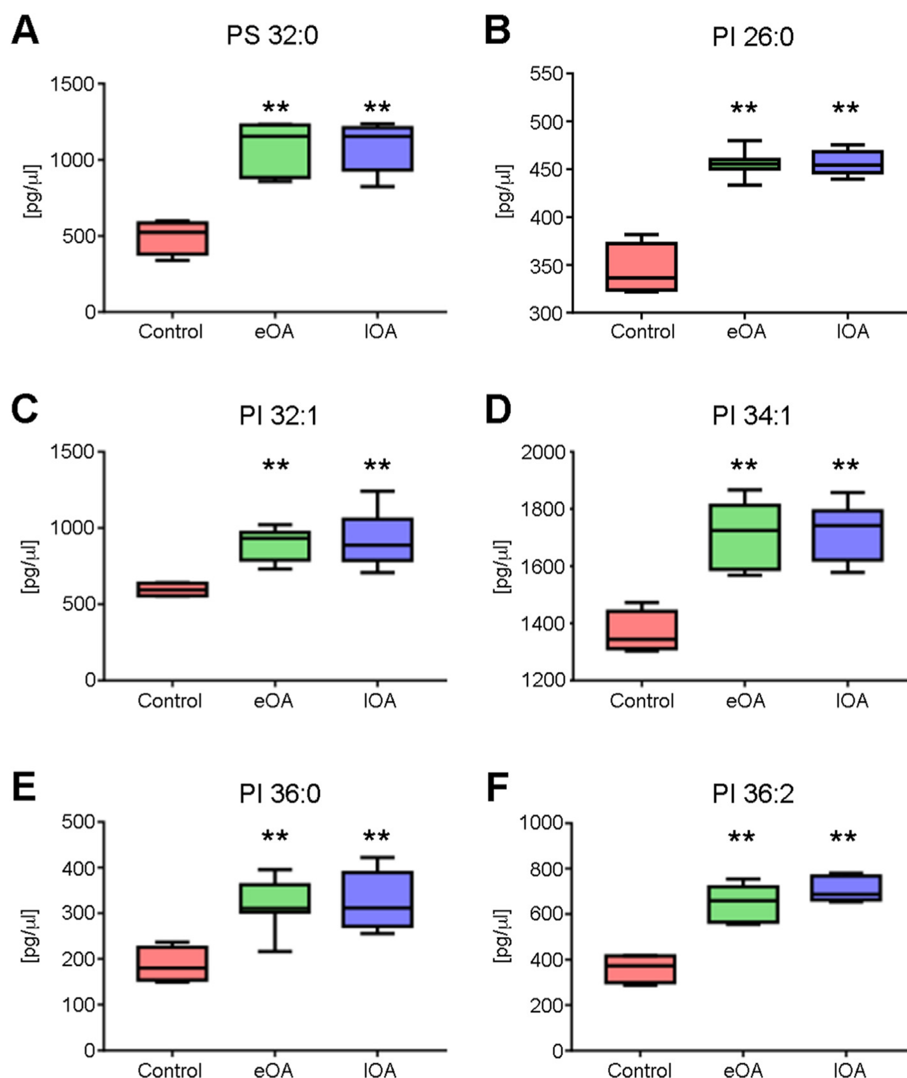

Fig. 1. Boxplots of representative lipids identified from univariate analysis and PLS-DA as significantly different between non-OA control and OA subgroups (***p*-value < 0.01). Y-axis represents concentrations in pg/μl. Boxes indicate the 25%–75% range, whiskers indicate the minimum and the maximum range. OA: osteoarthritis; SF: synovial fluid.

discrepancy could be explained by the modest correlation that has been found between blood plasma and SF metabolite concentrations such as PC in OA [29]. Altogether, our data demonstrate the potential of SF phospholipidomic profiling for OA monitoring. The combination of these profiles with other molecular (i.e. miRNA, proteomics, etc.), imaging or clinical data would be a highly promising approach to improve their predictive accuracy [30].

OA is a phenotypically heterogeneous disease and identifying approaches to stratify OA patients is crucial to facilitate personalized medicine strategies for OA management. This is particularly important at the early stages of the disease, when lifestyle changes and therapeutic interventions are more likely to succeed in slowing disease progression. Accordingly, we investigated if different phospholipidomic-based endotypes could be defined within the early stages OA SF, using unsupervised clustering to classify subgroups of patients. Interestingly, we were able to clearly identify two distinctive endotypes in early OA (E1 and E2). The main differences between them correspond to their PC profile, being the Endotype 2 characterized by greater levels of 7 specific PC species and two other phospholipids (PS 36:0 and PG 34:2). The biological significance and putative impact on the OA process of this molecular features remains to be elucidated. Previous metabolomic studies support the usefulness of the strategy followed herein for deep phenotyping of OA. Metabolomic analysis of SF samples obtained from 80 end-stage OA patients found that three metabolically distinct subgroups (A, B1 and B2) could be clearly discriminated in OA, where PC species were the main significant phospholipids that contribute to subgrouping [10]. More recently, the characterization of the global metabolomic profiling of SF from individuals with eOA and lOA reported different metabolomic phenotypes within early and late OA cohorts [24]. Latest, another metabolomic-based study performed in OA plasma also classified patients in three distinct subgroups based on specific metabolic profiles. Particularly, the third subgroup (56% of patients) was characterized by alterations in the metabolism of phospholipids [19]. Our results are in agreement with those findings and suggest that the changes of certain phospholipid species in OA SF, mainly variations in PC types, would allow unravelling distinct OA endotypes at early stages of the disease. Although these results must be validated in larger sample sizes, phospholipid-based SF profiling by targeted lipidomics provides a powerful approach for OA stratification and could facilitate the development of personalized early interventions on these subgroups of patients.

The small sample size is the major limitation of this study (n = 19 patients), especially regarding non-OA SF samples (n = 4), which are more difficult to obtain. This should be taken into account especially

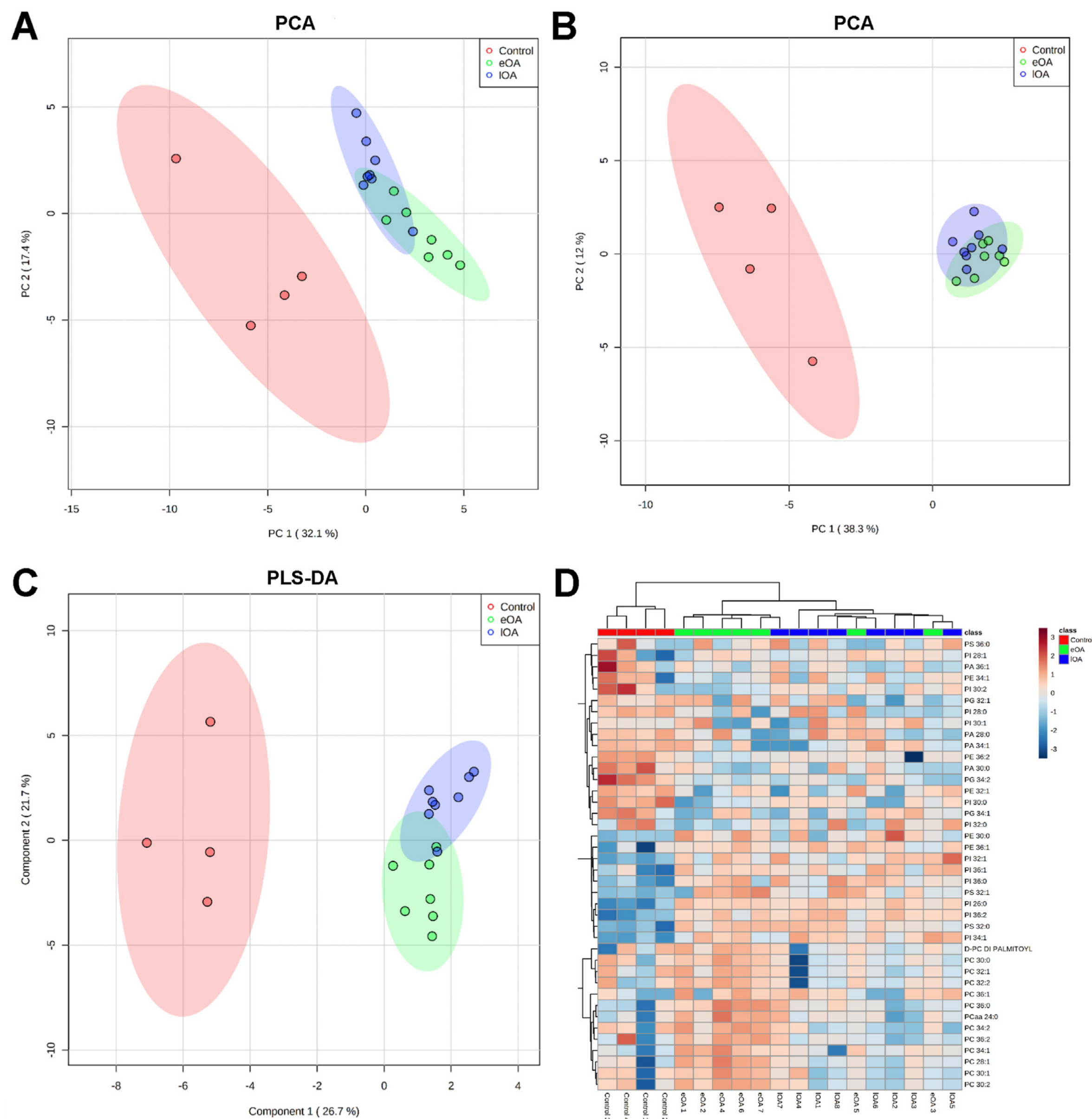

Fig. 2. Multivariate statistical analysis of the SF phospholipidome among sample groups. (A) Unsupervised PCA of all samples revealed good separation of non-OA control and OA SF. 95% confidence ellipses illustrate class separation. (B) Unsupervised PCA scores plot showing a clear separation between non-OA control and OA subgroups based on the phospholipid profiles quantified in negative ion mode. 95% confidence ellipses illustrate class separation. (C) Supervised multivariate analysis by PLS-DA discriminated non-OA control and OA SF in the component 1. The eOA group was separated from lOA in the component 2.95% confidence ellipses illustrate class separation. Scores plots are shown components 1 and 2. (D) HCA of all quantified lipid species of non-OA controls and OA subgroups. Clustering was assessed based on the Euclidean distance coefficient and average linkage method. Each column and each row represent individuals and lipids, respectively. Blue = low, red = high concentration. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

when interpreting the results, since it is unlikely that all phospholipid alterations and phenotypes could have been determined. In addition, control donors are not healthy individuals, therefore it is likely that they are not the most representative subset of control population. Furthermore, we lack follow up information and pain scores from most participants, which precludes to analyze the predictive potential of these lipid panels for disease progression and their association with pain. Importantly, the levels of total cholesterol have been identified as a potential confounder in this study, so we cannot exclude a bias in our findings due to the potential effect of this variable in the levels of phospholipids in SF, particularly in the eOA group. Dietary intake could also have a potential impact on SF phospholipid concentrations, but we do not have information about the nutritional behavior of our study participants. Therefore, further work is required to unequivocally distinguish disease-from putative diet-related lipid alterations. Finally, this targeted approach limits the coverage of the lipidome that has been quantified. Since our study is focused on a specific fraction of the lipid species that are known to be present in human SF, we might have missed other relevant molecules that could be associated with cartilage damage severity and be potentially useful to identify novel OA phenotypes or endotypes.

To our knowledge, this is the first study to employ a targeted MRM/MS-based phospholipidomic profiling of human SF to characterize phenotypes within OA patients at early stages of the disease. Our work clearly demonstrates the usefulness of this strategy to identify not only particular signatures between controls and different radiographic stages of OA, but also distinct endotypes that can be defined by specific phospholipid markers in eOA.

Table 3
Identification of 12 significantly modulated phosphatidylcholines between early- and late-stage OA SF by MRM/MS lipidomic analysis.

N	Lipid species	*m/z*	MS mode	eOA (n = 7)	lOA (n = 8)	FC	*p*-value
1	D-α-PC dipalmitoyl	734.60	POS	4420.00 ± 1451.00	2033.00 ± 743.80	2.44	0.001
2	PC 28:1	676.50	POS	829.40 ± 389.50	322.10 ± 126.70	2.58	0.003
3	PC 30:0	706.50	POS	1034.00 ± 385.50	454.40 ± 147.40	2.55	0.001
4	PC 30:1	704.50	POS	6789.00 ± 2060.00	4169.00 ± 1777.00	1.63	0.001
5	PC 30:2	702.50	POS	1413.00 ± 539.10	684.10 ± 317.10	2.07	0.004
6	PC 32:1	732.60	POS	4342.00 ± 1465.00	2057.00 ± 741.40	2.37	0.002
7	PC 32:2	730.50	POS	2490.00 ± 683.00	1098.00 ± 333.00	2.54	0.000
8	PC 34:1	760.60	POS	667.30 ± 285.40	311.80 ± 80.76	2.39	0.003
9	PC 34:2	758.60	POS	16386.00 ± 3784.00	9784.00 ± 3116.00	1.67	0.001
10	PC 36:0	790.60	POS	18282.00 ± 5341.00	12515.00 ± 3860.00	1.46	0.031
11	PC 36:2	786.60	POS	2682.00 ± 1084.00	1354.00 ± 621.90	1.98	0.011
12	PCaa 24:0	622.44	POS	16992.00 ± 4246.00	11444.00 ± 3658.00	1.48	0.017

eOA: early-stage osteoarthritis; lOA: late-stage osteoarthritis; POS: positive ion mode; FC: fold change; PC: phosphatidylcholine.
*significant difference from control SF.
#significant difference between eOA and lOA.

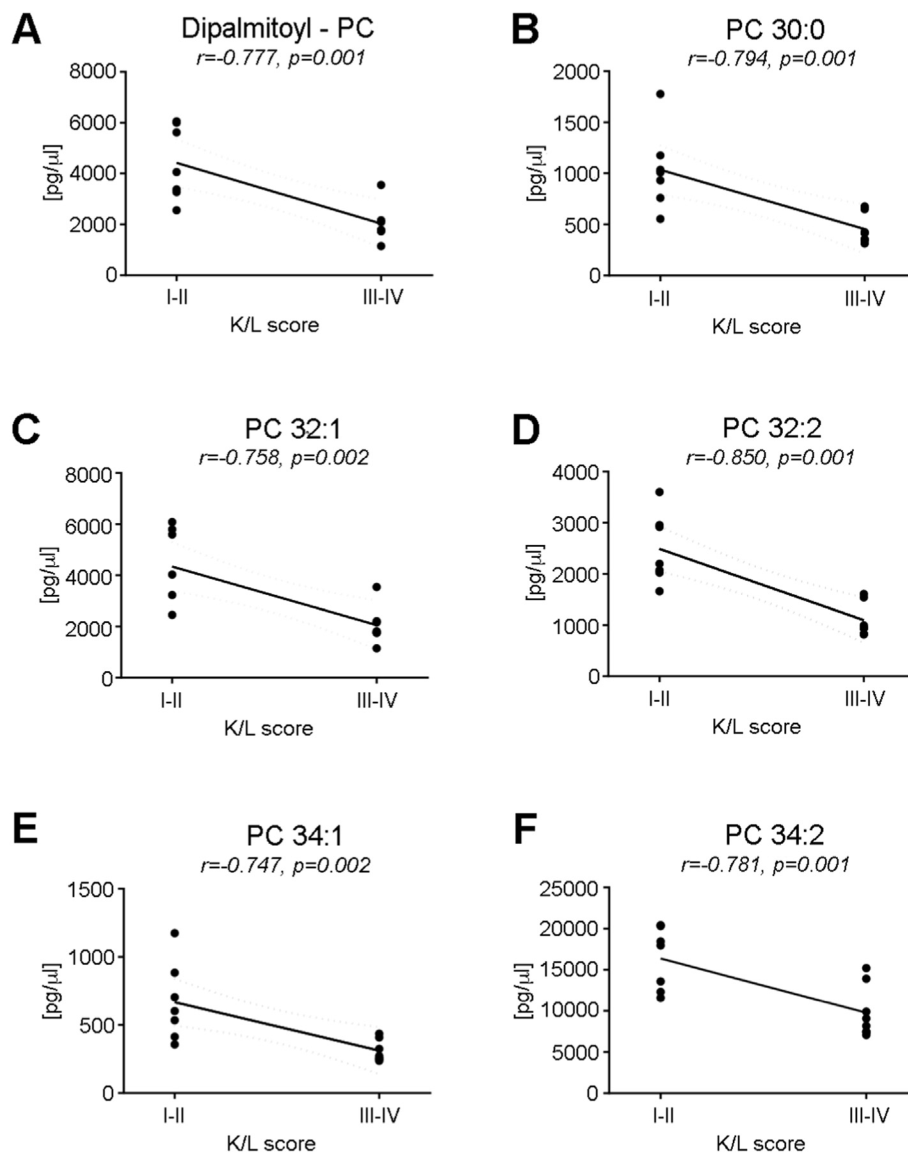

Fig. 3. Correlation of PC levels with cartilage damage in OA patients. Data were analysed by the Pearson correlation test. A negative correlation for all PC was observed. Pearson's correlation coefficients and *p*-values are shown. Concentrations of all PC are presented as pg/μl. PC: phosphatidylcholines; OA: osteoarthritis.

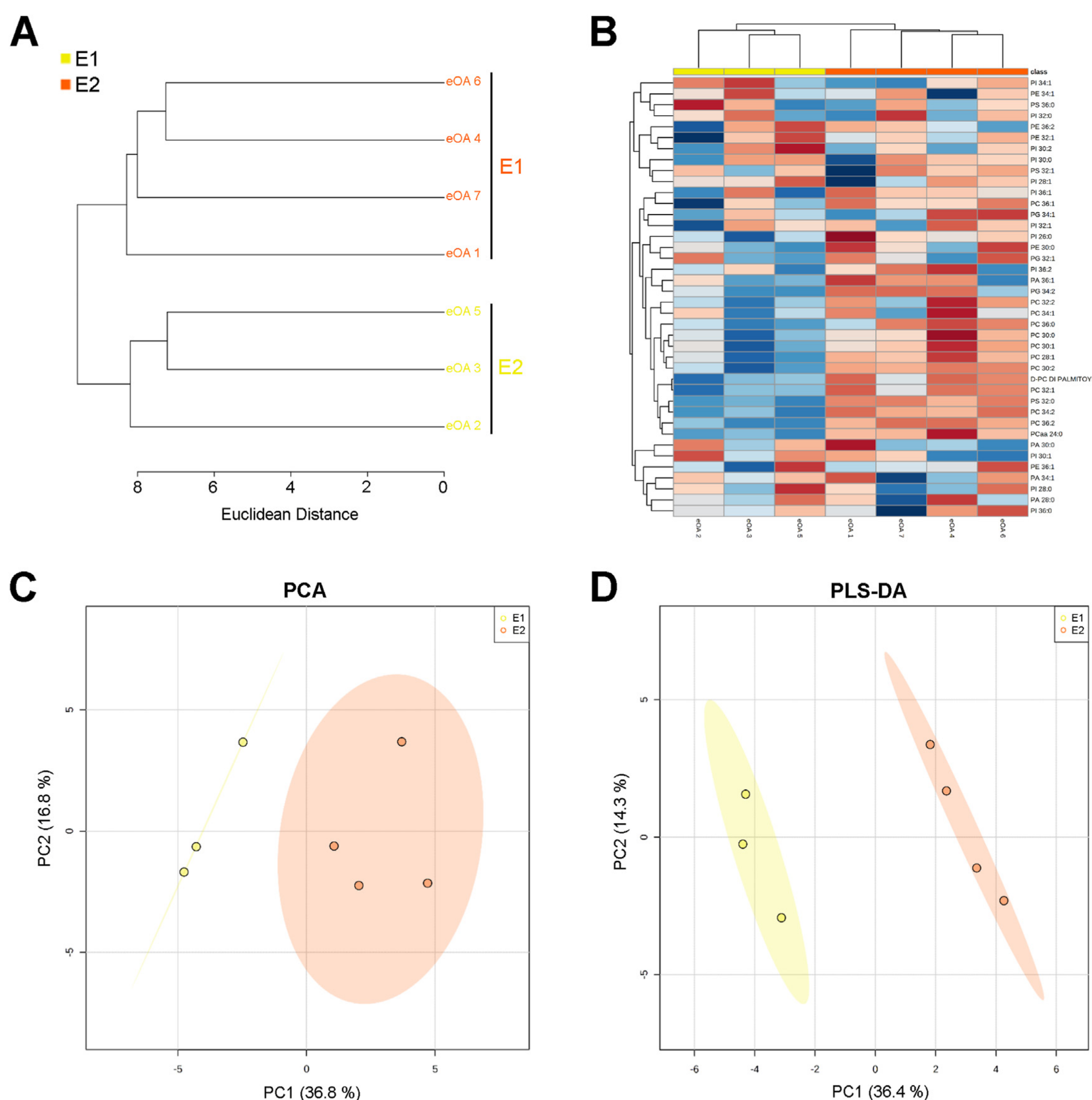

Fig. 4. Endotypes in eOA based on phospholipid SF levels. A) Unsupervised HCA of eOA participants. Two clusters of patients were identified: endotype 1 (E1, yellow) and endotype 2 (E2, orange). Line represents the Euclidean distance between patients and clusters. B) Heatmap illustrating the separation of eOA phenotypes based on specific phospholipid profiles. Unsupervised PCA (C) and PLS-DA (D) of eOA samples displayed a clear separation of both endotypes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Contributions

All authors have made significant contributions to the conception and design of this study, the acquisition of data, its analysis and interpretation. All authors were involved in drafting the article and approved the final version to be published. Detailed contribution: study conception and design (B.R., V-C., C.R.R. and F.J.B.), data acquisition (A.I., A.A., G.P.), data analysis, interpretation and drafting of the manuscript (B.R., V-C., A.I., C.R.R. and F.J.B.).

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Data statement

MRM/MS lipidomics data supporting the findings of this manuscript are available from the corresponding author upon request.

Declaration of competing interest

We certify that there is no conflict of interest to disclose regarding the materials and data discussed in this manuscript. The contents of this manuscript have not been copyrighted or published previously.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ocarto.2021.100219.

PCA: principal component analysis; PLS-DA: partial least squares discriminant analysis; SF: synovial fluid, eOA: early-stage OA; lOA: late-stage OA.

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