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Serum oxylipins profiling during the earliest stages of rheumatoid arthritis

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ABSTRACT

Objective: eicosanoids modulate inflammation via complex networks involving different pathways and downstream mediators, including oxylipins. Although altered eicosanoids are linked to rheumatoid arthritis (RA), suggesting an enhanced metabolization, the role of oxylipins in disease stratification remains unexplored. This study aims to characterize oxylipins networks during the earliest stages of RA and evaluate their associations with clinical features and treatment outcome.

Methods: 60 early RA patients (2010 ACR/EULAR criteria), 11 clinically suspect arthralgia (CSA) individuals and 28 control subjects (HC) were recruited. Samples were collected at onset, and treatment-naïve patients (n=50) were prospectively followed-up upon csDMARD treatment. A total of 75 oxylipins, mostly derived from arachidonic, eicosapentanoic and linoleic acids, were identified in serum by LC-MS/MS

Results: univariate analyses demonstrated differences in 14 oxylipins across HC, CSA and RA, exhibiting different trajectories. Network analyses revealed a strong oxylipins grouping pattern in RA, whereas a fuzzy network with high degree and closeness was found in CSA. In PLS-DA, 22 oxylipins had VIP scores>1, which allowed the identification of two clusters. Cluster usage differed among groups (p=0.003), and showed associations with disease severity and low rates of remission at 6 and 12 months in treatment-naïve RA. Pathway enrichment analyses revealed different precursors and pathways highlighting the relevance of AA and LOX pathway in CSA and RA, respectively. Distinct oxylipins signatures identified seropositive and seronegative RA subsets.

Conclusion: oxylipins networks differ across stages during earliest phases of RA. Oxylipins can inform on pathways with clinical relevance for disease progression, clinical heterogeneity and treatment response.

INTRODUCTION

Rheumatoid arthritis (RA) is an immune-mediated rheumatic condition characterized by chronic inflammation and joint destruction (1). Early diagnosis and prompt treatment guided by treat-to-target goals is crucial to ensure long-term disease control (2). Interventions during the early phase are associated with higher rates of remission, probably due to specific pathogenic mechanisms occurring at this point (3). Therefore, research into the early disease phase is of upmost scientific relevance. RA development is a multi-step process, where different phases can be distinguished (4). The recognition of the symptomatic phase preceding clinical arthritis, the so-called clinically suspect arthralgia (CSA), represents the first opportunity to identify patients at risk for progression to RA (5). This stage does not only open the window to characterize the changes underlying the shift from systemic autoimmunity to overt joint synovitis, but also to delineate potential targets to prevent progression (5,6). Although the cellular and proteomic characterization of these stages have been extensively pursued, the metabolomics and mainly, the lipidomics have received less attention.

In the 'omics' era, lipids are emerging as pivotal mediators for several biological processes (7). Poly-unsaturated fatty acids (PUFAs) and eicosanoids form one of the most complex networks in biology, controlling many physiological and pathological processes, often in opposing directions (8). The role of eicosanoids in RA dates back to 1970s and mid-1980s, with the description of the cyclooxygenase (COX) and lipooxygenase (LOX) pathways (9). However, these enzymes were not able to fully account for the underlying pathology and despite the profound improvements in clinical management, these pathways remained active (8). Consequently, a knowledge plateau was reached, in part due to technical limitations. It was not until recently that the CYP450-derived lipid species were described (10), although their role is still poorly characterized.

Lipidomics and high-throughput approaches have started a new period for the understanding of the control of local inflammation and its progression to either resolution or chronification (11). A number of novel lipid mediators, such as oxylipins, are now recognized as active players in controlling either resolution or fuelling inflammation (11–13). Oxylipins have been reported to promote polymorphonuclear cells migration, enhance vascular permeability, control cytokine production and modulate oxidative stress species (11), hence being attractive candidates to explain tissue injury and disease aggravation.

Previous results from our group revealed altered levels of different PUFAs in RA (14), which may reflect an accelerated metabolization towards their downstream mediators, including oxylipins. It is plausible that applying new analytical technologies will allow to answer the question whether

PUFA-derived oxylipins networks are altered in RA, and to comprehensively analyse how disturbed oxylipins networks may be associated with the clinical phenotype of the earliest stages of the disease. Therefore, the aims of the present study are (i) to analyse oxylipins levels and networks during the earliest stages of RA, including CSA, (ii) to evaluate whether oxylipins may identify patients with specific clinical features and treatment response and (iii) to evaluate whether oxylipins profiling may identify pathways related to disease heterogeneity.

MATERIAL AND METHODS

Study participants

The study was approved by the local institutional review board (Comité de Ética de Investigación Clínica del Principado de Asturias) in compliance with the Declaration of Helsinki. All study subjects gave written informed consent. Our study involved 60 early RA patients (2010 ACR/EULAR RA classification criteria (15)) from the early arthritic clinic of the Department of Rheumatology at Hospital Universitario Central de Asturias (HUCA) recruited at disease onset. A complete clinical examination (see Supplementary Materials) was performed on all patients during the recruitment appointment. Composite measures of disease activity were calculated: Disease Assessment Score using a 28-joint count and Erythrocyte Sedimentation Rate (DAS28-ESR), and Simplified Disease Activity Index (SDAI). Patients who had not been exposed to any disease-modifying antirheumatic drugs (DMARDs) or glucocorticoids (n=50) were considered as treatment-naïve early RA patients and were prospectively followed-up with complete clinical examinations at 6 months (n=46) and 12 months (n=40) upon csDMARD treatment. Clinical management was performed according to EULAR recommendations (16). Clinical response was evaluated by EULAR criteria at 6 and 12 months (17), and patients exhibiting a good response were considered as responders, whereas those with moderate or no response were classified as non-responders. Patients switching to a different conventional synthetic (cs)-DMARD during the first 12 months were also classified as non-responders. Subjects with CSA were recruited from the same clinic if they met at least 4 criteria of the EULAR definition of arthralgia suspicious for progression to RA (5), which allows a sensibility of 70% and specificity of 93.6%. Subjects without arthritis (HC) were recruited among age and gender-matched individuals from the same population without diagnosis of inflammatory rheumatic disease. Exclusion criteria for all the groups were pre-existing autoimmune or inflammatory conditions, medical prescription for non-steroidal anti-inflammatory drugs or coxibs, usage of fish oil supplements, and history of cancer or recent (<3 months) infection. A fasting blood sample was collected from all individuals by venepuncture, and serum samples were transferred to the laboratory, processed under controlled, standardized procedures and stored at -80ºC within less than 2 hours. In a subgroup of subjects (n=6), plasma samples were collected and processed in parallel.

Lipid extraction and LC-MS measurement of oxylipins

All sera samples at baseline were thawed once and immediately used for free fatty acid and oxylipins isolation as described (18,19). Briefly, 50 µl sera was spiked with a cocktail of 26 deuterated internal standards that also included some selected PUFAs, brought to a volume of 1

ml with 10% methanol and samples were then purified by solid phase extraction on Strata-X columns (see Supplementary Materials).

Oxylipins in sera were analysed and quantified by LC-MS/MS as previously described (18,19) (see Supplementary Materials).

Oxylipins and free fatty acids were quantitated by the stable isotope dilution method. Identical amounts of deuterated internal standards were added to each sample and to all the primary standards used to generate standard curves (see Supplementary Materials). Oxylipins levels are shown as pmol/ml.

Statistical analyses

Continuous variables were expressed as mean±standard deviation or median (interquartile range), whereas categorical variables were summarized as n(%). For each oxylipin, fatty acid precursor and pathway (first enzyme acting on the precursors) were retrieved from the literature. If a non-enzymatic mechanism was described in the literature, regardless of the experimental setting, it was included as well, in a conservative approach. Non-parametric tests were used to evaluate differences in oxylipins across groups, and p-values obtained were adjusted for multiple testing using the procedure of Benjamini-Hochberg (20). Linear regression models were used to control comparisons for potential confounders. Oxylipins levels were log-transformed, normalized to the median and scaled by range-scaling method (21). Correlograms and network analyses were built to analyse the correlations among oxylipins across conditions. Centrality measures were calculated for each metabolite and condition (22). Partial Least Squared Discriminant Analyses (PLS-DA) to identify discriminant metabolites controlling for multicollinearity, and cross-validation accuracy and permutation model statistics were retrieved. Oxylipins contributing to group discrimination in PLS-DA were selected on the basis of variable important projection (VIP) values>1 (22). VIP-selected metabolites were used in unsupervised cluster analyses. For two-group comparisons, orthogonal PLS-DA (OPLS-DA) models were performed to evaluate group discrimination. These models enhance the interpretation and discrimination based on intra- and interclass information, without a significant effect on prediction power (23). Cross-validation of OPLS-DA was performed by permutation analyses, and Q2 p-values were computed. Correlation analyses against pre-specified patterns were assessed and correlation coefficients (Spearman rank method), p-values and FDR were computed. Enrichment Pathway Analyses were performed using KEGG human library, global test for pathway enrichment analysis and betweenness centrality for pathway topology analysis. Raw p, (adjusted) Holm p and pathway impact were

obtained for each pathway. Statistical analyses were carried out under R v.3.6.3 and MetaboAnalyst v.4.0.

RESULTS

Serum oxylipins during the earliest stages of RA

Serum oxylipins were measured in 60 early RA patients (50 early treatment-naïve), 11 CSA individuals and 28 matched HC (Supplementary Table 1). A total of 74 oxylipins derived from arachidonic (AA, n=39), docosahexanoic (DHA, n=13), linoleic (LA, n=7), eicosapentanoic (EPA, n=7), dihomo-g-linolenic (DHGLA, n=3), a-linolenic (ALA, n=2) and oleic (OA, n=2) acids, were identified by LC/MS (Supplementary Table 2). A good correlation between oxylipins measured in serum and plasma was noted in the subgroup analyses (median correlation 0.870, range 0.750 – 1.000). Moreover, the peaks corresponding to LTB4 and 5S,12S-diHETE were compared in the samples. Both metabolites show similar fragmentation patterns by MS but show base-line separation under the LC conditions employed, excluding a potential overlap in the chromatogram. Further, a comparative analysis of both analytes allowed us to exclude the possibility of a significant platelet-neutrophil activation matrix during sample collection and preparation (Supplementary Figure 1), as 5S,12S-diHETE is considered a product of activated platelets and neutrophils.

A total of 14 oxylipins exhibited different levels across disease groups (Supplementary Table 3). Oxylipins exhibited different trajectories, some showing peaking levels in RA, whereas others did in CSA or HC (Supplementary Figure 2). Not always a gradual change was observed across groups, thus pointing to complex, individual patterns requiring a global approach. No associations with age, gender, BMI or traditional cardiovascular risk factors were observed in any of the study groups (all $p>0.050$). Similarly, excluding patients receiving medications at recruitment (n=10) did not change these results. Oxylipins were not observed to parallel disease activity in RA patients (all $p>0.050$). It must be noted that most of the patients were in high-disease activity status.

Correlograms showing the associations among oxylipins (Figure 1A) evidenced more defined groups in HC and RA, and a more widespread picture in CSA. Network graphs were generated to analyse the interactions among oxylipins (Figure 1B), confirming that the different clinical stages were hallmarked by distinct pictures. HC exhibited a well-defined group of oxylipins closely associated, mostly AA- and DHA-derived, the latter being in a central location. Overall, a relatively clear grouping pattern by precursors can be distinguished. RA was characterized by a smaller group of oxylipins strongly correlated, located in an eccentric location, and including EPA-, DHA- and AA-derived species, although a more diverse grouping was noted. Moreover, some nodes served as links between this group and smaller groups within the rest of the network. Finally, the CSA network exhibited a fuzzy pattern, with less clear groups, a more heterogeneous

distribution of nodes and a higher number of connections among them. Centrality measures (Supplementary Figure 3) supported these findings, since a higher degree and closeness was retrieved in CSA, whereas specific compounds exhibited a higher betweenness in RA.

These results confirm that quantitative and qualitative differences were present in oxylipins levels during the earliest phases of RA, event at the arthralgia stage.

Oxylipins profiling identified clinically relevant clusters

Multivariate approaches were conducted to capture the global picture of oxylipins disturbances. A PLS-DA with all identified metabolites (12.1% of the total variance explained, R2=0.461, 71.0% cross-validation accuracy and empirical permutation $p=5 \cdot 10^{-4}$) achieved a partial discrimination among groups (Figure 2A), although a certain overlap existed. Interestingly, the CSA group lie between HC and RA. These figures revealed that oxylipins may not be useful to accurately predict group classification but suggest the potential existence of oxylipins-based group similarities. A total of 22 oxylipins had VIP scores >1 (Figure 2B), which were used for heatmap visualization and cluster analysis (including all study subjects). A group-averaged heatmap (Figure 2C) confirmed the previous global differences in oxylipins, with an intermediate, 'transitional' profile observed in CSA, although some CSA-specific disturbances were also noted. Interestingly, RA and CSA showed a closer similarity.

The cluster analysis (Figure 2D) allowed the identification of two oxylipins clusters (cluster I and II). Clusters usage (Figure 2E) differed among groups (p=0.003), thus confirming the differences observed in network analyses and the partial overlap in the PLS-DA model, hence pointing to a potential clinical relevance of the oxylipins profiles. Whereas the HC were mostly grouped in the cluster I, CSA and RA patients were observed to use both clusters I and II. Therefore, we analysed whether the different clusters were related to clinical features in RA patients. RA patients exhibiting cluster I showed a higher VAS global assessment (p=0.016) and pain (p=0.003) scores than their cluster II-counterparts, whereas no differences were noted in other features (Table 1). Next, the clinical response upon csDMARD treatment (low-dose glucocorticoids and methotrexate) in treatment-naïve patients was compared between clusters. Interestingly, cluster I-patients were less likely to achieve a EULAR good response (5/16, 31.2%) than their cluster II-counterparts (21/30, 70.0%) (p=0.012) at 6 months. Equivalent results were observed for DAS28 (4/16 vs 20/30, p=0.007) and SDAI remission (3/13 vs 14/29, p=0.050) criteria. Furthermore, a similar picture was observed for the EULAR good response (4/13 vs 17/27, p=0.056) and DAS28 remission criteria (4/13 vs 17/27 vs 4/9, p=0.056) at 12 months. Two

patients in cluster I and three patients in cluster II were switched to a different csDMARD at 12 months. No patients were switched to bDMARD.

All these results suggest that oxylipins profiling can delineate disease clusters differently used across conditions, with CSA showing an intermediate profile, and associated with clinical features and early treatment response in RA, hence supporting their role in shaping the early RA clinical phenotype.

Oxylipins signatures identify pathways with clinical relevance for arthritis

Next, we aimed to identify whether oxylipins could delineate metabolic pathways related to disease progression or clinical heterogeneity at onset.

First, an OPLS-DA method was carried out to evaluate whether oxylipins can discriminate between HC and CSA individuals (Figure 3A) (permutation empirical Q2 p=0.014). Since a discrimination was achieved, a correlation analysis against the pattern HC→CSA was performed to identify those oxylipins with such linear increase (Figure 3B). A group of 8 oxylipins showing this pattern was identified, deriving from AA (4), LA (2), DHA (1) and DHGLA (1) precursors, and from LOX (4), CYP (3) and COX (1) pathways (Supplementary Table 4). A similar analysis for the pattern CSA→RA (Figure 3C/D) (permutation empirical Q2 p=0.054) retrieved 5 species, derived from LA (2), EPA (1), AA (1) and DHGLA (1), whereas almost all (4) originated from the LOX pathway (Supplementary Table 5). Furthermore, pathway enrichment analyses confirmed a higher impact of the AA metabolism for the HC/CSA comparison (Supplementary Table 6), whereas LA metabolism was ranked as the highest pathway for the CSA/RA one (Supplementary Table 7). Interestingly, the pattern HC→CSA→RA identified a group of 18 species, mostly deriving from AA (12) and from the COX pathway (8) (Supplementary Figure 4).

Additionally, we evaluated whether oxylipins can reveal differences between seropositive and seronegative (RF-/ACPA-) RA subsets. An OPLS-DA revealed a good discrimination between HC and seronegative RA patients (Figure 4A) (permutation empirical Q2 p=0.050). Correlation analyses identified a group of 7 oxylipins differentially expressed (Figure 4B), mostly deriving from AA (3) and OA (2), with COX (3) and nitration (2) pathways being the most important (Supplementary Table 8). On the other hand, HC and seropositive RA patients could be discriminated by an OPLS-DA, although a partial overlap was noted (Figure 4C) (permutation Q2 empirical p=0.054). In this case, a higher number of oxylipins (13) was found to be significant in the correlation analysis (Figure 4D), being AA (9) and 5-LOX (6) the most common precursors and pathways retrieved, respectively (Supplementary Figure 9). Accordingly, pathway enrichment

analyses revealed a higher relevance of AA metabolism pathway for the seropositive subset (Supplementary Table 10) than to the seronegative one (Supplementary Table 11).

In conclusion, oxylipins profiling inform on relevant pathways related to disease heterogeneity. Different species were related to the different disease stages underlying the early phase of RA. Similarly, oxylipins revealed divergent pathways between seronegative and seropositive RA subsets.

DISCUSSION

A growing body of studies supports the use of lipidomics to gain understanding towards complex diseases and unveil pathogenic circuits linked to disease progression and new targets. The results herein reported support, for the first time, the occurrence of alterations in PUFA-derived oxylipins during the earliest phases of arthritis. Oxylipins can identify subsets of patients with different clinical features and treatment response and may inform on specific metabolic pathways differentially expressed between disease subsets. Taken together, oxylipins profiling could be considered as an attractive source of biomarkers and potential targets for the early phase of inflammatory arthritis.

A remarkable finding from our study was the noticeable disturbance in oxylipins across groups, which delineated distinct global pictures among HC, CSA and RA oxylipins networks. Importantly, these patterns could not be attributed to class-specific (omega 3-derived, omega 6-derived) general impairments, but individual compounds seemed to follow distinct patterns. This notion has already been documented by our group (14) and others (24) at the level of fatty acid precursors, and underline the need of complex, global approaches to account for the heterogeneity of these species and urges a simultaneous assessment of the main pathways involved. Equivalent results were obtained in synovial fluid, hence supporting this notion (25). Moreover, oxylipins were not related to demographic features or traditional cardiovascular risk factors in all the study groups, as previously reported. Moreover, our findings from a treatment-naïve early arthritis cohort rule out the influence of disease duration and treatment exposure, thus pointing to oxylipins networks as actual disease players and not innocent bystanders.

The most interesting result of our work is the identification of the oxylipins alterations during the earliest stages of RA, including early RA as well as CSA. Preventive interventions at the CSA stage (26) are limited due to the poor characterization of the underlying pathogenic mechanisms. Although previous studies have identified some metabolites (27), the lipid compartment has been largely neglected. Our results support the role of oxylipins as potential players in this setting. These findings are in line with reports showing lower w3-PUFA levels in individuals with high risk of developing RA (28), and changes in gene expression related to lipid metabolism at this stage (29). Moreover, we have recently published that reduced circulating DHA, EPA and AA levels can be found at disease onset (14), suggesting a potential disturbed metabolism. The results herein presented reinforce this hypothesis and went further by identifying the actual species altered in the arthralgia stage downstream the main PUFA precursors. CSA individuals exhibited a genuine, widespread oxylipins network, with a higher degree and betweenness. This may be the result of a

transitional status where a high number of metabolic interactions are operating, or also a consequence of group heterogeneity. CSA is a very heterogeneous stage itself, with a number of possible clinical outcomes reported (30), from resolution to chronification. The fact that CSA individuals were equally represented in the two clusters identified and the overlap with the HC and RA groups in the PLS-DA support this idea. More importantly, absolute levels also revealed specific alterations in CSA which are not present in RA or HC. Furthermore, an important number of oxylipins exhibited decreased in CSA, with certain degree of recovery in RA, although other trajectories were also noted (see Supplementary Figure 2), which warrant further research.

Correlation analyses comparing HC/CSA or CSA/RA lead us to gain insight into potential changes occurring the multistep RA development. First, the HC/CSA analysis informed on 8 species originating from four different precursors and major pathways, whereas the CSA/RA comparison yielded less species, mostly coming from LA and the LOX pathway. Pathway analyses supported these differences. Overall, these findings suggest that distinct oxylipins alterations are associated with the different steps along RA course. A more diverse picture is observed in the first case, in line with the different risk factors and mechanisms associated with the first events in RA triggering (31), while a more convergent effect is observed for the CSA/RA comparison, where mechanisms are thought to be shared among disease subsets (32,33). Importantly, these discrepancies may be the result of the natural regulation of eicosanoid pathways, hallmarked by an initial production of pro-inflammatory species (mostly AA-derived) that prompts a class-switch to an anti-inflammatory, homeostatic response (34,35). However, anti-inflammation and pro-resolution are not equivalent (36), and it is plausible that a stronger, pro-resolving shift may be needed to control the CSA/RA phase.

Oxylipins showing a change between CSA and RA groups may be conceived as potential therapeutic targets to prevent disease progression. Actually, LOX pathway has been recently described to be upregulated in RA in comparison with osteoarthritis at the synovial level, while other enzymatic pathways are unchanged (25). Due to the relevance of LOX-derived species in this setting, LOX inhibition may be an attractive candidate. Actually, zileuton-mediated LOX inhibition has already been studied in RA, although no clinical efficacy was proved in established disease (37). However, in light of our results, further research on the effect of LOX inhibition in disease progression, rather than management, must be considered. This is supported by studies with animal models where treatment is administered very early (38). The fact that LOX expression is persistent along disease course, unchanged by conventional treatments (39), emphasises the need of an earlier intervention. Alternatively, and due to synergistic effects among oxylipins, dual COX/LOX inhibitors may be also considered (40).

Importantly, differences were noted between the two-step (HC/CSA and CSA/RA) and the global HC/CSA/RA analyses. Although the latter mostly supports the role of AA and LOX as a whole, a compartmentalization was noted in the two-step process, which aligns with the different oxylipins trajectories and allow to identify potential targets for tailored strategies, the main goal of personalized medicine (41), hence supporting the rationale of our analyses.

Our results shed new light into the potential role of oxylipins in early RA. Decreased EPA and DHA levels were observed at disease onset, hence strengthening our previous findings (14), linked to altered levels of their derived species. Cluster analyses revealed that two oxylipins profiles could be distinguished among RA patients. One of the clusters was predominantly present in HC, which may be a more 'homeostatic' profile, whereas the other cluster identified a group of patients with more severe clinical features, including higher pain scores. This is aligned with previous evidence from clinical trials with fish oils and omega-3 supplements reporting a protective effect on pain in RA (42,43). Importantly, eicosanoid metabolites are known to activate nociceptive pathways (44), but the actual mediators are unknown. Additionally, this cluster was also associated with csDMARDs treatment outcomes. Due to the importance of early remission in treat-to-target strategies (45,46), oxylipins networks deserve further research either as biomarkers or actionable mechanisms to facilitate clinical management.

Finally, oxylipins profiling lead us to identify differences between seronegative and seropositive RA subsets. Although previous metabolomics studies have observed distinct metabolomics signatures in seronegative RA patients, the exact compounds have not been elucidated (47). Our results confirm that whereas both subsets could be distinguished from controls based on oxylipins signatures, the precursors and pathways greatly differed between them. These results underscore the differences between these two subsets and add another layer of complexity by identifying oxylipins networks as potential contributors. Whereas AA metabolism clearly dominated the oxylipin signature in seropositive RA, less impact was observed in seronegative patients, as demonstrated in correlation and pathway analyses. Importantly, OA-derived nitrooleates, strong anti-inflammatory lipids (48), DHA- and EPA-derived species, in addition to COX products were associated with seronegative RA. Due to the complexity of the seronegative form, these findings warrant further research into these pathways.

In conclusion, serum oxylipins were altered during the earliest stages of RA, and specific alterations were found even at the arthralgia stage, which may reflect an altered PUFA metabolism. Oxylipins networks at onset were related to the clinical phenotype of the disease and can predict response to treatment. More importantly, oxylipins profiling helped to identify

metabolic pathways relevant to resolve disease heterogeneity. Our study has key strengths such as the recruitment of individuals comprehensively and characterized from the clinical point of view, the use of a robust targeted metabolomics platform and the appropriate statistical approach. Yet, this study has some limitations that must be remarked, including the cross-sectional design, which did not allow to prospectively follow CSA subjects, and lack of information on other lipid species. Although our approach included cross-validation and permutation tests, an important limitation of our findings is the lack of an external, validation cohort. Under these circumstances, it is unclear whether the results generalize beyond the profile of patients recruited. The potential effect of diet may be also considered, although our previous results failed to demonstrate a significant effect on PUFA levels (49,50). Moreover, the effect of the sample (serum/plasma) must be also taken into account to ensure comparability with other studies. Finally, pathway assignment was based on the existing literature. Whether a promiscuous oxylipin production by other enzymes or by non-enzymatic reactions exists in pathological conditions cannot be totally ruled out. However, the involvement of non-enzymatic pathways was extracted from the broad literature and it has not been proven that they were the main mechanisms in the setting of RA, so its relevance needs to be evaluated with caution. Nevertheless, the use of enrichment pathway analyses using a well-recognized library provides some degree of validation to our results. Taken together, this work has notably improved the understanding on eicosanoid networks in very early RA and paved the ground for future, larger, multicentric and prospective studies to address this topic. However, studies in larger, external cohorts of patients are needed to ensure the generalizability of our findings. In addition, to assess underlying metabolic changes in the inflamed joint, future research should also focus on synovial membrane, in line with the reports by other groups as well (25), including paired serum and synovial samples from RA patients. Expanding the lipid spectra analysed and a global integration of the lipid layer with the rest of the clinical and biological information must be in the research agenda.

CONFLICTS OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The funders had no role in study design, data analysis, interpretation or decision to publish.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the manuscript or revising it critically for important intellectual content and all the authors gave their approval of the final version of the manuscript to be published.

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TABLES

Table 1: Clinical features of RA patients at recruitment based on oxylipins clusters. Variables were expressed as median (interquartile range) or n(%), unless otherwise stated. Differences were assessed by Mann-Whitney or chi-square tests (or Fisher exact test, when appropriate), according to the distribution of the variables. * Among RA patients, 50 patients were recruited before being exposed to any treatment (early treatment-naïve RA patients).

	Cluster I n=23	Cluster II n=37	p-value
Clinical features			
Duration of symptoms (weeks)	24.00 (11.50-37.00)	20.00 (8.50-30.00)	0.472
Morning stiffness (minutes)	60.00 (15.00-90.00)	30.00 (12.50-120.00)	0.853
Tender Joint Count	9.00 (6.00-14.00)	8.00 (4.50-13.00)	0.478
Swollen Joint Count	6.00 (3.00-10.00)	5.00 (3.00-8.50)	0.337
ESR (mm/h)	19.00 (11.00-37.00)	20.00 (7.50-34.00)	0.616
CRP (mg/dl)	0.80 (0.30-3.20)	0.60 (0.20-1.65)	0.256
Patient Global Assessment (VAS 0-100)	70.00 (60.00-90.00)	50.00 (40.00-72.50)	0.016
Pain Assessment (VAS 0-10)	8.00 (7.00-8.00)	6.00 (5.00-8.00)	0.003
DAS28	5.66 (4.68-6.45)	5.05 (3.86-6.07)	0.163
SDAI	29.60 (23.12-39.37)	26.30 (18.15-35.05)	0.156
HAQ	1.50 (1.66-0.65)	1.10 (0.60-1.65)	0.432
Fatigue (VAS 0-10)	5.00 (0.00-8.00)	6.00 (1.50-8.00)	0.816
RF+	16 (69.5)	21 (56.7)	0.321
ACPA+	15 (65.2)	20 (54.0)	0.461
RF-/ACPA-	5 (21.7)	14 (37.8)	0.257
Traditional CV risk factors			
Hypertension	9 (39.1)	12 (32.4)	0.597
Diabetes	3 (13.0)	4 (10.8)	0.793
Dyslipidemia	8 (34.7)	11 (29.7)	0.735
Smoking	6 (26.0)	17 (45.9)	0.104
Treatments			
None*	18 (78.2)	32 (86.4)	0.406
Glucocorticoids	4 (17.3)	4 (10.8)	0.466
Methotrexate	2 (8.6)	3 (8.1)	0.936

FIGURE LEGENDS

Figure 1: Analyses of correlations among oxylipins. (A) Correlation matrices among oxylipins were plotted in correlograms. The tile colour is proportional to the strength of the correlation between each pair of oxylipins according to the legend at the bottom. (B) Network analyses based on the oxylipins concentrations across study groups. Each node corresponds to a single oxylipin, numbered as per the figure legend list. Node colours represent the precursor: red (AA), green (DHA), dark blue (EPA), light blue (LA), turquoise (DHGLA), orange (ALA), magenta (OA). The lines between nodes illustrate the strength (width) and type (green: positive, red: negative) of the correlations between each pair of oxylipins. The relative position of the nodes parallels its correlation: nodes more closely correlated locate closer to each other. ***1***(TXB2), ***2***(PGF2a), ***3***(PGE2), ***4***(PGD2), ***5***(TXB1), ***6***(PGE1), ***7***(TXB3), ***8***(PGE3), ***9***(20-OH-PGE2), ***10***(PGEM), ***11***(Tetranor 12-HETE), ***12***(12-HHTrE), ***13***(11-HETE), ***14***(11-HEPE, ***15***(13-HDoHE), ***16***(PGB2), ***17***(PGJ2), ***18***(9-HETE), ***19***(9-HEPE), ***20***(16-HDoHE), ***21***(20-HDoHE), ***22***(LTB4), ***23***(20-OH-LTB4), ***24***(12-oxo-LTB4), ***25***(LTE4), ***26***(5-HETE), ***27***(5-HEPE), ***28***(4-HDoHE), ***29***(9-HOTrE), ***30***(5-HETrE), ***31***(7,17-dhDPA), ***32***(PDX), ***33***(15-HETE), ***34***(15-HEPE), ***35***(17-HDoHE), ***36***(13-HODE), ***37***(13-HOTrE), ***38***(15-HETrE), ***39***(8-HETE), ***40***(10-HDoHE), ***41***(8-HETrE), ***42***(12-HETE), ***43***(12-HEPE), ***44***(14-HDoHE), ***45***(11-HDoHE), ***46***(9-HODE), ***47***(12-oxo-ETE, ***48***(9-oxo-ODE), ***49***(20-HETE, ***50***(18-HETE), ***51***(17-HETE, ***52***(16-HETE), ***53***(18-HEPE), ***54***(5,6-EET), ***55***(8,9-EET), ***56***(11,12-EET), ***57***(14,15-EET), ***58***(16,17-EpDPE), ***59***(19,20-DiHDPA), ***60***(9,10-EpOME), ***61***(12,13-EpOME), ***62***(5,6-diHETrE), ***63***(8,9-diHETrE), ***64***(11,12-diHETrE), ***65***(14,15-diHETrE), ***66***(9,10-diHOME), ***67***(12,13-diHOME), 68(AA), ***69***(AdA), ***70***(EPA), ***71***(DHA), ***72***(20-cooh-AA), ***73***(9-Nitrooleate), ***74***(10-Nitrooleate)

Figure 2: Oxylipins profiling across study groups. (A) PLS-DA showing discriminant capacity of all the oxylipins identified, based on the amount of variance explained by the first two components. (B) Top 25 oxylipins ranked based on the VIP scores of the PLS-DA model. The heatmap on the right indicate the concentration ranks across the different groups. (C) Group-averaged heatmap based on the 22 oxylipins with VIP scores >1. (D) Heatmap and cluster analysis based on the 22 oxylipins with VIP scores >1. Tiles were coloured based on oxylipins levels, red and blue indicating high and low levels respectively, as indicated in the legend (top right). Upper bar indicates group classes (HC: blue, CSA: green, RA: red). Heatmap allowed the

identification of two clusters (bottom). (E) Table indicating the number of individuals of each study group (HC, CSA and RA)) using the different clusters identified (clusters I and II).

Figure 3: Oxylipins signatures are associated with the earliest stages of RA. (A) OPLS-DA model evaluating the discriminant capacity of all oxylipins analysed between HC and CSA groups. (B) Analysis of the correlation pattern HC/CSA showing the top 25 oxylipins exhibiting that pattern based on Spearman rank correlation as distance measure. . (C) OPLS-DA model evaluating the discriminant capacity of all oxylipins analysed between CSA and RA groups. (D) Analysis of the correlation pattern HC/CSA showing the top 25 oxylipins exhibiting that pattern based on Spearman rank correlation as distance measure. Those oxylipins reaching statistical significance are highlighted in bold.

Figure 4: Oxylipins signatures are associated with seropositivity in early RA. (A) OPLS-DA model evaluating the discriminant capacity of all oxylipins analysed between HC and seronegative (RF-/ACPA-) RA groups. (B) Analysis of the correlation pattern HC/seronegative RA showing the top 25 oxylipins exhibiting that pattern based on Spearman rank correlation as distance measure. . (C) OPLS-DA model evaluating the discriminant capacity of all oxylipins analysed between HC and seropositive RA groups. (D) Analysis of the correlation pattern HC/seropositive RA showing the top 25 oxylipins exhibiting that pattern based on Spearman rank correlation as distance measure. Those oxylipins reaching statistical significance are highlighted in bold.

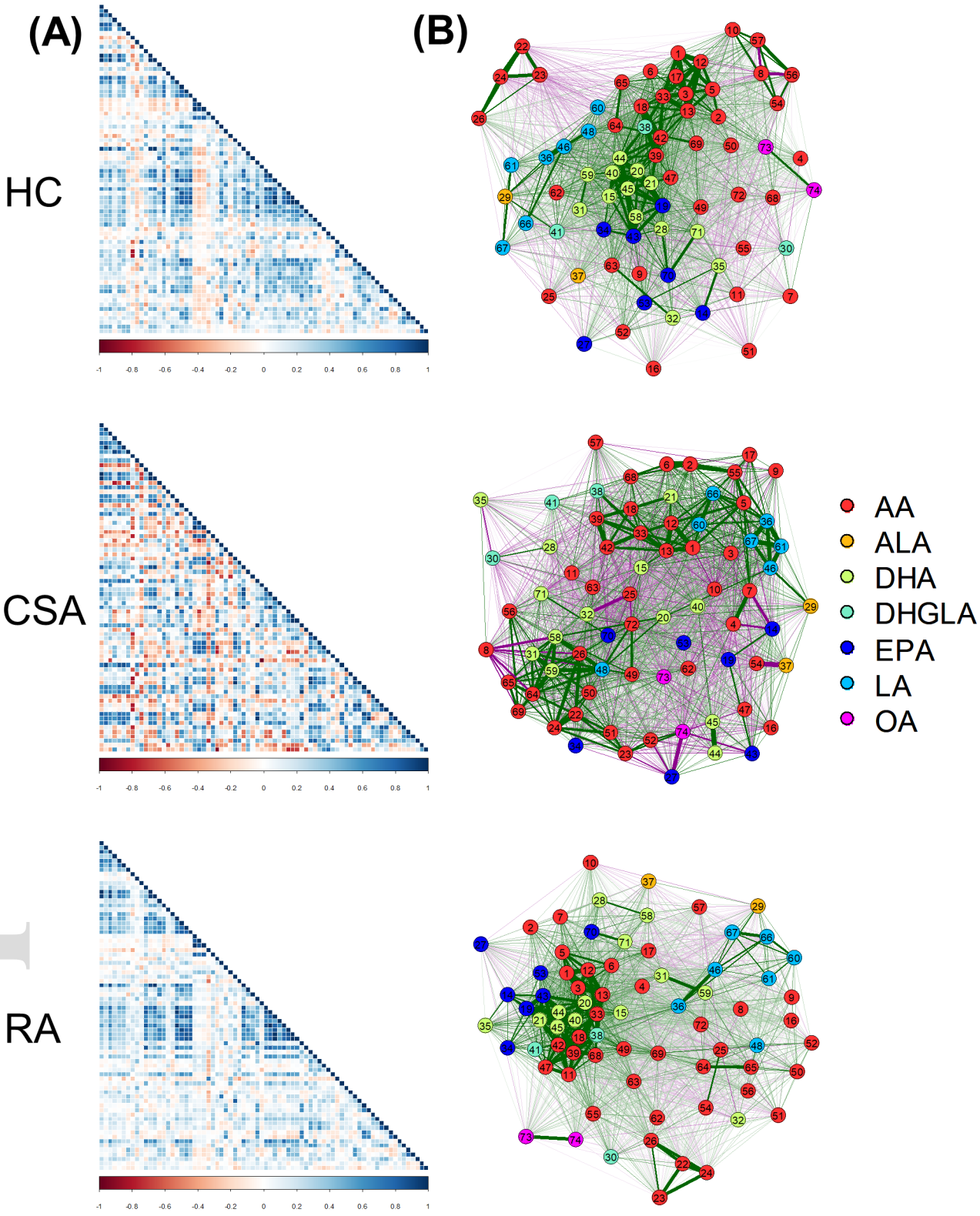
SUPPLEMENTARY FIGURES

Supplementary Figure 1: Comparative analysis of LTB4 and 5S,12S-diHETE in samples. Chromatograms showing LTB4 and 5S,12S-diHETE peaks in serum samples in two representative individuals. Distinct retention times for both metabolites were confirmed.

Supplementary Figure 2: Oxylipins trajectories across study groups. Oxylipins levels were summarized in a group-averaged heatmap to evaluate the different oxylipins trajectories observed among the three study groups. This heatmap include all the oxylipins analysed and it does not add information about the relevant of these differences, as it is only intended for visualization purposes. Oxylipins concentrations are indicated as per the legend (upper right). Upper bar indicates group classes (HC: blue, CSA: green, RA: red).

Supplementary Figure 3: Analysis of centrality measures on oxylipins networks. Centrality measures (degree, expected influence, betweenness and closeness) of the oxylipins networks (Figure 1B). Oxylipins are indicated in the vertical axes and centrality measures are represented in the horizontal axes for each study group (HC: blue, CSA: green, RA: red). Oxylipins are listed as per the Figure 1 legend list.

Supplementary Figure 4: Oxylipins signatures associated with the HC-CSA-RA pattern. (A) Analysis of the correlation pattern HC-CSA-RA showing the top 25 oxylipins exhibiting that pattern based on Spearman rank correlation as distance measure. (B) List of the top 25 oxylipins, including pathway information, precursors, correlation coefficients, p-values and FDR. Those reaching statistical significance are highlighted in bold and grey background. (*) depicts that the given oxylipin has been also described to be produced by means of non enzymatic mechanisms according to the broad literature.



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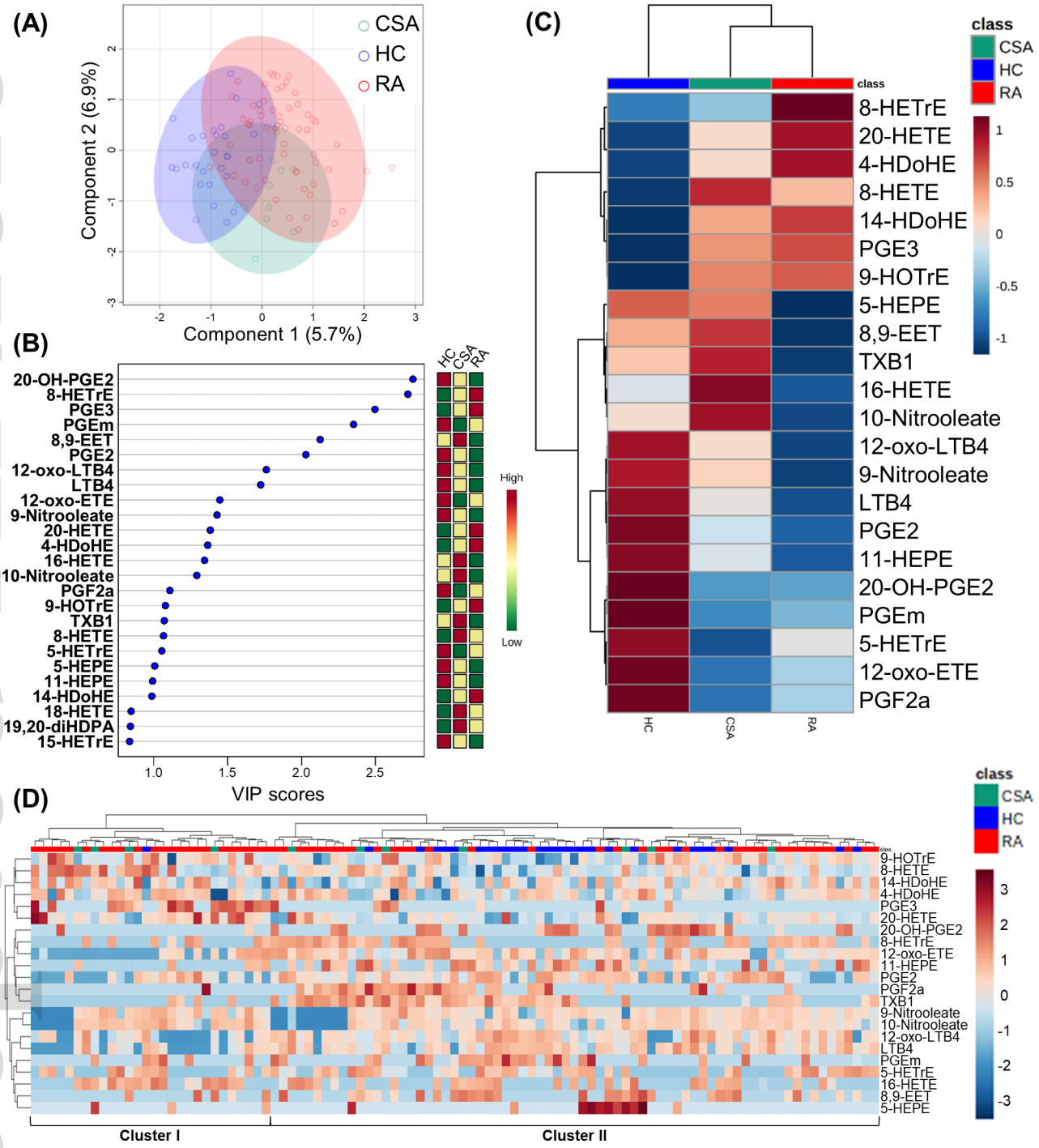

(E)

	Cluster I	Cluster II
HC, n(%)	1 (3.5)	27 (96.4)
CSA, n(%)	4 (36.3)	7 (63.6)
RA, n(%)	23 (38.3)	37 (61.6)

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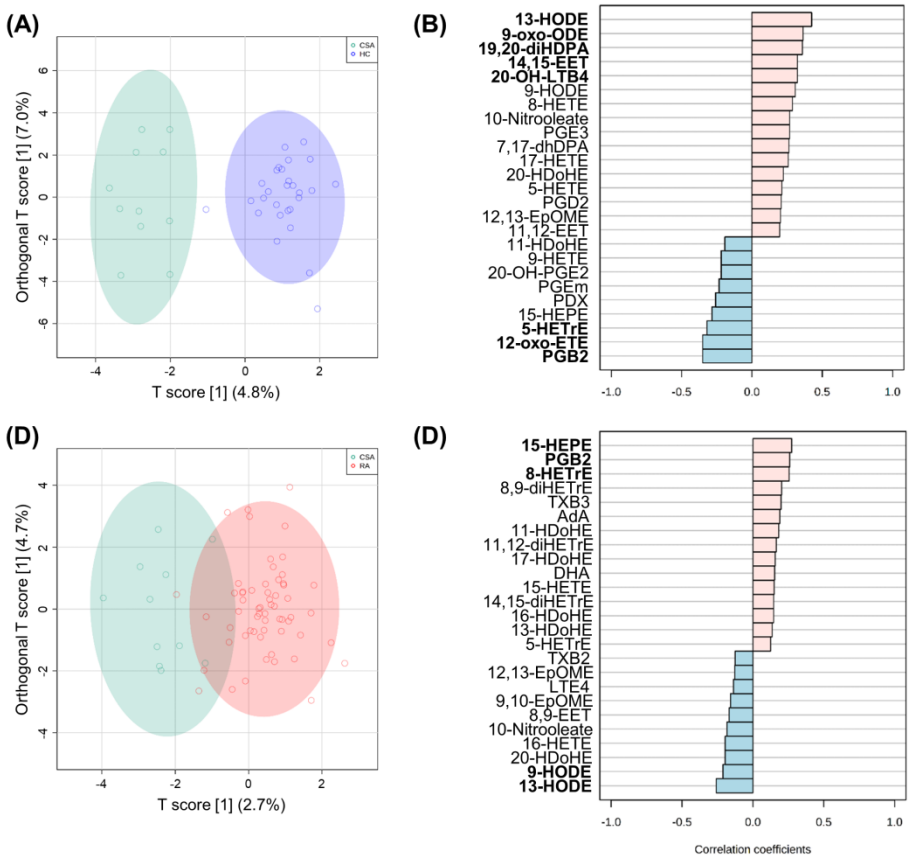

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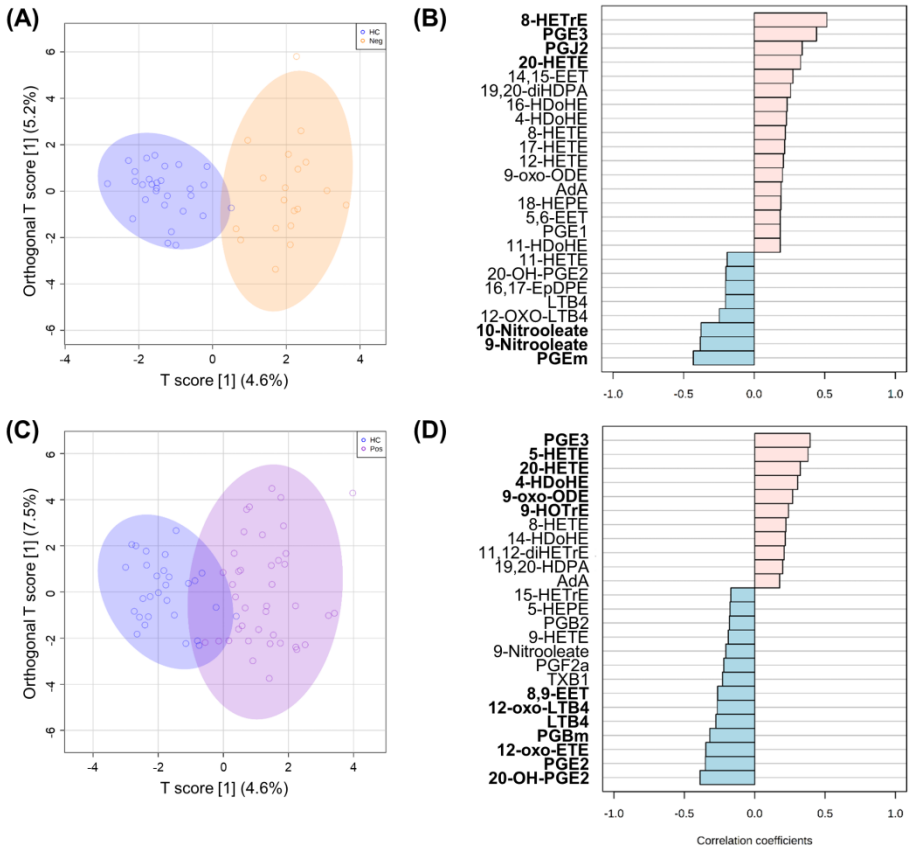

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