Characterization of synovial fluid metabolomic phenotypes of cartilage morphological changes associated with osteoarthritis

Authors: Alyssa K. Carlson, Rachel A. Rawle, Cameron W. Wallace, Ellen G. Brooks, Erik Adams, Mark C. Greenwood, Merissa Olmer, Martin K. Lotz, Brian Bothner, and Ronald K. June

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Characterization of metabolomic phenotypes of osteoarthritis morphological changes using global metabolomic profiling of human synovial fluid

Running title: Metabolomics of human synovial fluid

Alyssa K. Carlson
Rachel A. Rawle
Cameron W. Wallace
Ellen G. Brooks
Erik Adams
Mark C. Greenwood
Merissa Olmer
Martin K. Lotz
Brian Bothner
Ronald K. June
Abstract

Objective: Osteoarthritis (OA) is a multifactorial disease with etiological heterogeneity. The objective of this study was to classify OA subgroups by generating metabolic phenotypes of OA from human synovial fluid.

Design: Post mortem synovial fluids (n=75) were analyzed by high performance-liquid chromatography mass spectrometry (LC-MS) to measure changes in the global metabolome. Comparisons of healthy (grade 0), early OA (grades I-II), and late OA (grades III-IV) donor populations, as defined by macroscopic morphological assessment of the knee articular cartilages, were considered to reveal phenotypes throughout disease progression.

Results: Global metabolomic profiles in synovial fluid were distinct between healthy, early OA, and late OA donors. Pathways differentially activated among these groups included structural deterioration, glycerophospholipid metabolism, inflammation, central energy metabolism, oxidative stress, and vitamin metabolism. Within disease states (early and late OA), subgroups of donors revealed distinct phenotypes. Phenotypes of OA exhibited increased inflammation (early and late OA), oxidative stress (late OA), or structural deterioration (early and late OA) in the synovial fluid.

Conclusion: These results revealed distinct metabolic phenotypes of morphological grades of OA in human synovial fluid, provide insight into pathogenesis, represent novel biomarkers and assist in future development of personalized interventions for subgroups of OA patients.

Keywords

Osteoarthritis; metabolomics; mass spectrometry; synovial fluid; biomarkers; systems biology
Introduction

Osteoarthritis (OA) affects over 250 million individuals worldwide and is associated with an annual economic burden of at least $89.1 billion [1]. OA is the most common joint disease characterized by pain and loss of function resulting from the breakdown of the articular cartilage [2]. Pathologically, OA joints exhibit cartilage damage, osteophyte formation, subchondral bone sclerosis, and varying degrees of synovitis [3]. Altered joint metabolism, inflammation, increased joint loading, joint injury, and other factors contribute to the development of OA [4-8].

This multifactorial nature of OA contributes to a broad variation in presentation of symptoms, progression of disease, and response to treatments. In addition to the multiple contributing factors, the trajectory of OA prognosis is highly variable. Some patients rapidly progress into severe stages of disease, whereas others remain relatively stable for decades [9-12]. Similarly, the perception of pain is also variable, with some patients experiencing minimal pain despite obvious joint space narrowing and others experiencing extreme pain with minimal joint space narrowing. OA was recently described as having multiple phenotypes in which subsets of disease characteristics drive differences between subgroups of patients with distinct OA outcomes [8]. However, more data are needed to define these phenotypes. In this study, we find metabolomic phenotypes of OA.

OA heterogeneity poses many challenges for understanding pathogenesis, facilitating diagnosis and therapeutic interventions [13-15]. Defining phenotypes of OA is important for many reasons. First, this would provide insight into factors that contribute to the development of these distinct phenotypes [8]. Secondly, it would allow for development of targeted treatments for specific subgroups of OA [8]. Finally, given the heterogeneity of OA, defining phenotypes is crucial for identifying biomarkers for early diagnosis across all phenotypes or within specific subgroups once identified.

Metabolomics is a promising method for distinguishing phenotypes. Metabolomics analyzes large numbers of small-molecule intermediates [16]. Changes in the metabolome occur rapidly and reflect the overall biological response from changes in the genome, transcriptome, and proteome [17]. Metabolomic profiling generates a phenotype that characterizes functional cellular biochemistry [16, 17]. Global metabolomics is promising because it produces a global view of the metabolome with minimal bias. By focusing on all metabolite features in the sample, this analysis develops a network of pathways that illustrate metabolic perturbations with disease. Therefore, global metabolomic profiling is not only beneficial for identifying specific metabolites as potential biomarkers as demonstrated previously [18], but also providing insight into the underlying mechanism of disease.

The SF is an ultrafiltrate of the plasma containing additional molecules produced by the cells in joint tissue. SF provides lubrication between the articular cartilage surfaces and eliminates metabolic waste. The SF is in direct contact with other OA-affected tissues (i.e. articular cartilage, synovium, etc.) and will reflect local changes with disease [19]. This makes the SF a promising biofluid for phenotype identification given the heterogenous pathology of OA in the joint.

The objective of this study is to apply our established LC-MS-based global metabolomic profiling method to generate metabolic phenotypes of SF from post mortem knee joints from patients across all stages of OA (grades 0-IV) graded using the Outerbridge scale for cartilage damage. By characterizing global metabolomic profiles of early and late OA, this study seeks to (1) identify differences in metabolic pathways
throughout disease progression from healthy to late stage disease, and (2) classify patients within early and late OA into subgroups representative of potential OA phenotypes. To our knowledge, this is the first study to perform global metabolomic profiling of SF from donors with early and late stage OA to investigate metabolic perturbations throughout disease progression.

Methods

Human Synovial Fluid

Post mortem SF samples (n=75) from knee joints were used for this study under an IRB exemption with synovial fluids from the right or left knee joint chosen at random from each subject. Joints were graded based on severity of changes in the knee cartilage surfaces using the Outerbridge scoring system which grades joints from 0-IV based on macroscopic cartilage pathology [20]. The distribution of OA knees was as follows: grade 0 (n=7), grade I (n=28), grade II (n=27), grade III (n=13), and grade IV (n=4). SF samples were grouped in three cohorts: healthy controls (grade 0; n=7), early OA (grades I-II; n=55), and late OA (grades III-IV; n=17). These samples include both sexes and a variety of ages (Table 4.1). SF was frozen at -80˚C until analysis. All samples were de-identified and blinded prior to mass spectrometry and data analysis.

Donor Demographic Information

Age, sex, and OA grade were included for all donors (Table 1). Additional clinical data available for some but not all donors included donor height and weight, cause of death, pre-existing medical conditions, and history of OA. In this study, OA was based and classified based on the macroscopic assessment of the knee articular cartilages.

Global Metabolomic Profiling

Metabolites were extracted and analyzed by LC-MS analysis as previously described with slight modifications [18, 21]. SF samples were thawed on ice and centrifuged at 4˚C at 500xg for 5 minutes to eliminate cells and debris. The supernatant was resuspended in 50:50 water:acetonitrile at -20˚C for 30 minutes. The sample was vortexed for 3 minutes and centrifuged at 16100xg for 5 minutes at 4˚C. The supernatant was completely evaporated in a vacuum concentrator for ~2 hours, and the dried pellet was resuspended in 500 µL of acetone to precipitate proteins at 4°C for 30 minutes. The sample was then centrifuged at 16100xg for 5 minutes. The supernatant was completely evaporated by speedvac, and the pellet was resuspended in mass spectrometry grade 50:50 water:acetonitrile. Metabolite extracts were analyzed in positive mode using an Agilent 1290 UPLC system connected to an Agilent 6538 Q-TOF mass spectrometer (Agilent Santa Clara, CA). Metabolites were chromatographically separated on a Cogent Diamond Hydride HILIC 150x2.1 mm column (MicroSolv, Eatontown, NJ) using an optimized normal phase gradient elution method, and spectra were processed as previously described [18].

Statistical Methods and Analysis
Global metabolomic profiling generates a large multivariate dataset of thousands of mass-to-charge ratios (m/z) and their corresponding peak intensities [17]. The dataset was reduced by removing metabolite features (m/z values) with median intensity values of zero across all experimental groups. All data analysis steps were completed using MetaboAnalyst unless otherwise noted [22]. Data were log transformed using the base-2 logarithm (log2) to correct for non-normal distributions and standardized (mean centered divided by standard deviation). Standardized data were used for all analyses unless indicated otherwise.

All statistical tests used an a priori significance level of 0.05, and false discovery rate (FDR) corrections were applied when performing multiple comparisons per metabolite between groups [23]. The Kolomogorov-Smirnov test (KS-test) was used in MATLAB (MathWorks, Inc. Natick, MA) to compare cumulative median metabolite distributions between cohorts. This nonparametric test does not require assumptions about the underlying distributions and therefore is useful for metabolomics datasets that typically contain non-normal distributions. Specific differences between multiple groups were determined using analysis of variance (ANOVA) F-tests. Two-tailed Student’s t-tests examined specific pairwise differences. Differentially regulated metabolites between two groups were visualized by volcano plot to assess both significance and magnitude of change simultaneously. Metabolite features with a p-value (FDR corrected) less than 0.05 and greater than twofold change were considered both statistically significant and biologically important in these analyses.

Multivariate methods assessed variations in the metabolomic datasets. Unsupervised hierarchical clustering analysis (HCA) based on Euclidean distance and average linkage separated samples into groups of similar abundance patterns [24]. HCA assessed subgroups of donors exhibiting distinct OA phenotypes. HCA is visualized using heatmaps, known as a clustergrams, to analyze the overall metabolomic profiles. Clustergrams reveal both clusters of co-regulated metabolite features and the relative similarity between experimental groups [24]. Principal component analysis (PCA) is another unsupervised method used to analyze metabolomics data. PCA orthogonally transforms a set of observations into principal components that each represent a fraction of the overall variance within the dataset. Partial least squares-discriminant analysis (PLS-DA) is a supervised classification method that reveals the underlying source of distinction between known groups. PLS-DA scores each variable in each component indicating how important that variable was in contributing to the separation.

Metabolite features (m/z values) were matched to known metabolite identities and mapped to relevant pathways using the metabolite library and pathway enrichment tool, mummichog [25]. Mummichog predicts a network of functional activity based on the projection of detected metabolite features onto local pathways. Pathway libraries MFN and Biocyc were used for compound identification and pathway enrichment (mass tolerance: 0.1 ppm; positive mode). Pathways reported were significant by pathway overrepresentation analysis with an FDR-adjusted p-value less than 0.05.

To determine if cohorts or phenotypes were associated with any confounding variables, Student’s t-tests, logistic regression, and post hoc Chi Squared tests were employed to assess differences between groups based on the available clinical data including age, sex, and BMI were assessed between both groups and phenotypes.
**Results**

**Differences in Global Metabolomes between Healthy Knee Donors, Early and Late OA**

A total of 9903 metabolite features were detected in SF from donors with grade 0-IV OA. This dataset was refined to 1362 detected features by removing features with a median intensity of zero. ANOVA identified 39 differentially expressed metabolite features between healthy, early OA, and late OA SF (FDR-corrected p<0.05).

We first explored whether the global metabolomes were distinct between healthy, early, and late OA cohorts, as assessed by morphological grading of the knee cartilages. To examine differences between cohorts, three pairwise comparisons were made: healthy vs. early OA; healthy vs. late OA; and early vs. late OA. Between-group differences in global metabolomes were assessed using KS-tests, and this revealed significant differences between all pairwise comparisons \((p_{\text{KS}}<0.01\); Fig. 1). Taken together, these results indicate that the global metabolomes are significantly different between healthy, early, and late OA.

To visualize differences in metabolomic profiles and identify specific metabolite features with the greatest discriminative capabilities for separating cohorts, supervised PLS-DA was used. PLS-DA shows clear separation of healthy donors from disease donors, and minimal overlap between early and late OA donors (Fig. 1). By examining VIP scores, we found metabolite features that contribute the most to distinguishing between cohorts and are strong candidates for potential metabolite biomarkers (Supplemental Table 1).

Volcano plot analysis examined pairwise differences using both significance and fold changes (Fig. 1). 188 metabolite features were significantly different between healthy and early OA SF with 162 lower and 26 higher in early OA. 64 metabolite features were significantly different between healthy and late OA SF, with 39 decreased and 25 increased in OA. Within OA, 191 metabolite features were significantly different between early and late stage disease, with 9 lower and 182 higher in late stage disease. To infer metabolic activity, significantly different metabolite features were enriched using mummichog’s pathway analysis (Supplemental Table 2) presented below.

[Suggested location for Figure 1]

**Co-Regulated Metabolites Map to Differentially Regulated Metabolic Pathways with Disease**

Early and late OA profiles were distinct from healthy SF (Fig. 2). Unsupervised HCA of healthy and diseased SF showed that the early and late OA profiles were more similar to one another than healthy SF (Supplemental Fig. 1). From the clustering, six groups of co-regulated metabolites were identified based on consistency of clustered distance and assessed for enriched pathways associated with stage of OA. (Supplemental Table 3).

[Suggested location for Figure 2]
Cluster 1 contained 38 metabolite features that decreased throughout disease progression. These mapped to 14 of the previously identified enriched pathways (Supplemental Table 2) including amino acid metabolism (glycine, serine, alanine, threonine, lysine, arginine, and proline), the urea cycle, phosphatidylinositol phosphate metabolism, the carnitine shuttle, vitamin metabolism (B5 and C), and porphyrin metabolism (Supplemental Table 3).

Cluster 2 contained 135 metabolite features that decreased in OA compared to healthy SF. These metabolite features mapped to 20 enriched pathways including vitamin metabolism (E, C, B3, and B6), phosphatidylinositol phosphate metabolism, glutathione metabolism, leukotriene metabolism, butanoate metabolism, amino acid metabolism (similar to cluster 1 with the addition of tryptophan and histidine metabolism), and the carnitine shuttle (Supplemental Table 3).

Cluster 3 contained 188 metabolite features lowest in early OA compared to healthy and late OA. These mapped to 14 enriched pathways including porphyrin metabolism, galactose metabolism, fructose and mannose metabolism, vitamin metabolism (B5, B3, E), methionine and cysteine metabolism, N-glycan degradation, glycerophospholipid metabolism, and leukotriene metabolism (Supplemental Table 3).

Clusters 4-6 contained metabolism features higher in abundance in OA cohorts. Cluster 4 contained 64 metabolite features highest in late OA. These metabolite features mapped to 8 enriched pathways including keratan sulfate degradation, N-glycan degradation, fructose and mannose metabolism, leukotriene metabolism, and butanoate metabolism (Supplemental Table 3).

Cluster 5 contained 177 metabolite features with the greatest abundance in early and late OA SF. These mapped to 36 enriched pathways including amino acid metabolism (histidine, glycine, serine, alanine, threonine, tyrosine, glutamate, aspartate, valine, leucine, isoleucine, aspartate, asparagine, lysine, and tryptophan) urea cycle, keratan sulfate degradation, fatty acid metabolism, glycerophospholipid and glycosphingolipid metabolism, the TCA cycle, N-glycan metabolism, glutathione metabolism, tryptophan metabolism, and vitamin C metabolism (Supplemental Table 3).

Cluster 6 contained 60 metabolite features highest in abundance in early OA. These mapped to 33 enriched pathways included glycolysis and gluconeogenesis, the pentose phosphate pathway, sialic acid metabolism, N-glycan degradation, keratan sulfate degradation, tryptophan metabolism, glutathione metabolism, and vitamin B3 metabolism (Supplemental Table 3).

Unsupervised Clustering Suggests OA Phenotypes within Early and Late OA

To examine OA phenotypes, early and late OA were further analyzed by unsupervised HCA. In early OA, this revealed two clusters of donors, E1 and E2, containing 33 and 22 donors, respectively (Fig. 3A). There were 379 metabolite features differentially expressed between phenotypes E1 and E2 (FDR-corrected p<0.05). HCA of late OA also showed two distinct clusters of donors, L1 and L2, that may be representative of late OA phenotypes (Fig. 4A). 11 donors clustered in phenotype L1, and 6 donors clustered in phenotype L2. There were 187 differentially expressed metabolite features between phenotypes L1 and L2 (FDR-corrected p<0.05).

PCA, an unsupervised method, was used to examine the separation between potential phenotypes. Plotting the PCA scores of early OA donors shows the separation
between phenotypes, with PC1 and PC2 accounting for 27.1% of the overall variance (Fig. 3B). Separation of late OA donors into two distinct phenotypes is also supported by PCA, with PC1 and PC2 associated with 35.8% of the overall variance (Fig. 4B). PLS-DA, a supervised method, further supports distinct phenotypes within early and late OA as indicated by separation between E1 and E2 donors and L1 and L2 donors (Fig. 3C, 4C). Taken together, HCA, PCA, and PLS-DA support four distinct subgroups of donors in early and late stage disease that may be representative of metabolic OA phenotypes.

Distinct pathways were represented in the various phenotypes as determined by analyzing differentially expressed metabolites for enriched pathways. Volcano plot analysis found 254 metabolite features differentially expressed between the early OA phenotypes and 158 metabolite features differentially expressed between late OA phenotypes (Fig. 3D, 4D). Enrichment analysis was then employed to map differentially expressed metabolite features to pathways (Tables 2-3).

A subgroup of donors at each stage of OA (E2 and L2) exhibited evidence of glycosaminoglycan degradation and structural deterioration. E2 was associated with 25 significantly enriched pathways, including glycosaminoglycan degradation, sialic acid and N-glycan metabolism, tryptophan metabolism, and ascorbate metabolism (Table 2). L2 was associated with 4 significantly enriched pathways including keratan sulfate and N-glycan degradation, sialic acid metabolism, and galactose metabolism (Table 3).

The remaining OA phenotypes, E1 and L1, were associated with increased inflammation. Phenotype E1 was associated 14 significantly enriched pathways including metabolism of butanoate and leukotrienes—both of which play a role in inflammation (Table 2). L1 was associated with 30 significantly enriched pathways including arachidonic acid metabolism and leukotriene metabolism (Table 3). Phenotype L1 was also associated with glutathione metabolism, which may be suggestive of altered levels of oxidative stress (Table 3). Please see the Supplemental Data including figures S2-S5 for additional discussion of metabolomic phenotypes and associated pathways.

Confounding variables

We evaluated if differences in metabolomic profiles between healthy, early, and late OA were associated with age, sex, or BMI as possible covariates (Table 1). The ages and BMI of the healthy, early, and late OA cohorts were calculated and analyzed by Student’s t-test. Male:female ratios were analyzed by logistic regression and chi-squared tests. There were significant differences in ages between healthy, early, and late OA comparisons with early OA younger than late OA (p<0.05). However, there was little to no evidence of differences in BMI or male:female ratios (p>0.05). Therefore, any differences noted between cohorts besides being due to the presence or absence of OA may be associated with aging.
Discussion

To our knowledge, this is the first study to use LC-MS-based global metabolomic profiling of human SF to study OA phenotypes. The present study used an accurate morphological grading system [20] to quantify changes in the knee cartilages and used these scores to separate groups of patients based on the severity of changes. While several studies used metabolomics to analyze OA in various fluids [26-30], only a single prior study used a targeted approach based on 186 metabolites for this same goal and found that acylcarnitine and free carnitine levels were significantly different between subgroups [9]. In contrast, the global approach used here removes bias by not excluding metabolites a priori. By focusing on all detected metabolites, this study produced a network of pathways perturbed with OA. These data provide greater understanding of disease pathogenesis, therapeutic targets, and insight for biomarker discovery.

1362 metabolite features were detected in human SF by LC-MS analysis, and global metabolomic profiles were generated for healthy, early OA, and late OA SF. OA was associated with altered extracellular matrix component metabolism (glucosamine and galactosamine biosynthesis, ascorbate metabolism, keratin sulfate metabolism, and N-glycan metabolism), amino acid metabolism, fatty acid and lipid metabolism (glycosphingolipid and glycerophospholipid metabolism, the carnitine shuttle), inflammation (leukotriene metabolism), central energy metabolism (glycolysis and gluconeogenesis, the TCA cycle), oxidative stress (vitamin E, glutathione metabolism), and vitamin metabolism (C, E, B1, B3, B6, and B9).

Structural Deterioration

Diseased SF exhibited greater evidence of tissue damage compared to healthy SF. Keratan sulfate degradation, N-glycan degradation, sialic acid metabolism, and ascorbate metabolism were altered with OA. Keratan sulfate, chondroitin sulfate, and heparin sulfate are glycosaminoglycans (GAGs) that function as building blocks of articular cartilage. Their presence in the SF typically indicates increased cartilage turnover [31]. In OA, the articular cartilage is degraded reducing GAG content [32, 33]. These data are consistent with both synthesis and degradation of GAGs in the SF of both early and late stage donors. OA cartilage also exhibits collagen damage [34]. We identified hydroxyproline as a metabolite with the greatest ability in distinguishing early from late OA. Sialic acids and N-glycans are also important components of lubricin, a mucinous glycoprotein that lines the cartilage surfaces and acts as a lubricant [35]. These pathways were perturbed in diseased SF suggesting that the SF function in lubrication is compromised.

Vitamin Metabolism and Oxidative Stress

The physiological significance of vitamins E, B5, and C may relate to their roles as antioxidants to counteract the increased oxidative stress in the joint during OA [36]. Additional results from diseased SF suggest oxidative stress included glutathione metabolism. Furthermore, vitamin B3 is also a required cofactor for the production of nitric oxide (NO) by NO synthase. NO has been shown to have both catabolic and protective effects in OA by modulating a variety of inflammatory and anti-inflammatory mediators [37]. Thus, altered vitamin B3 metabolism may drive NO-related changes
during OA pathogenesis. The altered antioxidant metabolism exhibited in OA SF in this study further supports a role for oxidative stress in the development of OA [38].

Phenotypes of OA in Synovial Fluid from Early and Late Stage Disease

OA is a heterogeneous disease with varying presentation. Because of this, we investigated if distinct metabolic phenotypes existed within OA SF (i.e. early vs. late or within each). We identified two distinct phenotypes in early OA—E1 and E2 and two in late OA—L1 and L2. Between E1 and L1, 60.2% of the metabolites were the same, and between E2 and L2, 55.3% of metabolites were the same (Supplemental Figures 6-7).

Both inflammation and structural degradation are involved in OA. In early OA, a subset of donors (E1) was associated with greater inflammation, while the remaining donors (E2) exhibited evidence of greater structural deterioration. Similarly, in late OA, phenotype L1 was associated with inflammation and oxidative stress while L2 was associated with structural deterioration products. These data suggest that inflammation and degradation may not be as closely correlated as expected. Furthermore, because of the close relationship between inflammation and pain [39, 40], the inflammatory phenotypes E1 and L1 may be associated with increased pain.

As in late OA phenotype L1, oxidative stress and inflammation have been extensively studied for their role in OA pathogenesis, yet both contribute to OA by promoting cartilage degradation [41]. Despite this, phenotype L1 exhibited reduced structural deterioration products in the SF compared to L2. This suggests a structural damage phenotype at both early and late stage disease, an inflammatory phenotype in early OA, and an inflammatory and oxidative stress phenotype at late stage disease. Overall, these findings further support the heterogeneous nature of OA and suggest stage-dependent phenotypes that may drive differences in symptoms (Supplementary Discussion and Figures).

Limitations

This study has limitations and also opens opportunities for future research. First, we used a morphological definition of OA based on macroscopic changes on the knee cartilages. Future studies are necessary to confirm that the candidate metabolic markers revealed in the present study are able to distinguish patient cohorts that area classified on the basis of a clinical or imaging-based definition of OA. Also, the sample size for this study was relatively small (n=75). Some cohorts, such as healthy (grade=0), consisted of only 6 samples, whereas early OA contained 55. With a small sample size, it is unlikely that all metabolic phenotypes were represented. Furthermore, this sample did not contain complete clinical information. Age and sex were provided for all donors, BMI was provided for most, but others lacked cause of death, prior medical history, and/or ethnicity. Importantly, age was identified as a potential confounder in this study. Age-matching within experimental cohorts would avoid potential confounding by age. This was a cross-sectional study, and future research may improve upon this using a longitudinal study design. Lastly, this study analyzed post-mortem SF. However, studies have shown that metabolites in SF remain stable post-mortem [42, 43]. Targeting specific inflammatory metabolites or degradation products may yield further insight into OA phenotypes, and expanded sample sizes may allow detection of OA biomarkers.
Conclusions

This is the first study to generate global metabolomic profiles of early and late OA SF and identify OA phenotypes within early and late OA cohorts. The identified pathways in early and late OA provide insight into disease progression and provide several molecular pathways to further investigate as biomarkers of OA and as targets for drug discovery. Furthermore, the identification of specific metabolomic phenotypes in OA supports the heterogeneity of disease. Expansion of this study will identify candidate biomarkers of early and late OA in human SF and within OA phenotypes.

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Author Contributions

Conception and design: AKC, ML, RKJ
Analysis and interpretation of the data: all authors.
Drafting of the article: AKC, RKJ
Critical revision of the article for important intellectual content: all authors
Final approval of the article: all authors
Statistical expertise: MCG

RKJ (rjune@montana.edu) is responsible for the integrity of the research from inception to finished manuscript.

Conflict of Interest

RKJ owns stock in Beartooth Biotech, which was not involved in this study.

References


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Figure 1. Global metabolomes are distinct between cohorts. (A-C) The cumulative distribution of metabolites between groups were distinct from one another. KS-tests comparing the median metabolite intensity distributions between groups revealed significantly ($p_{KS}<0.01$) different metabolomic profiles. Mirrored metabolite distributions display differences between groups. (D-F) PLS-DA displayed differences in metabolomic profiles of between groups, revealing clear separation between healthy and OA donors and some separation between early and late OA donors. The first two components are plotted against one another with their contribution to the overall variance. 95% confidence ellipses illustrate class separation. (G-I) Volcano plot analysis between groups reveal metabolite features upregulated and downregulated by p-value and fold change analysis. Dashed lines indicate the p-value threshold of 0.05 (horizontal) and fold change threshold of 2 (vertical). The upper right and left quadrants contain significant ($p<0.05$) upregulated and downregulated features with a fold change greater than twofold. Metabolite features in the upper right and left quadrants were assessed for enriched pathways reported in Supplemental Table 2.
Figure 2. Metabolic changes in SF during early and late stage OA. Clustergram of median global metabolomic profiles of early and late OA SF normalized to healthy SF display patterns of metabolite expression with disease. Arbitrarily selected clusters of co-regulated metabolite features are boxed in black and enriched for relevant pathways in Supplemental Table 3.
Figure 3. Phenotypes in early OA synovial fluid. (A) Unsupervised HCA of all early OA donors. Two clusters of donors were identified and labeled as phenotype E1 (red) and phenotype E2 (blue). E1 contained 33 donors and E2 contained 22. Line length represents Euclidean distances between donors and clusters. (B) Unsupervised PCA of all early OA donors reveals separation of early OA phenotypes. The first two components are associated with 27.1% of the variation between phenotypes. (E1=red; E2=blue). (C) Supervised PLS-DA further illustrated the separation between phenotypes (E1=red; E2=blue) with PC1 and PC2 accounting for 24.3% of the variance. (D) Volcano plot visualization of differentially regulated metabolite features by Student’s t-test significance and fold change analysis (E1:E2). The p-value threshold is represented by the horizontal dashed line (FDR-corrected p<0.05), and the vertical lines represent the fold change threshold (greater than twofold change). Metabolite features in the upper right and left quadrants (p<0.05 and greater than twofold change) were enriched for relevant pathways reported in Table 3, with the full list of perturbed pathways in Supplemental Table 2.
Figure 4. Phenotypes in late OA synovial fluid. (A) Unsupervised HCA of all late OA donors. Two clusters of donors were identified and labeled as phenotype L1 (red) and phenotype L2 (blue). L1 contained 11 donors, and L2 contained 6 donors. Line length represents Euclidean distances between donors and clusters. (B) Unsupervised PCA of all early OA donors reveals separation of early OA phenotypes. The first two PCs are associated with 35.8% of the variation between phenotypes. (L1=red; L2=blue). (C) Supervised PLS-DA further illustrated the separation between phenotypes (L1=red; L2=blue), with component 1 and component 2 accounting for 34% of the overall variance. (D) Volcano plot visualization of differentially regulated metabolite features by Student’s t-test significance and fold change analysis (L1:L2). The p-value threshold is represented by the horizontal dashed line (FDR-corrected p<0.05) and the vertical lines represent the fold change threshold (greater than twofold change). Metabolite features in the upper right and left quadrants were assessed for enriched pathways reported in Table 4.6.
Figure 5 Metabolomic phenotypes of osteoarthritis. Cluster analysis of our data revealed distinct metabolic phenotypes (n=75). Within Outerbridge grades I and II (early OA), metabolite features clustered into an E1 phenotype associated with inflammatory pathways and an E2 phenotype associated with structural degradation pathways. Within Outerbridge grades III and IV, metabolites clustered into an L1 phenotype associated with oxidative stress and inflammation and an L2 phenotype associated with structural degradation. These data emphasize the heterogeneity of OA. Sketch of cartilage defects adapted with permission from Lasanianos and Kanakaris Traumatic and Orthopaedic Classifications 2014.
Table 1. Descriptive statistics for donor population. Descriptive statistics of donor population for each cohort including age, sex (as male % population), and BMI. All means are reported as mean +/- standard deviation. BMI was unavailable for some donors (BMI=body mass index).

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Table 2. Perturbed pathways in early OA phenotypes. Pathway enrichment of significant metabolite features upregulated and downregulated (p<0.05; greater than twofold change) with early OA phenotypes in Fig. 4.5F volcano plot analysis comparing phenotype E1 to phenotype E2 (E1:E2 fold change ratio). Significant metabolite features greater in abundance in the upper right quadrant of the volcano plot (higher in E1 compared to E2) in Fig. 3D were enriched to reveal corresponding upregulated pathways. Significant metabolite features reduced in abundance in the upper left quadrant of the volcano plot (lower in E1 compared to E2) in Fig. 3D were enriched to reveal corresponding downregulated pathways. Pathways are reported with the total metabolites in the pathway, the total detected metabolites in the pathway, and total significant (by volcano plot analysis) metabolites within that pathway. Only pathways with an FDR-corrected p-value less than 0.05 are reported. The full list of pathways identified in Fig. 3D volcano plot is reported in Supplemental Table 2.

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Table 3. Perturbed pathways in late OA phenotypes. Pathway enrichment of significant metabolite features upregulated and downregulated (p<0.05; greater than twofold change) with late OA phenotypes in Fig. 4D volcano plot analysis comparing phenotype L1 to phenotype L2 (L1:L2 fold change ratio). Significant metabolite features greater in abundance in the upper right quadrant of the volcano plot (higher in L1 compared to L2) in Fig. 4D were enriched to reveal corresponding upregulated pathways. Significant metabolite features reduced in abundance in the upper left quadrant of the volcano plot (lower in L1 compared to L2) in Fig. 4D were enriched to reveal corresponding downregulated pathways. Pathways are reported with the total metabolites in the pathway, the total detected metabolites within the pathway, and total significant (by volcano plot analysis) metabolites within that pathway. Only pathways with an FDR-corrected p-value less than 0.05 are reported. The full list of pathways identified in Fig. 4D volcano plot is reported in Supplemental Table 2.

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Supplemental Data Carlson et al

Supplemental Figures, Tables, and Discussion

Supplemental Figure 1

Distinct global metabolomic profiles of healthy, early OA, and late OA SF. Clustergram of median global metabolomic profiles of healthy, early OA, and late OA SF displays patterns of metabolite expression. HCA illustrates that early and late OA SF were more similar than healthy SF.

Please see separate files for supplemental tables.

Supplemental Table 1. Discriminative metabolites identified by PLS-DA for classifying SF as healthy, early OA, late OA, phenotype E1, phenotype E2, phenotype L1, or phenotype L2. Full list of discriminative metabolite features with VIP scores for the top two components for each PLS-DA plot in Figure 1D-F, 3C, and 4C. Potential metabolite identities are reported as compound matches using mummichog and the Biocyc pathway library.
**Supplemental Table 2.** Distinct pathways are perturbed between groups. Full pathway enrichment of volcano plots in Fig. 1G-I, 3D, and 4.D. Pathways are reported for the pathway library, MFN.

**Supplemental Table 3.** Full list of all pathways identified for each cluster in Figure 2 clustergram.

**Supplemental Table 4.** Analysis comparing metabolites from E1 and L1 metabolomic phenotypes

**Supplemental Table 5.** Analysis comparing metabolites from E2 and L2 metabolomic phenotypes

**Supplemental Discussion (including Supplemental Figures 2-7)**

Based on the detected metabolite features, pathways relating to extracellular matrix (ECM) structural components, lipid metabolism, inflammation, central energy metabolism, oxidative stress, and vitamin metabolism were altered in both early and late OA compared to healthy controls.

**Structural Deterioration.**

Diseased SF exhibited greater evidence of tissue damage compared to healthy SF. Keratan sulfate degradation, N-glycan degradation, sialic acid metabolism, and ascorbate metabolism were altered with disease (p<0.05). Heparan sulfate and chondroitin sulfate degradation were also altered with diseased, although they were marginally significant (p=0.06). Keratan sulfate, chondroitin sulfate, and heparin sulfate are glycosaminoglycans (GAGs) that function as the building blocks of the articular cartilage. Their presence in the SF is typically indicative of increased catabolism of GAGs [1]. In OA, the articular cartilage is degraded and thus, decreased GAG content [2, 3]. These pathways support cartilage degeneration as a
hallmark of OA, as shown by increased GAG degradation products in the SF of both early and late stage disease.

OA cartilage also exhibits collagen damage [4]. Ascorbate (vitamin C) is required to convert proline to hydroxyproline, a required component in collagen [5]. In this cross-sectional study, ascorbate metabolism increased over the course of disease, consistent with numerous studies showing increased collagen synthesis in OA [6, 7]. N-glycans also function to provide support to the extracellular matrix (ECM) of the articular cartilage. One study found that alterations in N-glycan concentrations were associated with early OA before the development of histological changes [8]. Our findings support altered N-glycan metabolism in both early and late stage disease. These structural components are typically studied for their roles in the cartilage ECM, and thus their increased presence in the SF suggests that structural deterioration is occurring in both early and late stage disease.

**Glycerophospholipids and Inflammatory Lipid Mediators.**

Glycerophospholipid metabolism was altered in both early OA and late OA in comparison to healthy SF. Clustergram analysis (Figure 2) revealed a cluster of co-regulated metabolites higher in diseased SF compared to healthy SF that mapped to glycerophospholipid metabolism and glycosphingolipid biosynthesis. Volcano plot analysis revealed that metabolites lower in OA (both early and late OA) in comparison to healthy also mapped to glycerophospholipid metabolism. Lipidomic analyses of OA SF have previously found elevated levels of glycerophospholipids in late OA in comparison to healthy SF [9, 10]. Glycerophospholipids are important structural components of the lipid bilayer of cell membranes and have roles in signal transduction and transport. Important to OA pathogenesis, glycerophospholipids serve as the upstream source of arachidonic acid, the precursor for prostaglandin and leukotrienes [11]. Prostaglandins and leukotrienes are eicosanoid lipid
mediators released from arachidonic acid to regulate inflammatory processes. Variable levels of prostaglandins and leukotrienes have been measured in human OA synovial membrane explants and OA osteoblasts, and many studies have demonstrated the role of eicosanoids in structural degradation in OA [12]. Furthermore, levels of prostaglandins can distinguish between two types of patients with OA [12]. In this study, prostaglandin biosynthesis and leukotriene metabolism were identified in concurrence with glycerophospholipid metabolism in all analyses. Taken together, these findings suggest that altered glycerophospholipid metabolism may regulate inflammation in diseased SF. Further studies are needed to determine the role of glycerophospholipids in OA pathogenesis.

**Central Energy Metabolism**

Coenzyme A catabolism, the TCA cycle, the carnitine shuttle, and vitamin B5 biosynthesis were reduced in diseased SF compared to healthy controls, suggesting altered central energy metabolism with disease. Here, coenzyme A catabolism was downregulated in early OA in comparison to healthy SF, vitamin B5 biosynthesis was downregulated in late OA in comparison to healthy SF, and the TCA cycle and the carnitine shuttle were downregulated in both early and late OA in comparison to healthy SF. Vitamin B5, also known as pantothenic acid, is required to synthesize coenzyme A, an important cofactor in the production of acetyl CoA for the start of the tricarboxylic acid (TCA) cycle. Coenzyme A also plays an important role in the oxidation of fatty acids, in which it acts as a carrier from the cytoplasm to the mitochondria. Similarly, the carnitine shuttle transports long-chain fatty acids across the inner mitochondrial membrane for fatty acid oxidation. The oxidation of fatty acids produces energy for ATP generation by producing acetyl-CoA for entry into the TCA cycle.

Importantly, one cluster of co-regulated metabolites was reduced over the course of disease and mapped to the TCA cycle (Fig. 2). This suggests that altered energy metabolism
may be associated with disease severity. The joint is already a hypoxic environment, and oxygen levels are further reduced in the joint cavity of OA as there is reduced oxygen delivery to the SF due to fibrosis of the joint capsule and subchondral bone sclerosis [13-15]. During hypoxia, hypoxia-inducible factors (HIFs) are activated and alter energy homeostasis by stimulating anaerobic glycolysis and inhibiting mitochondrial aerobic metabolism, including the TCA cycle [16]. The results of this study suggest the products of altered energy metabolism are present in diseased SF, with greater aberrant energy metabolism activity associated with disease progression.

**Oxidative Stress**

The hypoxic environment in the joint is associated with increased reactive oxygen species (ROS) as the main source of oxidative stress [17]. In this study, many pathways relevant to oxidative stress in OA were altered in diseased SF compared to healthy. In particular, many antioxidants were reduced with disease including vitamin E, vitamin A, and glutathione metabolism. Vitamin A metabolism was reduced in early OA compared to healthy SF. Both vitamin E metabolism and glutathione metabolism were reduced over the course of disease, while ascorbate (vitamin C) metabolism, another antioxidant, was increased with disease. One study reported a decrease in levels of ascorbate and glutathione in OA SF, although this decrease may be associated with age-related oxidative stress [18]. Another study found that of the antioxidants measured, only vitamin E was significantly reduced in OA SF, with no significant difference in the levels of ascorbate or glutathione compared to control SF [19].

Antioxidants have the capacity to modulate oxidative stress by neutralizing ROS in the joints. A greater understanding of antioxidant levels during OA progression may provide insight into slowing or ameliorating oxidative stress-induced damage in the joint. The altered
antioxidant metabolism exhibited in diseased SF in this study further supports oxidative stress in the development of OA [20].

**Vitamin Metabolism.**

Vitamin (B6, B5, B9, B3, B1, E, C and A) metabolism was altered in diseased SF. Vitamin C (ascorbate) metabolism, as described above, was increased with disease (e.g. increased in both early and late OA compared to healthy controls). Metabolism of vitamin B6, B5, B9, B1, and E were reduced in early and late OA in comparison to healthy SF. Vitamin B3 and A metabolism were reduced in early OA in comparison to healthy controls. Metabolism of vitamin B5, B3, and B9 were higher in late OA than early OA SF. The physiological significance of vitamin E, B5, A, and C were described above for their roles as antioxidants. Vitamin C (ascorbate) is not only an antioxidant, but also plays an important role in the synthesis of collagen, as mentioned above. Therefore, vitamin E, B5, A, and C metabolism may be implicated in OA pathogenesis for their roles in oxidative stress and ECM maintenance.

Vitamin B6 (pyridoxine) has been frequently studied in the context of rheumatoid arthritis (RA) [21]. RA is a chronic inflammatory arthritis in which reduced vitamin B6 levels have been associated with inflammation [21-24]. Vitamin B6 metabolite pyridoxyl 5’-phosphate has been associated with increased levels of pro-inflammatory cytokines and is thought to act as a coenzyme for the production of pro-inflammatory cytokines and mediators [25]. The results herein may suggest that OA is also associated with reduced vitamin B6 similar to RA, although further studies are needed to determine its role in inflammation and OA.

Vitamin B3 (nicotinate and nicotinamide) metabolism was reduced in early OA compared to healthy SF, but higher in late OA than early OA. Vitamin B3 (niacin) is converted to nicotinamide, the pyrimidine ring of nicotinamide adenine dinucleotide (NAD) and nicotinamide
adenine dinucleotide phosphate (NADPH), which are important cofactors in oxidative phosphorylation and the pentose phosphate pathway. Similarly, vitamin B1 (thiamin) was reduced with disease. Vitamin B1 is a cofactor for the conversion of pyruvate in glycolysis, the conversion of alpha-ketoglutarate in the TCA cycle, and the transketolase reaction in the pentose phosphate pathway [26]. Therefore, reduced vitamin B3 and B1 metabolism further support altered energy metabolism as discussed above. Vitamin B3 is also a required cofactor for the production of nitric oxide (NO) by nitric oxide synthase. NO has been shown to have both catabolic and protective effects in OA by modulating a variety of inflammatory and anti-inflammatory mediators [27]. Thus, altered vitamin B3 metabolism may also support nitric oxide’s role in OA pathogenesis.

Vitamin B9 (folate) is most notable for its role in bone health. Vitamin B9 plays an important role in detoxifying a methionine metabolite, homocysteine. Homocysteine, in excess, promotes osteoporosis and atherosclerosis and altered collagen synthesis [28]. Reduced vitamin B9 (as shown in this study) may lead to increase homocysteine in the joint cavity, resulting in the deleterious effects of homocysteine. Additional studies are needed to further elucidate homocysteine’s role in OA, although the results herein suggest that vitamin B9 may be implicated in OA pathogenesis.

Changes from Early to Late Stage OA

Despite many similarly regulated pathways in early and late OA, these data show that the metabolomic profile of early OA is distinct from late stage disease (Supplemental Figure 1, above). Focusing on volcano plot analysis of late OA compared to early OA (Figure 1I), few pathways were downregulated with late stage disease. Pathways pertaining to inflammation (leukotriene, arachidonic acid, and glycerophospholipid metabolism), altered central energy metabolism (CoA catabolism, and vitamin B3 and B5 metabolism), oxidative stress (vitamin E
metabolism), and structural degradation (N-glycan degradation and sialic acid metabolism) were all upregulated in late OA compared to early OA. Interestingly, the majority of these differentially regulated pathways were also identified within the cluster of co-regulated metabolites lower in disease (both early and late OA) compared to healthy SF. This suggests that in early OA, there may be an initial reduction in activity of these pathways in comparison to healthy SF, but these slowly regain limited activity by late stage disease.

Supplemental Figure 2

Metabolomic phenotypes in early OA. Using hierarchical clustering, we found two phenotypes (E1 and E2) in early OA.
Supplemental Figure 3

Pathways associated with phenotypes of early OA. Based on volcano plot analysis, metabolite features associated with early OA were used to identify pathways associated with metabolomic phenotypes E1 and E2.

Metabolomic Phenotypes of OA in Synovial Fluid from Early and Late Stage Disease

OA is a heterogeneous disease, known to present in a variety of phenotypes (e.g. erosive hand OA compared with idiopathic knee OA). Because of this, we investigated if distinct metabolic phenotypes existed within OA SF aside of disease state (i.e. early vs. late) using HCA to identify clusters of donors within early and late OA. We identified two distinct phenotypes in early OA, labeled as E1 and E2 (Supplemental Figure 2-3). Phenotype E2 exhibited greater chondroitin sulfate, heparan sulfate, N-glycan, and keratan sulfate degradation, sialic acid metabolism, and ascorbate metabolism than phenotype E1. Despite the observations of similar macroscopic damage between phenotypes E1 and E2 (e.g. no differences in Outerbridge grades), these pathways suggest that phenotype E2 had greater structural degradation products in the SF. The second phenotype, E1, exhibited increased butanoate metabolism, galactose metabolism, and leukotriene metabolism. Leukotrienes, as previously mentioned, are eicosanoid inflammatory mediators, and butanoate is a short chain fatty acid known to modulate
inflammation. This suggests that phenotype E1 is associated with greater inflammation than phenotype E2.

Previous studies show that inflammation precedes structural changes in early OA [29], with the metabolomic phenotype of E1 supporting this finding. However, the structural degradation pathways identified in E2 suggest that structural changes are occurring even early in disease progression. Taken together, these results suggest that early OA in this donor population can be divided into two distinct phenotypes, one exhibiting early structural degradation and the other exhibiting increased inflammation.

**Supplemental Figure 4**

Metabolomic phenotypes in late OA. Using hierarchical clustering, we found two phenotypes (L1 and L2) in late OA.
Supplemental Figure 5

Pathways associated with phenotypes of late OA. Based on volcano plot analysis, metabolite features associated with early OA were used to identify pathways associated with metabolomic phenotypes L1 and L2.

We also identified two distinct phenotypes in late OA, L1 and L2 (Supplemental Figure 4). Phenotype L2 exhibited greater keratan sulfate, N-glycan, and sialic acid degradation than phenotype L1 (Supplemental Figure 5). Late stage OA is associated with articular cartilage degradation, and all late OA donors received similar OA grades based on macroscopic joint damage. Despite this, L2 exhibited greater evidence of degradation in the SF compared to phenotype L1. Phenotype L1 exhibited increased arachidonic acid metabolism, leukotriene metabolism, and glutathione metabolism. This suggests that phenotype L1 is associated with a phenotype of oxidative stress and inflammation. Oxidative stress and inflammation have been extensively studied for their role in OA pathogenesis, yet both have been shown to contribute to OA by promoting cartilage degradation [30]. Despite this, phenotype L1 exhibited reduced structural deterioration products in the SF compared to phenotype L2. This suggests that OA pathogenesis may present distinct phenotypes of late OA pertaining to either greater damage or greater oxidative stress and inflammation.
Overlap between Metabolomic Phenotypes of OA

Unsupervised clustering methods identified several clusters within the samples of OA synovial fluid. In both early (Outerbridge 1 and 2) and late (Outerbridge 3 and 4) OA samples, two metabolomic phenotypes were clearly present. We defined the early OA phenotypes as E1 and E2 and the late OA phenotypes as L1 and L2. There is interesting overlap in the metabolites between each group (Supplemental Figures 6-7). E1 and L1 share 60.2% of total detected metabolites, and pathways associated with these metabolites generally represent inflammation. E2 and L2 both include metabolites representing structural deterioration, and these phenotypes share 55.3% of the total metabolites. These data are consistent with the current paradigm of OA phenotypes research [31-35].

Supplemental Figure 6
Comparison of metabolites between L1 and E1 phenotypes. Magenta-colored metabolites are both significantly different between group and have a fold change greater than 2. Using this approach, the majority of the metabolites (60.2%) were similar between the L1 and E1 phenotypes.
Comparison of metabolites between L2 and E2 phenotypes. Magenta-colored metabolites are both significantly different between group and have a fold change greater than 2. Using this approach, the majority of the metabolites (55.3%) were similar between the L2 and E2 phenotypes.

**Conclusion**

These results provide important insight for biomarker discovery for OA. With distinct phenotypes existing in both early and late OA SF, it is possible that unique biomarkers will be identified within each phenotype. Furthermore, the distinct discriminative metabolites found by VIP scores in PLS-DA further support a greater understanding of OA phenotypes for biomarker discovery.

**Supplemental References**


