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.
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.
Supplemental Figure 7

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


