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Application of Global Metabolomic Profiling of Synovial Fluid for Osteoarthritis Biomarkers

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Abstract

Osteoarthritis affects over 250 million individuals worldwide. Currently, there are no options for early diagnosis of osteoarthritis, demonstrating the need for biomarker discovery. To find biomarkers of osteoarthritis in human synovial fluid, we used high performance liquid-chromatography mass spectrometry for global metabolomic profiling. Metabolites were extracted from human osteoarthritic (n=5), rheumatoid arthritic (n=3), and healthy (n=5) synovial fluid, and a total of 1233 metabolites were detected. Principal components analysis clearly distinguished the metabolomic profiles of diseased from healthy synovial fluid. Synovial fluid from rheumatoid arthritis patients contained expected metabolites consistent with the inflammatory nature of the disease. Similarly, unsupervised clustering analysis found that each disease state was associated with distinct metabolomic profiles and clusters of co-regulated metabolites. For osteoarthritis, co-regulated metabolites that were upregulated compared to healthy synovial fluid mapped to known disease processes including chondroitin sulfate degradation, arginine and proline metabolism, and nitric oxide metabolism. We utilized receiver operating characteristic analysis to determine the diagnostic value of each metabolite and identified 35 metabolites as potential biomarkers of osteoarthritis, with an area under the receiver operating characteristic curve > 0.9. These metabolites included phosphatidylcholine, lysophosphatidylcholine, ceramides, myristate

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Competing interest statement

Dr. June owns stock in Beartooth Biotech. This company was not involved in this study. The remaining authors have no competing interests in this work.

derivatives, and carnitine derivatives. This pilot study provides strong justification for a larger cohort-based study of human osteoarthritic synovial fluid using global metabolomics. The significance of these data is the demonstration that metabolomic profiling of synovial fluid can identify relevant biomarkers of joint disease.

Keywords

osteoarthritis; metabolomics; mass spectrometry; biomarkers; joint disease

Introduction

Osteoarthritis (OA) is the most common degenerative joint disease involving the breakdown of the extracellular matrix in cartilage. OA affects over 250 million individuals, with the majority being adults over the age of 65[1]. Currently, symptoms include joint pain and loss of function. Unfortunately, diagnosis at the onset of symptoms typically occurs after irreversible structural degeneration. Joint replacement is commonly utilized for advanced disease[1]. There are currently no available tests for diagnosing early OA or tracking its progression, demonstrating the need for biomarkers of OA. Previous studies have attempted identifying biochemical biomarkers in urine[2, 3], blood serum[4, 5], or joint synovial fluid (SF)[6–8] samples using proteomics, genomics, and most recently, metabolomics[2–7, 9, 10].

Among the studies utilizing metabolomics for OA biomarker identification, many use urine and blood serum samples[2, 3, 5]. While obtaining a blood sample is less invasive than SF, metabolites from the diseased joint must move across the synovium prior to entering the serum for detection. This dilutes potential biomarkers and leaves them susceptible to potential degradation in the circulatory compartment. One study showed that the composition of metabolites and their corresponding concentrations were altered in osteoarthritic SF[7]. The SF is also in direct contact with the diseased joint tissue, making it a promising candidate for biomarker discovery in OA.

Many studies have used ^{1}H NMR for metabolite biomarker discovery[11]. While ^{1}H NMR has very little sample preparation and high reproducibility, it is mainly used for metabolites that exist in high concentrations due to sensitivity limitations[7]. Alternatively, mass spectrometry has a much higher sensitivity than ^{1}H NMR and is inherently capable of detecting larger numbers of metabolites. Liquid chromatography coupled to mass spectrometry (LC-MS) shows great promise for analyzing complex biofluids such as SF because it provides chromatographic separation of these heterogeneous samples prior to reaching the mass analyzer.

Here, we characterized global metabolomic profiles from SF of patients with various joint diseases using mass spectrometry. Metabolomic profiles of rheumatoid arthritis (RA) synovial fluid were used to validate the method by confirming established findings in RA. To our knowledge, this is the first study to perform LC-MS-based global metabolomic profiling of osteoarthritic SF for biomarker discovery. The long-term goal of this research is to identify biomarkers of OA phenotypes for earlier diagnosis and quantification of disease

progression. The objective of this study was to evaluate global LC-MS-based metabolomic profiles as a tool for quantifying biomarkers within SF. We hypothesized that (1) RA profiles would be distinct from OA with RA showing increases in inflammatory metabolites and (2) OA patients would exhibit diverse metabolomics profiles suggesting distinct disease phenotypes that correlate with clinical symptoms.

Methods

Patients and Synovial Fluid Samples

OA (n=5) and RA (n=3) SF samples were obtained under IRB approval. Healthy SF samples (n=5) were purchased from Articular Engineering (Northbrook, IL) *post mortem* with partial clinical data including age, gender, race, and cause of death. All samples were frozen at −80°C after harvest until analysis.

Metabolite Extraction

Metabolites were extracted from 100 μL of SF prior to analysis by LC-MS. Samples were thawed on ice for 3–5 minutes prior to centrifuging at 4°C at 500 × *g* for 10 minutes to remove cells and debris. The supernatant was collected and evaporated, and dried samples were re-suspended in 100 μL of 50:50 water:acetonitrile. 500 μL of acetone was added and the sample was mechanically shaken for 3 min to precipitate polymers. The samples were then refrigerated for 10 minutes prior to centrifuging at 16100 × *g* for 5 min. The supernatant was transferred to a new tube and further evaporated to remove acetone. The remaining sample volume was doubled with acetonitrile for a final 50:50 water:acetonitrile LC-MS injection buffer.

Metabolomic Profiling and Data Processing

Metabolites extracted from SF samples were analyzed using an Agilent 1290 UPLC system connected to an Agilent 6538 Q-TOF mass spectrometer (Agilent, Santa Clara, CA). The samples were run in normal phase, using a Cogent Diamond Hydride HILIC 150 × 2.1 mm column (MicroSolv, Eatontown, NJ). The HPLC solvents used were 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The elution gradient consisted of 100 to 25% solvent B over 13 minutes, and each run began with 2 minutes wash (15 minutes total run time).

Metabolite detection was performed in positive mode with a resolution of ~20,000 and accuracy of ~5 ppm. Agilent MSConvert converted data from Agilent's proprietary file format, .d files, to mzXML files to filter data in MZMine 2.14 for retention time, noise level, and peak detection[12]. An intensity threshold of 1000 was applied to detect peaks above the noise. The processed chromatograms were then normalized and aligned for metabolite (m/z value) detection. Metabolites with median intensity values of zero across the OA, RA, and healthy datasets were removed from the analysis. To identify the metabolites, untargeted metabolite mass to charge (m/z) values were loaded into METLIN, a database containing over 80,000 metabolites[13]. The batch search used a mass tolerance of 15 parts per million (ppm), including positively charged molecules with $+1H^+$ or $+1Na^+$ adducts but excluding peptides, toxins, and drugs. All m/z values detected were crosschecked against our library of

metabolite standards. To confirm metabolite identities, both m/z value and retention time had to match within a m/z value tolerance of 0.01 or 30 ppm and retention time tolerance of 0.25 minutes[14].

Statistical analyses

Statistical analysis was performed in Matlab (Mathworks, Natick, MA USA). To identify metabolites differentially expressed between OA, RA, and healthy SF, we used Student's T-tests and one-way analysis of variance (ANOVA) with false-discovery rate (FDR) corrections at a significance level of 0.05. Additionally, we used volcano plots to visualize metabolites significantly upregulated and downregulated in OA or RA in comparison to healthy SF[15]. The negative $\log_{10}$ of the p-value (y-axis) for each m/z value and the fold change ($\log_2$(ratio)) of the median intensity between disease state and healthy SF (x-axis) were plotted against one another to yield both significance and magnitude of change. M/z values with zero intensity for either group were excluded from the volcano plot analysis. Principal component analysis (PCA) was applied to fold change normalized, log-transformed metabolites to examine sources of underlying variation in the dataset. To visualize the unique metabolites in each dataset, we created a scatterplot of median metabolite intensities from each group against one another (+/− standard deviation). Where applicable, error bars show standard error.

Hierarchical cluster analysis (HCA) was used to provide a visual representation of global metabolomic profiles and co-regulated metabolites. Co-regulated metabolites within clusters were identified using METLIN and subsequently analyzed for pathway enrichment using IMPaLA[16]. Based on the metabolites in those clusters, IMPaLA identifies potential pathways implicated by over-representation analysis using established biochemical pathway databases (*i.e.* KEGG, Reactome, HumanCyc, etc.).

Potential biomarkers were determined using receiver operating characteristic (ROC) analysis. By analyzing the area under the receiver operating curve (AUC), we identified metabolites that could accurately classify between two cases, such as OA and healthy. We focused on metabolites with an AUC>0.9 as potential biomarkers.

Results

Metabolomic Profiling of Osteoarthritis, Rheumatoid Arthritis, and Healthy Synovial Fluid

We detected a total of 1233 metabolite features across all OA, RA, and healthy SF samples using LC-MS analysis. HCA of all metabolites in OA, RA, and healthy SF demonstrated that OA and RA have more similar metabolomic profiles than healthy SF, as indicated by the dendrogram (Fig. 1A). PCA of all three datasets revealed clear separation between diseased and healthy SF metabolomic profiles, with the first two principle components associated with 52.1% of the total variation (PC1=34.2%, PC2=17.9%, Fig. 1B). 26 metabolites were significantly different between OA, RA, and healthy SF as determined by one-way ANOVA (p_{fdr} <0.05). 15 metabolites were confidently identified by both m/z value and retention time using our library of standards, with 6 of these being significantly different (p_{fdr}<0.05). Citric acid, D-lactic acid methyl ester, hydroxyl-L-proline, L-isoleucine, and L-methionine were

significantly upregulated in healthy SF in comparison to diseased. In contrast, L-citrulline was significantly upregulated in OA over both RA and healthy SF.

Metabolomic Profiles of OA Synovial Fluid

1098 metabolites were detected in OA and healthy SF. 58 metabolites were significantly different between OA and healthy SF ($p_{fdr}<0.05$). HCA was performed on all 318 significantly different metabolites without FDR corrections to remain consistent with the RA and healthy analysis (Supplemental Figure 1). HCA showed 2 distinct clusters of co-regulated metabolites, with cluster 2.1 containing 132 metabolites downregulated in OA and cluster 2.2 containing 186 metabolites upregulated in OA (Fig. 2A). Enrichment of cluster 2.1 identified similar pathways as cluster S1.1 (metabolites downregulated in healthy compared to RA) in Supplemental Figure 1A, including purine metabolism, tRNA charging, and gene expression. Enrichment of cluster 2.2, however, yielded arginine and proline metabolism, chondroitin sulfate degradation, amino oxidase reactions, COX reactions, and creatine biosynthesis (Supplemental Table 1, Supplemental Table 6).

PCA showed clear separation between OA and healthy SF, with the first two principle components (PC1=35.4%, PC2=22.9%) associated with 58.3% of the variation (Fig. 2B). Of the 1098 metabolites detected in OA and healthy SF, 390 metabolites were exclusively detected in OA SF and 301 metabolites were exclusively detected in healthy SF (Fig. 2C). Volcano plot analysis identified 76 metabolites upregulated and 28 metabolites downregulated in OA, with a p-value<0.05 and fold change>2 (Fig 2D). Enrichment of these metabolites yielded similar pathways implicated in clusters 2.1 and 2.2 (Supplemental Table 2). Heatmap clustering, PCA discrimination, scatterplot visualization, and volcano plot analysis clearly demonstrate that OA and healthy SF represent distinct populations with phenotypic differences.

Differences between Pathological and Healthy Synovial Fluid

OA and RA metabolite intensities were normalized to healthy SF to determine how metabolomic profiles of SF change with disease. HCA of the normalized metabolites exhibited distinct clusters of co-regulated metabolites (Fig. 3A). Cluster 3.1 included 276 co-regulated metabolites downregulated in diseased compared to healthy (Fig. 3A). These metabolites mapped to SLC-mediated transport, gamma-glutamyl cycle, tRNA aminoacylation, and various amino acid transporters (Supplemental Table 1, Supplemental Table 7). Cluster 3.2 included 258 co-regulated metabolites upregulated in OA in comparison to healthy and RA (Fig. 3A). These metabolites mapped to creatine biosynthesis, alpha-linolenic acid metabolism, galactose metabolism, vitamin B6 metabolism, chondroitin sulfate degradation, arginine biosynthesis, urea cycle, and nitric oxide metabolism (Supplemental Table 1, Supplemental Table 7). Cluster 3.3 included 305 co-regulated metabolites upregulated in diseased compared to healthy (Fig. 3A). These metabolites mapped to phospholipid biosynthesis, mTOR signaling, arginine and proline metabolism, organic cation transporters, synthesis of PS, acyl chain remodeling, glycerophospholipid metabolism, and phosphatidylcholine biosynthesis (Supplemental Table 1, Supplemental Table 7). Cluster 3.4 included 152 co-regulated metabolites upregulated in RA over healthy and OA, mapping to histidine, lysine, phenylalanine, tyrosine, glycine,

proline, and tryptophan metabolism, ibuprofen metabolism, steroid biosynthesis, glucocorticoid and mineralocorticoid metabolism, alpha-linolenic acid metabolism, and leukotriene biosynthesis (Supplemental Table 1, Supplemental Table 7). Select metabolites implicated in each cluster are displayed in Figure 3B.

Biomarker Candidates for Osteoarthritis

We used ROC analysis to determine the potential diagnostic value of each detected metabolite as an OA biomarker. ROC analysis identified 65 m/z values as potential biomarkers of OA, all having an AUC>0.9. 30 of these 65 m/z values were also identified as potential biomarkers of RA and were consequently removed from the analysis. To identify the remaining 35 m/z values as metabolites, we searched METLIN for a full list of potential metabolites that match these m/z values. Of these 35 m/z values, 16 did not have any METLIN database matches, thus classifying them as potentially novel metabolites for future analysis. The remaining 19 metabolites with an AUC>0.9 mapped to 91 putative metabolites identities (Supplemental Table 3). Of those 91 identified metabolites identities, over half were phospholipids (PC, PAF, PG, PE, PS, and ceramides). Other potential biomarkers for OA included 2,3,6-trihydroxypyridine, myristate derivatives, arachidonyl carnitine, N-methyl arachidonyl amine, oleanolic acid acetate, vitamin D3 derivatives, sulfonic acid derivatives, and ursolic acid derivatives (Supplemental Table 3). Additional studies are needed to validate these metabolites as OA biomarkers and determine the implications of their involvement in OA pathogenesis.

Discussion

This is the first study to perform global LC-MS-based metabolomic profiling on human OA synovial fluid. We used LC-MS for both (1) biomarker discovery and (2) understanding disease pathophysiology. PCA and HCA of metabolite data distinguished between OA and healthy SF, creating distinct metabolomic profiles with identifiable features. We detected a total of 1098 metabolites in OA and healthy SF, more than any previous study we know of[7, 17–20], with 58 significantly different metabolites between OA and healthy SF. Enrichment analysis of metabolites that were upregulated in OA identified nitric oxide production and chondroitin sulfate degradation both of which have previously been linked to OA pathogenesis[5, 21, 22]. Chondroitin sulfate is a glycosaminoglycan that plays an important structural role in the cartilage matrix. Altered levels of chondroitin sulfate have previously been found in OA cartilage[21, 22]. Nitric oxide aids in OA disease progression by promoting expression of proinflammatory cytokines, inhibiting proteoglycan and collagen synthesis, mediating apoptosis, and activating metalloproteinases[5].

ROC analysis is valuable for determining the diagnostic value of each metabolite as a potential biomarker. ROC analysis identified 35 potential biomarkers of OA, including phosphatidylcholines (PC), lysophoshatidylcholines (lysoPC), ceramides (Cer), myristate derivatives, and carnitine derivatives. Phospholipids are important components of the SF that contribute to the mechanical function of the SF to lubricate articular cartilage surfaces[6]. Altered composition and concentration of lubricating agents in the SF are associated with damaged articular cartilage surfaces in both OA and RA[23–25]. However, the small sample

size of this pilot study prevents broader interpretation about these metabolites as potential biomarkers and their implications in OA pathogenesis.

Global metabolic profiles of RA patient SF found anti-inflammatory drug pathways, amino acid metabolism, leukotriene biosynthesis, alpha-linolenic acid metabolism, glucocorticoid and mineralocorticoid metabolism, and steroid biosynthesis implicated in RA (Supplemental Table 1, Supplemental Table 2). Given that rheumatoid arthritis is highly inflammatory, the identification and upregulation of anti-inflammatory drug pathways is expected in RA SF and validates the use of our LC-MS global metabolomic profiling approach.

While this pilot study holds great promise for biomarker discovery, this is discovery phase research with important limitations. First, healthy SF samples were harvested *post mortem*. In contrast, the SF from OA and RA joints was harvested from living patients. While *post mortem* SF may differ from that of living patients, previous studies found that *post mortem* SF maintained normal lubricant components, composition, and function[26, 27]. Second, patient information was limited. Thus, the ability to decipher effects of age, BMI, or gender on metabolomic profiles was not possible. The third, and main limitation of this study is the small sample size. This was a pilot study meant to assess global metabolomic profiling of human SF for biomarker discovery. Future studies will increase the sample sizes of all patient groups as well as age and gender match samples to identify potential biomarkers of OA.

In summary, this study demonstrates the advantages of global metabolomic profiling for both biomarker discovery and further understanding of OA pathogenesis. Despite extremely small sample sizes, we were able to detect a number of potential metabolites for future validation studies. Global metabolomic profiling via LC-MS may provide physicians with a high sensitivity method for tracking treatment effectiveness, disease progression, and biomarker discovery for OA and other diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

OA	osteoarthritis

RA	rheumatoid arthritis
SF	synovial fluid
m/z	mass-to-charge ratio
LC-MS	liquid chromatography mass spectrometry
ROC	receiver operating characteristic curve
PCA	principal component analysis
HCA	hierarchical cluster analysis

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Highlights

- Untargeted metabolomics using liquid chromatography-mass spectrometry clearly discriminates between osteoarthritis, rheumatoid arthritis, and healthy synovial fluid, showing distinct metabolomic profiles with key features of each disease state.
- Global metabolomic profiling confirmed previously identified pathways implicated in osteoarthritis including chondroitin sulfate degradation and nitric oxide metabolism.
- Of the 1233 metabolite features identified in human synovial fluid using our method, 65 features are potential biomarkers of osteoarthritis based on receiver operating characteristic analysis (AUC>0.9) including various phospholipids, vitamin D3 derivatives, carnitine derivatives, and oleanolic acid acetate.

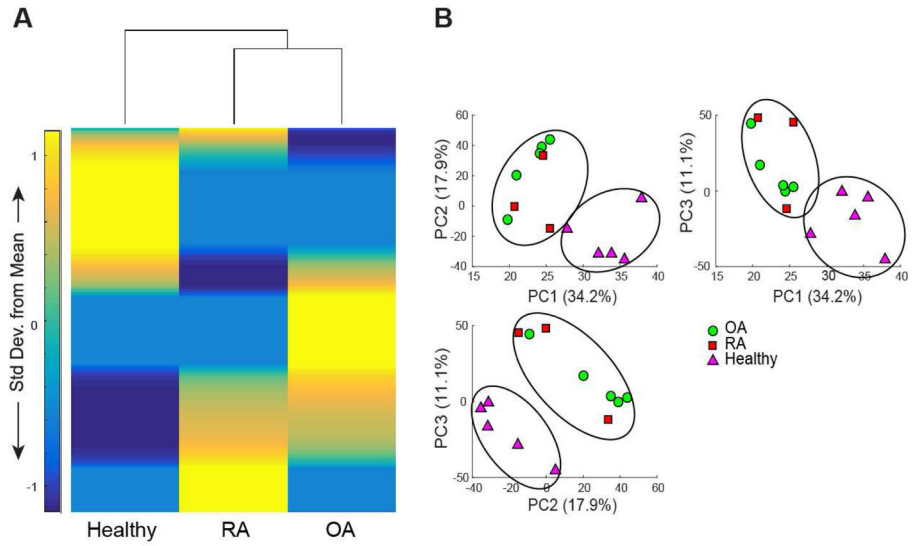

Figure 1.

Global metabolomic profiles of diseased (OA and RA) SF are distinct from healthy SF. **(A)** Clustered heatmap of median metabolite intensities from healthy (n=5), OA (n=5), and RA (n=3) SF. 1233 metabolites, represented by the rows in this heatmap, were detected in human SF. Dendrogram of HCA demonstrates that OA and RA metabolomic profiles are more similar to one another than healthy SF. **(B)** PCA of metabolite intensities in OA, RA, and healthy SF, comparing PC1 (34.2%), PC2 (17.9%), and PC3 (11.1%). PCA shows clear discrimination between diseased and healthy metabolomic profiles.

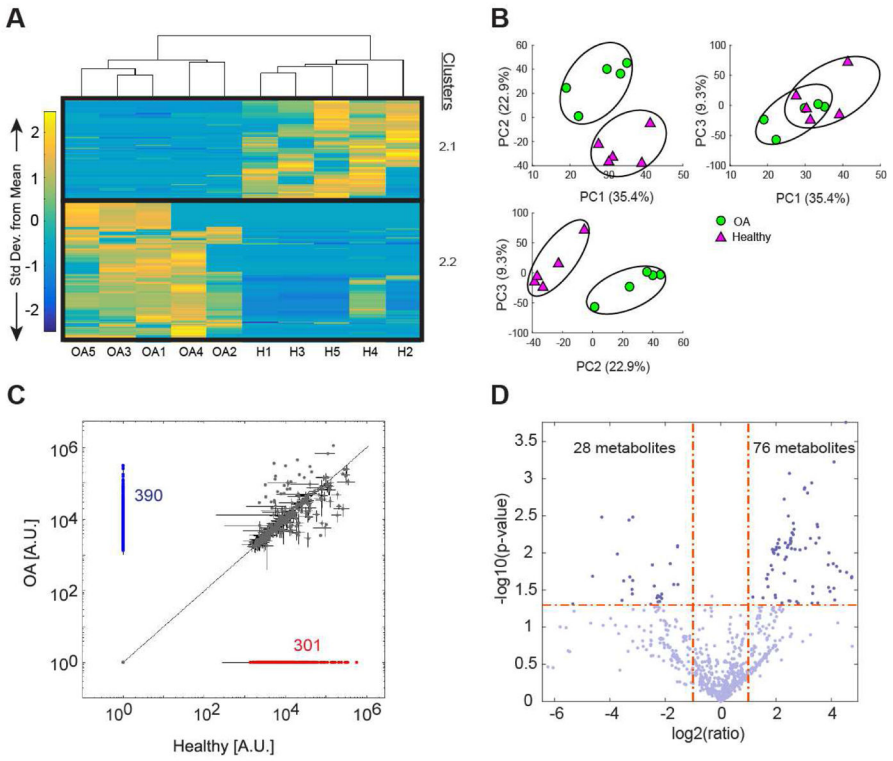

Figure 2.

OA and healthy SF exhibit distinct metabolomic features. **(A)** Clustered heatmap of OA (n=5) and healthy (n=5) SF median metabolite intensities (H=healthy). 1098 metabolites were detected in OA and healthy SF, with 318 being significantly different as represented in this heatmap. Clusters of co-regulated metabolites are outlined in black and labeled as 2.1 and 2.2. Cluster 2.1 contains 132 metabolites downregulated in OA and cluster 2.2 contains 186 metabolites upregulated in OA. **(B)** PCA of OA and healthy SF metabolite intensities. PC1 (35.4%), PC2 (22.9%), and PC3 (9.3%) were associated with 67.6% of the variation within the dataset. Comparison of PC1, PC2, and PC3 show clear separation between OA and healthy SF metabolomic profiles. **(C)** Scatterplot comparing OA and healthy metabolite intensity levels (A.U.=Arbitrary Units). 301 metabolites were exclusively detected in OA SF (blue) and 290 metabolites were exclusively detected in healthy SF (red). **(D)** Volcano plot of OA and healthy SF, plotting the −log(p-value) against the fold change (log2(ratio)) of individual metabolites. Vertical dashed lines mark the twofold change and the horizontal line marks the cutoff p-value of 0.05. Metabolites in the upper right and left quadrants represent metabolites with a p-value less than 0.05 and a greater than twofold change. 76 metabolites were significantly upregulated in OA (upper right quadrant) and 28 metabolites were significantly downregulated in OA (upper left quadrant) in comparison to healthy (p-value<0.05; fold change>2).

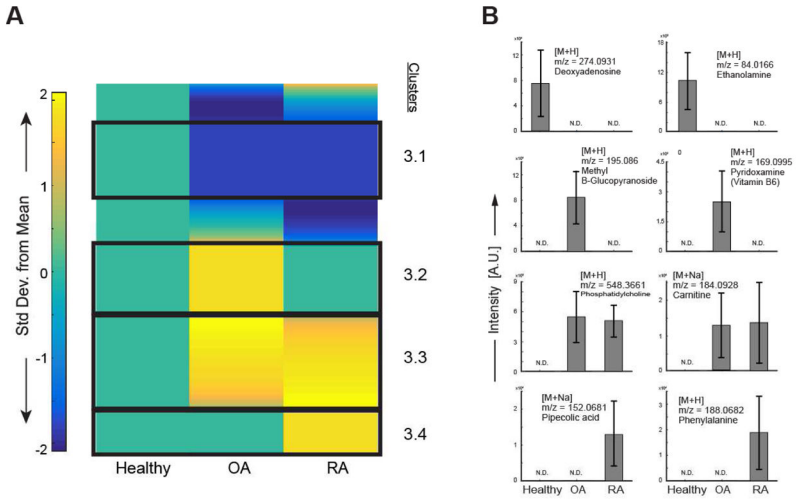

Figure 3.

Metabolomic profiling reveals features of each disease state with co-regulated metabolites upregulated or downregulated in comparison to healthy SF. **(A)** Clustered heatmap of 1233 median metabolite intensities normalized to healthy. Four clusters of interest were identified as clusters 3.1–3.4, with co-regulated metabolites upregulated or downregulated in OA and RA. **(B)** Bar graphs of median metabolite intensities +/− standard deviation (A.U. = arbitrary units, N.D. = not detected) for 2 individual metabolites implicated in each of the 4 clusters. Row 1 corresponds to cluster 3.1, row 2 corresponds to cluster 3.2, and so on. Each bar graph contains the m/z value, the metabolite identification, and its suspected adduct.