

**Identifying Metabolic Differences in The Synovial Fluid of Males and Females  
Affected by Osteoarthritis**

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## **Abstract**

Osteoarthritis (OA) is the most common joint disease in the body, characterized by limited mobility due to the degradation of cartilage. Age, weight, and genetic profiles have been identified as clear risk factors for OA; women are also more likely to suffer from OA compared to men. While previous research indicates that postmenopausal women have an increased likelihood of developing OA due to decreasing estrogen, few studies have investigated metabolic differences between diseased males and females. This study seeks to understand the differences between global metabolic profiles of males and females with OA. In order to investigate metabolic discrepancies, metabolites were extracted from joint-effusion-related OA synovial fluid. Global metabolomic profiling by liquid chromatography-mass spectrometry (LC-MS) was then employed to identify significant metabolic differences between sexes. The results of this study suggested a distinct metabolic response to OA in males and females, most notably a significant difference between fatty acid and amino acid metabolism. This study provides a greater understanding of metabolism in OA pathogenesis and suggests opportunities for more individualized, therapeutic interventions that target sex differences. In the future, researchers should replicate this experiment with a larger sample size and employ targeted metabolomics to further elucidate the role of fatty acid and amino acid metabolism in OA responses.

## Table of Contents

Introduction.....	5-8
Methods.....	8-10
Results.....	10-18
Discussion.....	18-22
References.....	23-26

## Introduction

Osteoarthritis (OA) is the most common form of arthritis<sup>1</sup>. Characterized by pain, limited mobility, and cartilage degradation, OA affects over 250 million people worldwide including 12.4 million adults over the age of 65<sup>1</sup>. In the United States, the overall economic burden of OA accounts for an estimated \$136.8 billion annually<sup>2</sup>. Osteoarthritic joints exhibit cartilage damage, osteophyte formation, subchondral bone sclerosis, and variable synovitis<sup>3</sup>, significantly lowering the quality of life for affected patients.

The multifactorial nature of OA contributes to its variable phenotypes and prognosis, with disease progression ranging from rapid cartilage breakdown to long-term cartilage matrix instability<sup>4</sup>. On a biochemical level, OA is characterized by an upregulation of matrix-degrading enzymes, including Aggrecanases (which break down the proteoglycan aggrecan) and Matrix Metalloproteases<sup>3,4,5</sup>. Additionally, the progression of pain is also variable and unpredictable, with some patients experiencing extreme pain despite minimal joint space narrowing while others experience minimal pain with evident joint space narrowing<sup>3</sup>. Along with the variability of prognosis and development, OA treatment is also multifactorial, ranging from activity modification to total knee arthroplasty. Hyaluronic acid (HA) injections have also shown potential benefits in combating OA by enhancing the viscoelasticity of cartilage and preventing excess force from damaging the underlying bone<sup>6-8</sup>. Despite these advances in treatment, OA does not have a cure, thereby prioritizing the importance of research into preventive mechanisms.

There are several risk factors associated with OA development including age, abnormal loading conditions, altered joint metabolism, inflammation, and genetic factors<sup>9</sup>; however, gender and sex discriminants are poorly understood<sup>9</sup>. Previous studies reveal that women are up to 3.5 times more likely than men to suffer from OA and to develop more severe joint narrowing of the knee<sup>9</sup>. In general, women have an increased risk of knee OA and tend to present for treatment in more advanced stages with more debilitating pain than their male counterparts<sup>9</sup>. Research also indicates a sex discrepancy in OA expression and disproportionate joint effects such as knee cartilage volume<sup>10</sup>. Hame et al. observed that men had significantly greater total tibial and patellar cartilage volume than women and that women had a significantly higher prevalence of patellar cartilage defects at baseline.<sup>7</sup> Over time, women showed more knee cartilage loss compared to their male counterparts. Lower baseline cartilage and more aggressive volume loss are likely contributors to a higher prevalence of OA in women. While anatomical differences are currently known to contribute to the disproportionate

presentation of OA between men and women, little is understood about how hormonal differences exacerbate sex-specific variation in OA progression.

In conjunction with anatomical discrepancies, hormonal variations between men and women may also serve as major contributors to osteoarthritis and osteoporosis. Males and females differ in their estrogen, progesterone, and testosterone serum concentrations, production sites, and interactions with different organs. These hormonal differences also play a major role in OA discrepancies<sup>10</sup>. Studies indicate that estrogen may be beneficial to cartilage maintenance<sup>11</sup>. This finding is supported by the presence of estrogen receptors in articular cartilage, which implies a possible relationship between estrogen and cartilage health<sup>12</sup>. One study in particular found that estrogen concentrations are shown to modulate OA by inhibiting the degradation of the extracellular matrix<sup>13</sup>. Furthermore, estrogen appears to benefit cartilage remodeling by promoting proteoglycan and insulin-like growth factor binding proteins, which are involved in chondrocyte production<sup>12</sup>. Therefore, postmenopausal women have an increased likelihood of developing OA due to the decrease in estrogen production and modulation. To combat this issue, Zhang et al. demonstrated that postmenopausal women who take estrogen replacement therapy have a reduced chance of OA development<sup>14</sup>. Demonstration of estrogen's role in cartilage protection is an important advancement, but more research is needed to understand the molecular basis involving hormonal changes and OA development.

Articular cartilage plays a vital role in not only joint mobility but also in maintaining the structural integrity of underlying structures, such as the subchondral bone<sup>15</sup>. Furthermore, abnormal loading conditions can alter cartilage maintenance and produce metabolic changes within surrounding joints. Previous studies have discovered OA-associated metabolic changes that are involved in amino acid metabolism, the urea cycle, phosphatidylinositol phosphate metabolism, the carnitine shuttle, vitamin metabolism, and porphyrin metabolism<sup>3</sup>. Research also indicates an upregulation of glycolysis, gluconeogenesis, and the pentose phosphate pathway, which suggests an increase in energy expenditure for cartilage remodeling<sup>4</sup>. Additionally, variations in inflammatory mechanisms serve as potential targets for investigating the biochemical response to OA. Although biochemical pathways are perturbed in diseased cartilage, research elucidating sex differences at the molecular level in OA development is lacking. In comparison to males, females demonstrate a higher level of macrophage stimulators and pro-inflammatory mediators (interleukins)<sup>9</sup>. Research also illustrates an upregulation of butanoate and leukotrienes, metabolites involved in inflammatory pathways<sup>4</sup>. While the exact pathophysiological mechanism is not well understood, cartilage fragments from diseased cartilage may be seen as foreign bodies and therefore trigger a secondary immune and inflammatory response<sup>16,17</sup>. Biochemical and inflammatory responses are

shown to be altered in OA pathogenesis, but the exact metabolic mechanisms need to be investigated to better understand global metabolic shifts associated with cartilage degradation.

Although estrogen and testosterone modulate OA, more research is necessary to understand the underlying mechanisms behind sex disparities and cartilage regulation. Metabolomics serves as a promising method for investigating metabolic shifts between healthy and osteoarthritic patients within male and female subgroups<sup>3,4,19</sup>. Metabolomics investigates the small, intermediary molecules within biochemical pathways<sup>19</sup>. Elucidating these molecules is vital in understanding how OA manifests itself as a metabolic perturbation. Furthermore, global metabolomics is useful because it produces a global view of metabolic responses with minimal bias, allowing for the investigation of many biochemical pathways<sup>20,4</sup>. Global metabolic profiling is important in identifying specific metabolites as well as overall shifts in metabolism, providing insight into underlying disease mechanisms<sup>3,4,21</sup>.

Global metabolomics proves important in identifying and distinguishing metabolites within various mediums, such as synovial fluid. Although the underlying subchondral bone supplies vital nutrients and support to the synovial joint, the primary source of nutrients to the cartilage is synovial fluid (SF). The SF is an ultrafiltrate of plasma containing various molecules produced by joint tissue cells<sup>4,22</sup>. SF provides lubrication, reduces friction between the articular cartilage surfaces, and eliminates metabolic waste. Because SF is in direct contact with osteoarthritic tissue, studying the metabolic shifts in diseased SF can elucidate local alterations within cartilage and indicate metabolic changes following cartilage degradation<sup>4</sup>. The use of global metabolic profiling on SF will help fill the knowledge gap regarding sex-associated metabolism alterations.

More data are needed to fully elucidate the role of sex in OA pathogenesis. In particular, research is lacking in understanding the sex differences at the biochemical level. While previous studies on OA provide evidence of metabolic alterations in diseased SF, the exact molecular mechanisms underlying sex-specific metabolic changes are yet to be elucidated. We aim to fill this gap in knowledge using global metabolic profiling to assess metabolic alterations in male versus female OA synovial fluid. The use of global metabolic profiling will help identify metabolic biomarkers of OA, specifically those biomarkers involved in hormonal discrepancies between male and female populations. OA progression has been shown to alter metabolic pathways; however, there is limited information regarding sex differences in OA and how hormones impact disease progression and associated phenotypes. Additionally, few studies have investigated metabolic perturbations to tease out differential biomarker expression that might drive

clinical research toward sex-orientated diagnosis and treatment protocols<sup>7,9</sup>. Therefore, the overall goal of this project is to expand the field's understanding of sex differences in OA development and phenotypes and to use metabolomics to discover sex-specific biomarkers as potential therapeutic targets for OA diagnosis and treatment.

## **Methods**

### *Experimental Design*

A total of 11 samples were used in this experiment. The experimental group (OA SF) contained seven total replicates: three female and four male diseased synovial fluid samples. The control group contained four total replicates (healthy SF): two female and two male samples of healthy synovial fluid. Samples were collected from Performance and Injury Care, Helena, MT. These samples are normally discarded tissues and were obtained with IRB approval. There were three female and four male samples that were affected by joint-effusion-related OA. Females ranged from 63 to 67 years old; males ranged from 68 to 72 years old. While most of the samples were collected from the knee, the sample from the 72-year-old male was collected from the right shoulder. The control group consisted of two female and two male post-mortem samples obtained from Performance and Injury Care, Helena, MT. The two female control samples were obtained from patients aged 47 and 53 years old. Their cause of death was not joint-related. The two male control samples were obtained from patients aged 59 and 71 years old. Neither died of a disease that would affect the physiological function of joint structures.

### *Metabolite Extraction*

The metabolite extraction protocol was adapted from Carlson et al.<sup>3</sup>. Both aqueous acetonitrile and methanol were mass spectrometry grade, and were purchased from Sigma Aldrich (St. Louis, MO). Methanol used in this experiment was 80% v/v made up in reverse osmosis water. The aqueous acetonitrile was 50% v/v stored at 0°C for at least 30 minutes before the start of the extraction protocol. One hundred microliters of each sample was thawed on ice for 3 to 5 minutes to prevent change in cellular metabolism within the synovial fluid. Once thawed, samples were centrifuged at 500xg at 4°C for 5 minutes (Sorvall Legend RT, Thermo Electron Corporation) to separate cellular debris and metabolites based on density. Cell debris, being denser than metabolites, pelleted to the bottom of the centrifuge tube, leaving metabolites in the supernatant. The supernatant was collected and transferred to a new microcentrifuge tube. 80% v/v methanol was added to the supernatant and stored at -20°C for 30 minutes to pull out metabolites. Samples were then vortexed for 3 minutes to ensure sample mixture.

Subsequently, samples were centrifuged at 16,100xg at 4°C for 5 minutes to further separate the metabolites from potential debris that was not eliminated during the first centrifugation step. The supernatant was then recollected and placed in the vacuum concentrator (Savant AES 1010, ThermoFisher Scientific, Waltham, MA) for 2 hours to evaporate the solvent. After the sample had been dried, metabolites were resuspended with 5 volumes (500 µL) of 50% aqueous acetonitrile. The aqueous acetonitrile lysed the hydration shell surrounding the proteins, causing them to precipitate out of the solution. Aqueous acetonitrile and the resuspended metabolites were fully mixed via centrifugation at 16,100xg for 5 minutes. Following centrifugation, the proteins formed a pellet at the bottom of the centrifuge tube, and the supernatant was collected and transferred to a new centrifuge tube. The supernatant was again dried via vacuum concentrator for 2 hours and then stored at -80°C until analysis by LC-MS.

### *Liquid Chromatography-Mass Spectrometry*

Metabolites were resuspended in 50:50 water: acetonitrile solution for analysis by liquid chromatography-mass spectrometry (LC-MS) in positive mode. The LC-MS system used for data acquisition was an Agilent 1290 UPLC and Agilent 6538 Q-TOF mass spectrometer (Montana State University; MSU). Metabolites were separated on a Cogent Diamond Hydride HILIC (Hydrophilic Interaction Liquid Chromatography) 150 x 2.1 mm column using a normal phase gradient elution method. The elution gradient was established as the mobile phase and became increasingly polar over 15 minutes due to the addition of water. Subsequently, the Agilent 6538 Q-TOF (Quadrupole-time-of-flight) mass spectrometer (Agilent, Santa Clara, CA) was used to determine mass-to-charge ( $m/z$ ) ratios. The mass spectrometer detects metabolites with a resolution of ~20,000 FWHM (full-width half-maximum) with an accuracy of 5 ppm.

### *Statistical and Pathway Analyses*

Data generated by the LC-MS were normalized to the median, log-transformed, and standardized prior to analysis by univariate and multivariate statistical tests in MetaboAnalyst. Metabolites from diseased SF identified via fold change analysis were mapped to metabolic pathways to identify those that were upregulated or downregulated in response to OA. Unsupervised principal component analysis (PCA), supervised partial least squares discriminant analysis (PLS-DA), and supervised orthogonal partial least squares discriminant analysis (OPLS-DA) were applied to visualize overall variation within the dataset. Metabolites with the highest variable importance in projection (VIP) scores from OPLS-DA were mapped to metabolic pathways using the functional analysis feature in MetaboAnalyst. The significance for pathway analyses was determined with a false discovery rate (FDR) corrected p-value threshold < 0.05.

Data from the MSU LC-MS analysis was returned and underwent data clean-up. Sample profiles that contained an N/A were replaced with 0s, replicates were removed, and the retention time column was removed, after which the file was saved as a CSV file. The cleaned file was uploaded to MetaboAnalyst to undergo statistical and functional analysis. During the data integrity check, MetaboAnalyst replaced the 0s with  $\frac{1}{5}$  of the minimum peak intensity, as it could not be determined whether metabolites were absent from the sample or within the noise of the LC-MS signal. Furthermore, data was normalized against the median and auto-scaled to account for intragroup variation between metabolites (rows) and samples (columns).

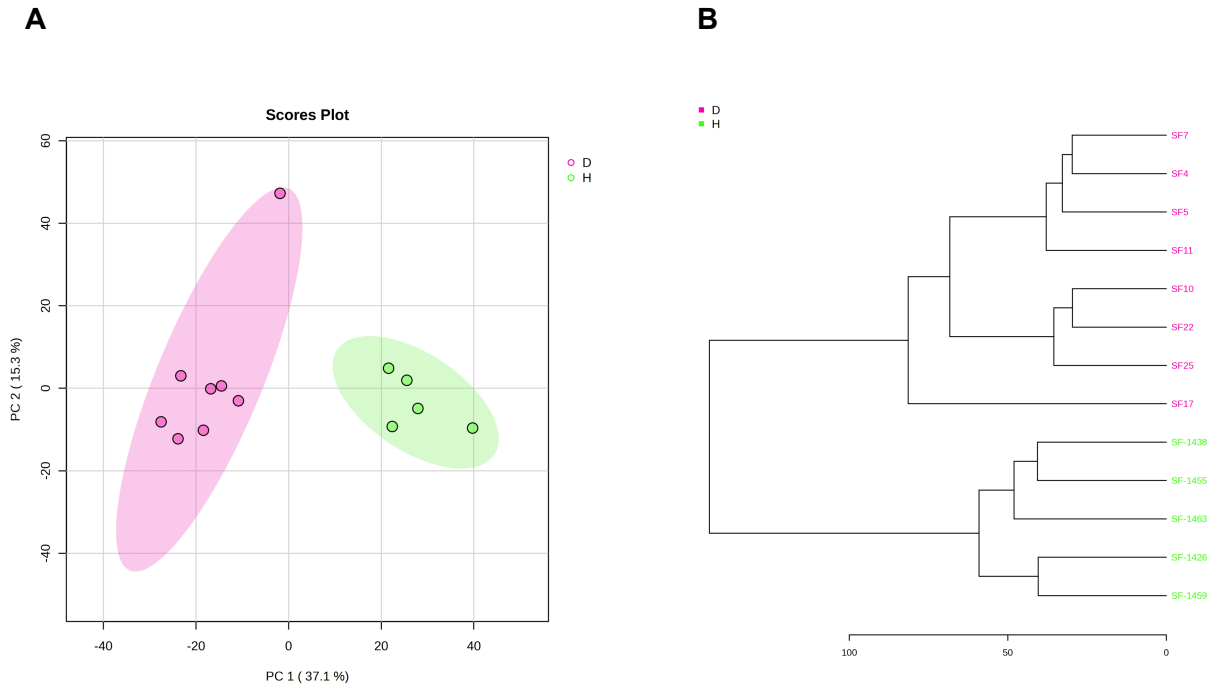
Univariate analyses were used to determine statistically significant differences between male and female samples in the control and experimental groups. The control and diseased synovial fluid metabolites were analyzed by an ANOVA test with post-hoc comparison. ANOVA tests identify significant differences among the groups within the data set; furthermore, a post-hoc comparison was employed to determine which groups, specifically, have a significant difference in cellular metabolism. Post-hoc comparison elucidated differences within the synovial fluid sample cohorts as a whole. Diseased male and female SF samples were analyzed using a volcano plot, which established overall up and downregulations within the two cohorts and identified sex-specific differences in metabolism.

The data was further analyzed through multivariate analysis. Principle component analysis (PCA) was utilized to establish global or local metabolic shifts within the diseased patient cohorts. PCA plots examined the cluster pattern of healthy versus OA groups and further distinguished sex-based metabolic alterations. Orthogonal partial least squares discriminant analysis (OPLS-DA), which contains a predictive component, was employed to further identify metabolic differences between diseased males and females. OPLS-DA plot analysis generated a list of featured metabolites via variable importance projection (VIP) scores which were used in the functional analysis and metabolic mapping of the dataset via Mummichog. Mummichog analyzed the most important metabolic features, in combination with background information, to determine which metabolic pathways are most affected by OA in males versus females. Metabolites were ranked in order of significance based on their gamma p-value. When connected to the metabolic map, the clustering of metabolites revealed significantly enriched metabolic pathways.

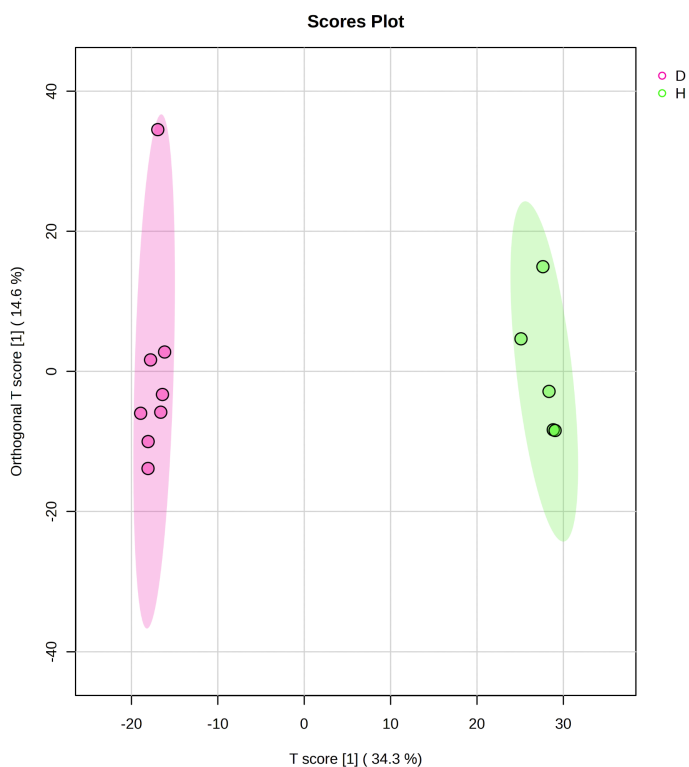
## Results

The initial unsupervised PCA and dendrogram analysis demonstrated a distinct separation between healthy and diseased OA, illustrating a global shift in SF metabolism between healthy and osteoarthritic cohorts (**Figure 1**). The PCA plot showed a clear

separation between OA and healthy SF, with the first two principal components (PC1=37.1%, PC2=15.3%) associated with 52.4% of the variation (**Figure 1A**). Furthermore, the unsupervised dendrogram illustrated a clear separation between diseased and healthy groups, demonstrating distinct metabolic changes between osteoarthritic and healthy SF (**Figure 1B**). OPLS-DA plot analysis demonstrated the separation between healthy and diseased OA, illustrating preliminary metabolic differences between and within patient cohorts (**Figure 2**). This analysis highlighted discriminatory metabolic shifts between healthy and OA SF, allowing for the identification of specific metabolic features that are altered in response to OA. Altogether, dendrogram, PCA discrimination, and OPLS-DA plot analysis clearly demonstrated distinct metabolic profiles between OA and healthy SF with distinct phenotypic differences. Results indicated a global metabolic shift between healthy and diseased individuals (**Figures 1 and 2**), which was further analyzed by investigating sex-specific metabolic alterations.

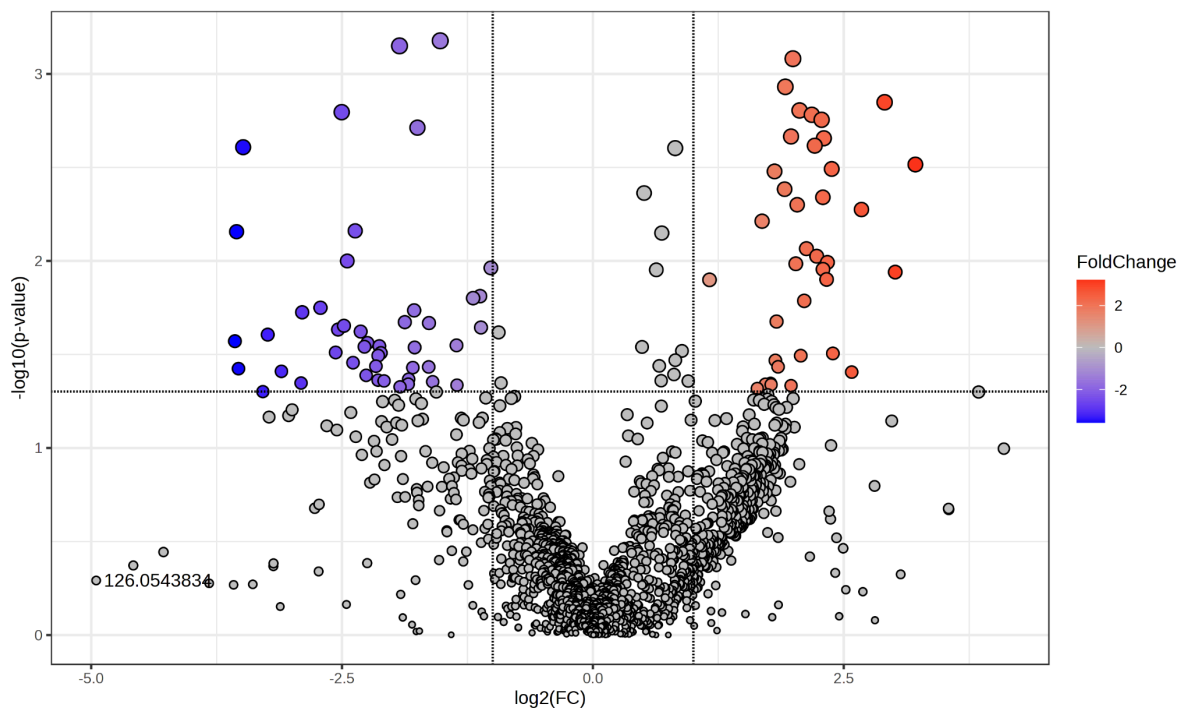


**Figure 1.** Unsupervised statistical plots illustrating variation within and between cohorts for healthy versus diseased (OA) SF. Cohorts correspond to their respective color: pink=diseased SF, green=healthy SF. **(A)** Unsupervised PCA depicts a significant separation between healthy and diseased SF. The x-axis and y-axis Principal Components sum to 52.4% of the variation between cohorts, with PC1=37.1% and PC2=15.3%. **(B)** Unsupervised dendrogram using hierarchical clustering comparing healthy and diseased SF. Pink and green indicate diseased (D) and healthy (H), respectively.



**Figure 2.** OPLS-DA highlights a distinct separation between diseased (D) and healthy (H) cohorts along the predictive component (x-axis), with the T score accounting for 34.3% of the variation between healthy and diseased cohorts. Pink and green indicate diseased (D) and healthy (H), respectively.

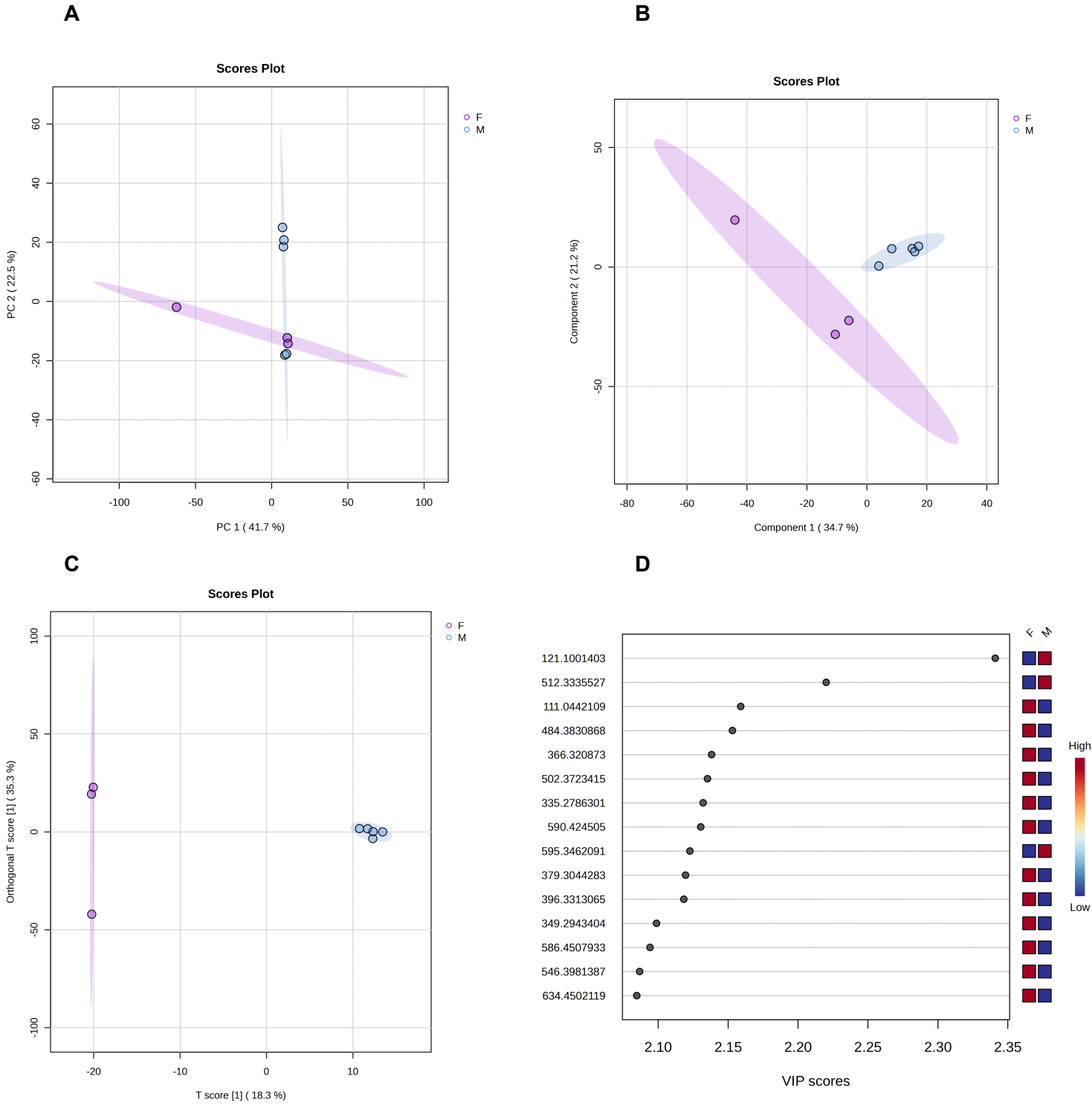
Volcano plot visualization demonstrated the most significant upregulated and downregulated metabolites between diseased male and female SF (**Figure 3**). The volcano plot with fold change analysis was used together with PLS-DA to identify specific metabolic shifts between diseased SF cohorts. Fold change (FC) analysis involved a two-group comparison of the ratio of metabolite intensities. Results identified 37 significantly upregulated ( $FC > 2$ ) and 46 significantly downregulated ( $FC < 0.5$ ) metabolites between diseased male and female cohorts (**Figure 3**). PLS-DA plot analysis demonstrated group variation between male and female diseased synovial fluid. The clear separation between the cohorts demonstrated global shifts in detectable metabolic features. The diseased male cohort demonstrated more compact grouping in comparison to the diseased female cohort. The more widespread metabolic profile of the female cohort demonstrated greater variability within the group when compared to the diseased male population (**Figure 4**).



**Figure 3.** Volcano plot of diseased (OA) male vs. female SF, plotting  $-\log(p\text{-value})$  against  $\log_2(\text{fold change ratio})$  of individual metabolites. Vertical dashed lines mark the twofold change, and the horizontal dashed line marks the cutoff  $p$ -value of 0.05. Metabolites in the upper right and left quadrants represent metabolites with a  $p\text{-value} < 0.05$  and greater than twofold change. 46 metabolites were significantly downregulated (upper left quadrant), and 37 metabolites were significantly upregulated (upper right quadrant) in diseased female SF compared to diseased male SF ( $p\text{-value} < 0.05$ ; fold change  $> 2$ ).

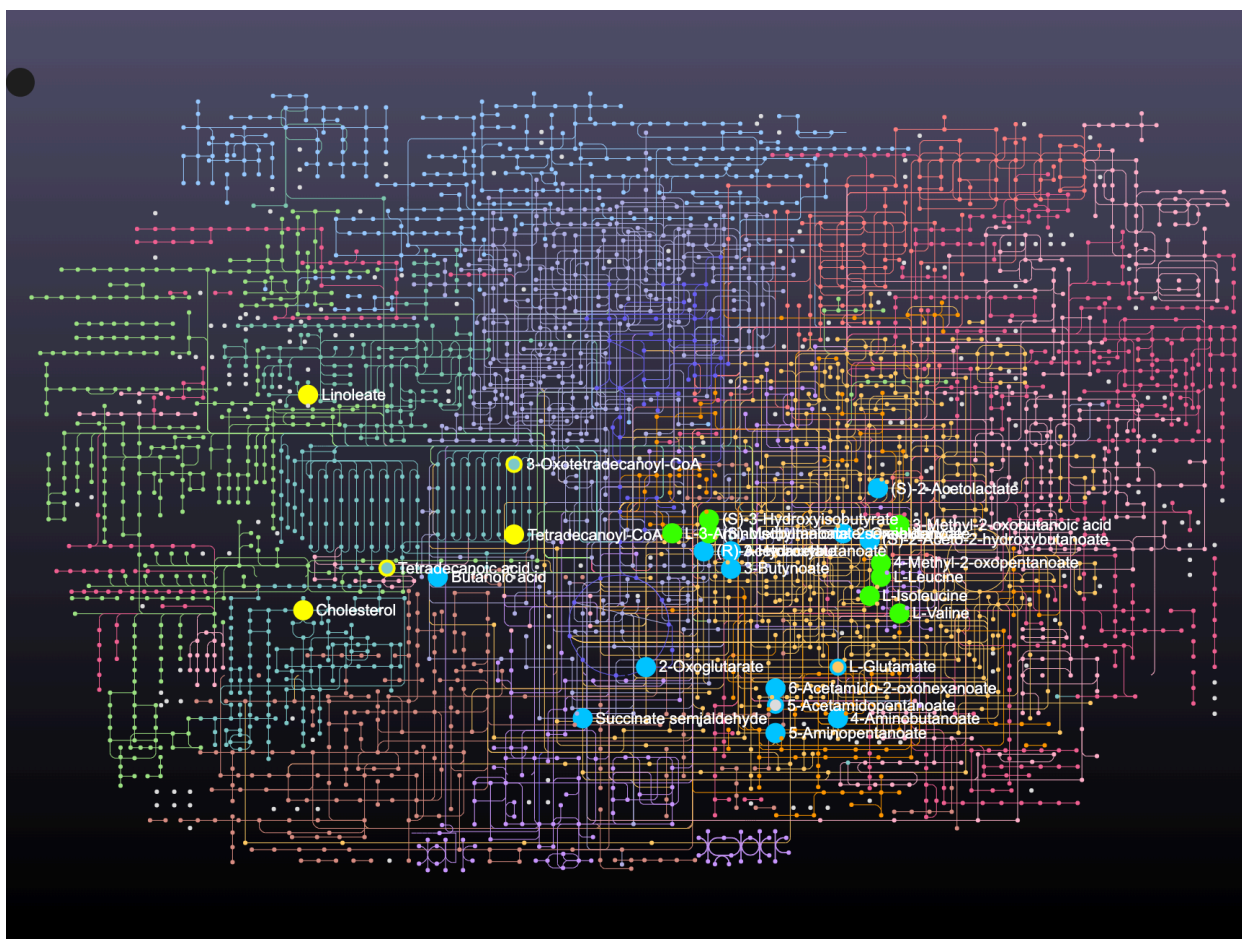
The diseased male and female SF showed distinct group separation between male and female osteoarthritic SF, with a larger variation among female metabolites. Diseased male SF samples illustrated tighter grouping in comparison to diseased female feature grouping, as shown by PCA analysis (**Figure 4A**). The diseased male and female cohorts were largely separated with no apparent overlap, demonstrating sex discrepancies in metabolism within OA synovial fluid. The PLS-DA plot comparing OA male vs. female samples highlighted a distinct separation between cohorts along the predictive component (x-axis), with the predictive component accounting for 18.3% of the variation between healthy and diseased cohorts. Results of the PLS-DA plot analysis (**Figure 4B**) were further supported by OPLS-DA plot analysis, demonstrating distinct within and between group separation between diseased male and female cohorts (**Figure 4C**). The OPLS-DA plot was used to visualize sex discrepancies within and between diseased SF, and

Variable Importance of Projection (VIP) scores were used to identify a list of the most important discriminatory metabolites between diseased male and female SF (**Figure 4D**).



**Figure 4.** Statistical plots illustrating distinct metabolic profiles between OA male and female SF cohorts. **(A)** Unsupervised PCA plot of metabolite intensities within and between diseased males and females. Metabolite clusters show distinct separation and minimal overlap between male and female SF. Cohorts correspond to their respective colors: purple=diseased female SF, blue=diseased male SF. The x-axis and y-axis Principal Components sum to 64.2% of the variation between cohorts, with PC1=41.7% and PC2=22.5%. **(B)** Supervised PLS-DA plot depicting distinct metabolic profiles and discriminatory metabolites between male and female SF. Components 1 and 2 account for 34.7% and 21.2% of the variation, respectively, totaling 55.3% variation between cohorts. Different colors represent their respective groups with purple=diseased female SF and blue=diseased male SF. **(C)** Supervised OPLS-DA analysis further separates diseased male and female cohorts via important discriminatory metabolites illustrating distinct metabolic shifts. **(D)** Variable Importance in Projection (VIP) scores, shown on the x-axis, were obtained from OPLS-DA to generate a list of top discriminatory metabolites (y-axis). Red and blue indicate high and low intensities, respectively, in females (F) or males (M).

VIP scores obtained from the OPLS-DA plot were entered into Mummichog for functional analysis, with results indicating a significant upregulation of metabolites involved in fatty acid metabolism, amino acid metabolism, and butanoate metabolism (**Figure 5**). The metabolic map illustrates three of the most significant metabolic pathways contributing to the sex-specific metabolic discrepancies between diseased SF cohorts, with fatty acid metabolism, valine, leucine, and isoleucine degradation, and butanoate metabolism being upregulated in diseased females. The dots illustrate the metabolites associated with their respective pathways, with three of the most significantly upregulated pathways being mapped through functional analysis. These pathways included fatty acid metabolism (yellow), valine, leucine, and isoleucine degradation (green), and butanoate metabolism (blue) (**Figure 5**), highlighting their role within the complexity of the human metabolic network.



**Figure 5.** Metabolic map for diseased males versus females generated from VIP scores obtained from OPLS-DA. The different colored dots represent the respective metabolic pathways: yellow=fatty acid metabolism, green=valine, leucine, and isoleucine metabolism, and blue=butanoate metabolism.

Through pathway enrichment and functional analysis of the generated VIP scores, **Table 1** was generated (FDR corrected  $p$ -value $<0.05$ ) to show discriminatory metabolic pathways between male and female cohorts. The most notable differential metabolic pathways between males and females with OA included fatty acid metabolism, valine, leucine, and isoleucine degradation, butanoate metabolism, fatty acid biosynthesis, urea cycle/amino group metabolism, arginine and proline metabolism, lysine metabolism, omega-3 fatty acid metabolism, aspartate and asparagine metabolism, fatty acid oxidation, peroxisome, phytanic acid peroxisomal oxidation, fatty acid activation, and tyrosine metabolism.

**Table 1.** Pathway enrichment analysis of OPLS-DA VIP scores comparing diseased SF between male and female cohorts, including the number of metabolites detected and the

false discovery rate (FDR)-adjusted p-value. Pathways enriched in female compared to male diseased SF.

Pathway	Metabolites Detected	FDR p-value
Fatty acid metabolism	9	0.01544
Valine, leucine and isoleucine degradation	10	0.01584
Butanoate metabolism	11	0.016167
De novo fatty acid biosynthesis	9	0.018407
Urea cycle/amino group metabolism	14	0.022774
Arginine and proline metabolism	10	0.028128
Lysine metabolism	9	0.028429
Omega-3 fatty acid metabolism	5	0.029938
Aspartate and asparagine metabolism	19	0.030361
Fatty acid oxidation, peroxisome	4	0.030451
Phytanic acid peroxisomal oxidation	4	0.030451
Fatty acid activation	12	0.0413
Tyrosine metabolism	26	0.048851

Global metabolic profiling detected 1528 distinct metabolite features across all osteoarthritic male and female SF samples. Unsupervised analysis allowed for unbiased analysis of all metabolite changes, whereas supervised plots such as OPLS-DA allowed for the identification of specific metabolites that differed between diseased male and female cohorts. Osteoarthritic male and female SF was analyzed via PCA, OPLS-DA, and a dendrogram, all demonstrating distinct metabolic shifts between male and female cohorts (**Figure 1 and Figure 4**). Unsupervised analysis, such as PCA, showed overall variation within the data as well as depicted distinct cohort separation between healthy and diseased SF (**Figure 1 and Figure 4**). In comparing healthy and diseased SF, PCA plots indicated more variability (**Figure 1**), while OPLS-DA analysis demonstrated more compact clustering between healthy and OA synovial fluid samples (**Figure 2**). Additionally, PCA plots demonstrated further group distinction between diseased male and female SF (**Figure 4A**). Variability within the data set was visualized by the compact clustering of data points within each cohort, with more widespread cohort separation highlighting greater variability. Similarly, in the subpopulation comparison of SF from diseased males and females, PCA plots indicated distinct cohort separation with larger

variability compared to the corresponding OPLS-DA plot. OPLS-DA analysis demonstrated tighter cohort groupings and more noticeable separation, indicating global shifts in metabolism (**Figure 4C**). These results illustrate a detectable shift in metabolism between healthy and OA SF, with even further metabolic separation between male and female diseased SF, specifically linked to fatty acid metabolism, amino acid metabolism, and inflammatory pathways (**Table 1**).

## **Discussion**

To our knowledge, this study is the first to analyze the sex-specific global metabolic differences in osteoarthritic SF. This investigation aimed to elucidate the global metabolic shifts between diseased male and female SF and to identify sex-specific biomarkers of OA. Furthermore, this work sought to discover sex-specific perturbations in metabolism to gain insight into OA pathogenesis and progression in male and female cohorts.

Overall, we detected a total of 1528 discriminatory metabolic features between diseased male and female cohorts. These metabolites were mapped to over 10 significant metabolic pathways (**Table 1**), suggesting a sex-specific response to OA. As evidenced by the unsupervised (PCA) and supervised (OPLS-DA, PLS-DA) plot analysis, healthy and osteoarthritic SF showed significant global metabolic shifts between cohorts. This separation allowed for the investigation of the metabolic differences between diseased male and female SF, specifically. Supervised plots illuminated specific differences in metabolite features in response to OA. The obtained VIP scores, which illustrated the most significant discriminatory metabolites between cohorts, were used to map the metabolic pathways perturbed in response to OA. The most notable metabolites were linked to specific biological pathways including pathways involving fatty acids, amino acids, urea, and peroxisomes, among others. This study elucidated the physiological effects on SF metabolism in response to Osteoarthritis. More specifically, the map of metabolic pathways from the SF of Osteoarthritic individuals identified metabolites that were linked to fatty acid synthesis, amino acid metabolism and extracellular matrix (ECM) remodeling, and inflammatory responses.

### *Amino Acid Metabolism and the Extracellular Matrix*

Amino acid metabolism refers to the assembly and degradation of the primary unit of proteins. When metabolized, amino acids may then participate in the generation of ATP, glucose, and fatty acids<sup>23</sup>. Importantly, this is directly related to other energy pathways, which have also been shown to be affected by OA. Additionally,

branched-chain amino acid (BCAA) metabolism is upregulated in those affected by OA. Using OA modeled in sheep, researchers found that changes to the anterior cruciate ligament transaction (surgically modeled OA) induced physiological changes in metabolism associated with OA<sup>19</sup>. These findings depict knee-associated changes that play a role in the presentation and progression of OA. Interestingly, previous research on amino acid metabolism suggests sex-specific responses in amino acid metabolism. A recent study found that BCAA to histidine metabolism was upregulated in females<sup>17</sup>; therefore, it is likely that there will be an upregulation in amino acid metabolism overall, but it will be most upregulated in the synovial fluid samples from females.

Notable amino-acid-related metabolic pathways altered in osteoarthritic synovial fluid indicate extracellular matrix (ECM) remodeling, which was expected to be higher in female subjects due to their elevated energy requirements<sup>24</sup>. These pathways included aspartate and proline metabolism; valine, leucine, and isoleucine degradation; and tyrosine metabolism. ECM regeneration reconstructs the collagen network and proteoglycans, which places increased energy demands on chondrocytes. Central metabolism and tissue repair rely on three main intracellular pathways: glycolysis, the pentose phosphate pathway, and the TCA (tricarboxylic acid) cycle<sup>22</sup>. In order to maintain proper cartilage, musculoskeletal cells, such as chondrocytes, require amino acids and other preliminary biomolecules for synthesis. The expected perturbations in valine, leucine, and isoleucine metabolism were also distinct between diseased male and female SF, demonstrating an underlying sexual dimorphism in the metabolic response to OA and the ability of cartilage to repair itself. Furthermore, functional amino acids are beneficial and would play an anti-inflammatory role in healthy tissues. OA development was linked to alterations in amino acid metabolism that included glutamate and arginine amino acids as well as their related metabolites which are known to be involved in cartilage remodeling, such as creatinine and hydroxyproline<sup>16,25</sup>. Therefore, these results are consistent with ECM remodeling as amino acid metabolism showed significant variation between diseased male and female synovial fluid. VIP scores generated from OPLS-DA analysis further demonstrated tyrosine, aspartate, and arginine metabolism as significantly enriched pathways in female OA synovial fluid, indicating sex-based discrepancies in the TCA cycle within diseased SF<sup>22</sup>.

#### *Fatty Acid Metabolism and Energetic Considerations*

Energy metabolism in humans confers sex-specific functions that may be indicative of the unique requirements in females for gestation and lactation<sup>24</sup>. This variation is largely due to the actions of sex chromosomes and sex-specific hormones such as estrogen and progesterone in females as well as testosterone in males<sup>24</sup>. Studies indicate a major difference between sexes regarding body-fat distribution and energy substrate utilization patterns<sup>24</sup>. Females tend to store more lipids and have higher

whole-body insulin sensitivity compared to males. These results support the significant variation in lipid-specific metabolic pathways between diseased male and female synovial fluid. Such pathways included fatty acid metabolism, de novo fatty acid biosynthesis, and omega-3 fatty acid synthesis. Results indicated an increase in energy-related metabolites across all diseased individuals compared to healthy SF. This distinction was further analyzed with results indicating a significant difference in metabolic features between diseased male and female SF. Because females tend to have higher energy requirements, we expected the diseased female cohort to illustrate greater metabolic alteration in tissue maintenance and repair pathways when compared to diseased males. The tricarboxylic acid (TCA) cycle is an energy-related series of biochemical reactions that releases energy in compounds to produce ATP. TCA metabolism is an essential step in cellular respiration and the generation of ATP. Previous studies have shown that glutamine is upregulated in OA<sup>20</sup>. Significantly, glutamine can be converted into alpha-ketoglutarate, an essential TCA intermediate; and increased levels of glutamine suggest an elevated production of ATP. This often marks extracellular matrix repair which is associated with OA. Furthermore, glutamine is required for glycosaminoglycan (GAG) production. Elevated levels of glutamine in the synovial fluid could potentially suggest reduced GAG production because, if glutamine is not being used for the production of GAGs, it will remain in the synovial fluid<sup>26</sup>. Another TCA-related pathway responsible for the alteration of SF metabolism in response to OA was butanoate metabolism. Butanoate is a short-chain fatty acid involved in pyruvate and acetyl-CoA synthesis, which are important in cellular respiration<sup>22</sup>. Furthermore, results indicated perturbations in the urea cycle, amino group metabolism, and butanoate metabolism, all of which are indicative of TCA cycle activation to generate more ATP in response to an increased energy demand<sup>17</sup>.

Glucose metabolism is the increase of sugar breakdown by cells, and glucose is a necessary component of cellular respiration. Previous studies demonstrate a significant increase in glucose metabolism that coincides with the severity of OA<sup>16,17,18</sup>. One study found that the pyruvate kinase M2 (*PKM2*) gene was reported to be upregulated in OA-affected chondrocytes<sup>17,18</sup>. *PKM2* codes for pyruvate kinase and is a pivotal part of glycolysis regulation. The increased presence of *PKM2* suggests an upregulation in glycolysis; therefore, we expected an upregulation in metabolites associated with glucose metabolism.

Changes in fatty acid metabolism and oxidation may be indicative of increased energy demands required to regenerate osteoarthritic cartilage constituents. Studies have previously shown that OA patients have lower levels of fatty acid in serum when compared to healthy individuals. Because females tend to have higher rates of triglyceride anabolism, female osteoarthritic SF was expected to contain even lower

levels of circulating fatty acids, which would lead to more noticeable metabolism alterations between male and female osteoarthritic samples.

Females tend to have increased energy demands and higher levels of adipose tissue<sup>24</sup>. Our data indicates a significant difference in fatty acid metabolism between diseased male and female cohorts, indicating a potential increase in energy required to incorporate free fatty acids into triglycerides, as supported by previous research<sup>16,18</sup>. Therefore, women are expected to have an increased energy expenditure. Furthermore, previous research indicates that when energy requirements increase (such as during ECM remodeling), females oxidize more lipids relative to carbohydrates, further supporting significant differences in fatty acid metabolism between diseased male and female cohorts<sup>16,18,27</sup>.

A fundamental sex difference exists in the utilization of carbohydrates and lipids as a fuel source<sup>16,27</sup>. Previous research shows that females tend to incorporate free fatty acids into triacylglycerides, while males under the same conditions tend to oxidize circulating free fatty acids. Results of our study indicated an increase in energy metabolism between diseased males and females. Despite the increased energy requirements of females and differential energy sources, the abundance of estrogen further plays a role in modulating OA progression. Currently, more attention has been drawn to the role of sex hormones in OA pathogenesis, with studies indicating that estrogen modulates changes in OA by inhibiting the degradation of the ECM. Reduced estrogen levels, specifically in menopause, may further exacerbate the phenotypes of OA and further degrade cartilage integrity<sup>29</sup>. The diseased female cohort in this research contains post-menopausal females who have decreased estrogen levels. Therefore, estrogen deficiency may contribute to the overall variation in energy-related metabolites such as fatty acids and amino acids.

### *Metabolites Linked to Inflammatory Responses*

Based on mapped metabolic pathways, synovial fluid from osteoarthritic patients contained expected metabolites consistent with the inflammatory nature of the disease. These metabolites were then mapped to fatty acid metabolism and omega-3 fatty acid metabolism via pathway enrichment, which are involved in the inflammatory response<sup>3</sup>. Specifically, eicosanoid pathways are associated with the innate immune response and produce lipid-based biomarkers of inflammation<sup>29,30</sup>. These inflammatory phenotypes are consistent with OA pathogenesis and demonstrate a metabolic response to cartilage damage. Previous studies have shown that cartilage fragments from diseased cartilage may be seen as foreign bodies and therefore trigger a secondary immune and inflammatory response<sup>29,31</sup>; therefore, it follows that eicosanoid metabolism is

upregulated. Furthermore, when observing leukocyte biomarkers in symptomatic knee OA, researchers found significant upregulation of prostaglandins E2 (PGE2) and 15-hydroxyeicosatetraenoic acid (15-HETE) in diseased samples<sup>31</sup>. Fatty acids commonly act as cell surface and intracellular receptors and signaling molecules that control inflammation. In summary, an upregulation in metabolites that link to the eicosanoid pathway is expected within the diseased synovial fluid. There have been no studies that examine the difference in eicosanoid metabolism between males and females; however, based on the current research, it is likely that females will have a more severe response to OA and will have a larger upregulation of the eicosanoid pathway in comparison to males. Upregulation of fatty acid oxidation, butanoate metabolism, and peroxisomal oxidation are all indicative of an upregulation of the inflammatory response.

### *Limitations*

Although this study has identified significant metabolic shifts between diseased male and female SF, there are important limitations to consider. First, the small sample size employed in this study makes it difficult to draw broader conclusions regarding the perturbed metabolic pathways. Furthermore, these patients were all older than 60 years old. Specifically for the female cohort, these individuals are all in the post-menopausal age range, which would exacerbate their estrogen deficiencies. Thus, this study is limited by potentially confounding variables. Future research should utilize donor samples of all ages to determine if similar metabolic pathways are altered. Additionally, follow-up studies should analyze larger sample sizes to confirm sex-related differences in metabolism within the diseased synovial fluid. Finally, this study used only global metabolomics to map metabolic features to their potential identities and pathways. Follow-up analyses should employ targeted metabolomics to confirm the results of this study, particularly regarding specific metabolites associated with fatty acid metabolism, amino acid metabolism, and the immune response.

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