RESEARCH ARTICLE

Preliminary Study for the Early Diagnosis of Osteoarthritis in Human Synovial Fluid Using ATR-FTIR Combined with Chemometrics

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Abstract

Objectives: Osteoarthritis (OA) is the most common form of arthritis resulting in joint deterioration. Currently, OA treatment primarily focuses on symptom management. The development of tools suitable for diagnosis is needed to support a paradigm shift towards the prevention of OA rather than treatment. Therefore, having effective diagnostic tools for early detection of OA is crucial.

Methods: The present study aimed to develop a rapid, inexpensive, and reliable detection method using attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy and chemometrics using synovial fluid (SF) for the early diagnosis of osteoarthritis (OA). A preliminary sample consisting of 10 participants in the OA group and 10 in the non-OA group was used to establish the feasibility of the method. All synovial fluid samples were handled uniformly, using fresh drops of 50 µL from each sample ten times, resulting in a total of 200 infrared spectra collected and analysed, revealing significant differences that effectively separated the OA and non-OA groups, demonstrating the potential of this approach for future larger-scale studies.

Results: A significant discrepancy seen in distinguishing SF samples from different categories via variance spectra specifically highlighted by wavenumber 551 cm-1. The predictive model achieved an accuracy rate of 85%, demonstrating promising results.

Conclusion: Our findings suggest that a discriminative model using the ATR-FTIR spectrum could enhance early diagnosis of human OA, providing superior results compared to using serum. This approach reflects the localized joint condition more accurately than serum, which reflects systemic condition.

Level of evidence: IV

Keywords: Chemometrics, FTIR, Infrared spectroscopy, Osteoarthritis, Synovial fluid

Introduction

OA is the most common degenerative joint disease, which has the pathological changes including progressive loss and destruction of articular cartilage, thickening of the subchondral bone, associated with pain and disability.[1] OA is considered incurable as there are currently no medications available to halt or reverse the loss of cartilage or bone, and treatment options primarily focus on pain management and end stage surgical intervention such as total knee replacement which is the gold standard procedure for OA as there are no effective therapeutic treatments for OA.[2] While OA is the most common joint disease in the world,[3] clinics still lack a sensitive and accurate diagnostic procedure. Currently, the diagnosis and grading of OA frequently rely on the

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combination of clinical indicators, physical evaluation, and assessment via X-rays. This includes observing features like space narrowing, osteophyte formation, and bone-end irregularities. However, X-ray imaging offers restricted insight into non-mineralized tissues, such as cartilage, which is crucially affected in this condition.[4] Researchers have been consistently exploring potential biomarkers in the early stages that could indicate the onset of the disease; however, no robust biomarkers have been identified so far.[5] Thus, there is an unmet clinical need for studies of the alternative treatments for early-stage OA.

Many physiological and pathological alterations may manifest in the initial phase of OA, serving as crucial indicators for early detection.[6] Wang et al.(2024)[7] reported that currently, sensitive biomarkers for early-stage of OA are lacking, resulting in clinical diagnosis being made during the later stages of OA. Studies have shown a correlation between serum molecules and both the presence and severity of OA.[8] Building on this, Aziz et al.(2023)[2] conducted a study to verify the effectiveness of using serum-based screening as an early diagnostic tool for OA. Measurement of these serum biomarkers aids healthcare professionals in evaluating the status of OA patients, monitoring disease progression, and evaluating the effectiveness of treatments. Besides that, molecules found in SF also hold relevance to OA.[7] Given that SF envelopes the entire joint cavity, it serves as a vital diagnostic tool for evaluating tissue turnover, providing a more direct or localized assessment compared to blood.[9] Nevertheless, the utilization of human SF for early OA diagnosis remains uncertain, yet it holds promise as a potential detection tool.

Fourier transform infrared (FTIR) spectroscopy is a valuable tool for studying biochemical variations by providing detailed information about the molecular composition and structure of biological samples[4] and how they change when a pathological state arises. The advantages of FTIR include its non-destructive nature, rapid data acquisition, and the ability to analyze complex biological materials without extensive sample preparation. These features make FTIR particularly suitable for investigating diseases such as OA.

While the application of FTIR for osteoarthritis analysis shows promise, it presents several challenges. One major concern is the potential interference from water in biological samples, such as synovial fluid. Water has strong absorption bands that can overlap with the spectral features of other biomolecules, which may obscure critical indicators of osteoarthritis. To address this, utilizing chemometric methods to analyse spectral features enables the detection of differences that are characteristics of a disease or natural state of a biological sample.[10] It can be used to detect biological patterns based on the infrared-active organic molecular bonds in biological fluids.[11] This approach has demonstrated promising outcomes as a potential screening tool for diagnosing human arthritic disorders based on SF.[12] By establishing a baseline spectrum from healthy samples, we can effectively compare it to the spectra of OA samples. This comparison improves the accuracy and reliability of the diagnostic process, allowing us to identify significant differences that indicate the presence of OA.

This study is preliminary in nature, and the use of a smaller sample size is justified as a preliminary step before scaling up to a larger cohort. Previous research has also employed small sample sizes in preliminary studies to establish foundational data before broader implementation. For instance, Hou et al. (2016)[13] conducted a chemometric analysis of canine synovial fluid for osteoarthritis detection using a small sample size of 10, justifying this approach by the need for preliminary data prior to scaling up. Similarly, Panizzi et al. (2022)[14] utilises infrared spectroscopy using 9 samples of serum from equine model. SF was prioritized in this study as it directly reflects biochemical changes within the joint microenvironment, offering a localized and early indication of OA progression. While serum and urine are more accessible, they primarily capture systemic changes and may lack the sensitivity to detect early joint-specific alterations. Cartilage biopsies, though informative, are highly invasive and not practical for early-stage diagnosis. Previous studies, such as Kalogera et al. (2023),[9] have shown that biomarkers detected in SF exhibit stronger associations with joint pathology compared to serum. Building on this, our study explores the use of ATR-FTIR spectroscopy combined with chemometrics to analyze SF, aiming to enhance early detection of OA. Thus, the aim of this study is to evaluate the potential of SF as tool to differentiate between OA and non-OA associated with early detection of OA using FTIR combined chemometrics.

Materials and Methods

Ethics approval

The Medical Research Ethics Committee, University Malaya Medical Center (MREC ID No: 2016927-4288) approved this prospective observational cohort research.

Study Setting

The study took place at the University Malaya Medical Center (UMMC), a large teaching hospital in Malaysia. Patient recruitment and sample collection were conducted between May-July 2024 in the Orthopedic Clinic, UMMC.

Sample size

The sample size for this study was set at 20 participants (10 with osteoarthritis [OA] and 10 without OA), determined using G*Power analysis to achieve a power of 95%, based on a medium effect size (Cohen's d = 0.5). The choice of a 95% power level, which exceeds the traditional 80% used in medical research, was made to enhance the robustness of the study's findings despite the relatively small sample size.[4,6]

Patient recruiting

Inclusion criteria for the OA group included average age of 40-60, gender (female or male), OA stage (end-stage/stage 4), primary OA, location of interest (knee joint), and we are focusing on a patient needing a knee replacement. Meanwhile, the exterior criteria include inflammatory diseases (rheumatoid arthritis), age below 40, early stage of OA, secondary OA, location of interest (hips and hands), and secondary OA. Ten patients underwent an arthroscopy of the knee were recruited for OA group. Prior to arthroscopy, the degree of arthritis in the surgical knee was evaluated on a posterior anterior (PA) radiograph by the orthopaedic surgeon, using the

Kellgren-Lawrence (KL) classification. To recruit patients for the non-OA group, individuals with ACL injuries were selected. The control group (n = 10) underwent thorough physical and orthopaedic examinations, confirming the absence of orthopaedic abnormalities associated with OA.

Sample Preparation

Under IRB (Internal Review Board) protocol, SF was withdrawn from consented patients following the induction of anesthesia and before the arthroscopy and ACL procedure from multiple clinically normal joints in each patient and stored at -80 °C in plain cryovials for later batch IR spectroscopic analysis. Each group used a volume of 50 µL SF for spectroscopic analysis. All synovial fluid samples were obtained intraoperatively from patients undergoing planned arthroplasty (for the OA group) or ACL repair procedures (for the non-OA group). Collection was performed after induction of anesthesia and prior to surgery, ensuring that no additional invasive procedures were carried out solely for research purposes. This approach minimized any added patient risk while allowing access to synovial fluid directly from the joint environment.

Minimizing Bias Strategies

To minimize selection bias, the control group (non-OA) was carefully chosen from patients undergoing ACL surgery, as these individuals have a low likelihood of developing osteoarthritis. To address measurement bias, all synovial fluid samples were handled uniformly, using fresh drops of 50 µL from each sample ten times to enhance reliability. Additionally, all scans underwent consistent pre-processing, including baseline corrections and smoothing, to further reduce bias. The FTIR operator was blinded to the sample group (OA vs. non-OA) to minimize observer bias. To mitigate technical bias, we used the same FTIR machine and consistent parameters for all samples, ensuring that the analytical conditions remained uniform throughout the study.[15,16]

ATR FT-IR Spectral Acquisition

This process was conducted using an ABB MB3000 FTIR spectrometer equipped with a diamond crystal of the ATR GladiATR platform to enhance reliability. An amount of 50 µL SF was put in touch with the diamond crystal at 20°C. The scanning was carried out in the mid-infrared range of 4000 cm^{-1} to 450 cm^{-1} to at 4 cm^{-1} resolutions. A total of 200 produced absorbance spectra were pre-processed using Horizon MB FTIR software version 3.0.13.1 to facilitate the differentiation process among samples. All spectra were baseline and smooth corrections to obtain a better spectrum. The spectra were analysed for chemo metrics analysis.

Variance spectra

Spectral data were pre-processed to ensure uniform formatting and organization. Variance across spectral wavelengths was calculated to quantify the dispersion of data points. Variance values were optionally normalized for comparative analysis. These values were then plotted against wavelength to create a Variance Spectrum Plot, facilitating the identification of spectral regions exhibiting significant variability. Peaks and dips in the plot indicated wavelengths where spectral features or characteristics of interest may be present. The plot was annotated with labels and titles for clarity and interpretation. This approach provided a visual tool to analyze and interpret spectral data variability, guiding subsequent analyses and insights into sample characteristics. Prior to variance calculation, the spectral data were pre-processed using baseline correction and smoothing. No further normalization (such as min-max scaling or area normalization) was applied before variance spectra computation. Variance was computed at each wavenumber across the replicates within each group (OA and non-OA) to quantify the variability of absorbance intensities. The resulting variance spectra represent the raw variance without additional scaling or normalization to preserve the true magnitude of biochemical differences between groups. The variance values were plotted against wavenumbers to visualize spectral regions with significant variability. Following variance spectra analysis, statistical validation of key wavenumbers was performed. At each wavenumber, an independent two-tailed Student's t-test was conducted to compare absorbance intensities between the OA and non-OA groups. A significance threshold of $p < 0.05$ was used. Effect sizes were also calculated using Cohen's d to quantify the magnitude of differences, where d values of 0.2, 0.5, and 0.8 correspond to small, medium, and large effects, respectively.

Chemometrics

Data pre-processing

The infrared spectrum data from all samples were converted into CVS and imported to the dataset table in XLSTAT 2016 software. At first, the functional group (4000 to 1701 cm^{-1}) and fingerprint group (1700 to 450 cm^{-1}) were separated. A total of 200 infrared spectrum data from each set of samples were utilized for the first DA to select the most significant wavenumbers from these groups before they were combined.

Kaiser–Meyer–Olkin (KMO) Test

The dataset was analysed for dataset adequacy by KMO test. Adequate dataset determines the ability of a generated model to extract latent variables from the dataset. In this study, the KMO test was employed at a significant level (α) of 0.01. The calculated KMO was ranked as: KMO < 0.5 = inadequate, 0.5 < KMO < 0.7 = mediocre, 0.7 < KMO < 0.8 = good, 0.8 < KMO < 0.9 = very good and KMO >0.9 excellent to indicate the dataset adequacy.[13]

Dataset transformation

To ensure that the dataset follows a normal distribution before performing Principal Component Analysis (PCA), the normality of the dataset was tested using the Shapiro-Wilk test at a significance level of $\alpha = 0.01$. If the data did not meet the normality assumption, normalization was applied using the standard deviation method (n-1) to transform the dataset. Data normalization was performed only if the initial dataset did not conform to a normal distribution, allowing for flexibility in the pre-processing steps based on the data's characteristic.

Dataset exploratory by Principal component analysis (PCA)

In this study, PCA was employed to investigate the Contribution of molecular structures in synovial fluid to differentiate between OA and non-OA groups, and to identify the variance among intercorrelated molecular structures at an α level of 0.05. The dataset was transformed into principal components (PCs), which are new sets of independent variables, and the results were visualized in the form of correlation biplots and molecular structure plots. The cumulative variability (CV) of the two-dimensional PCs, specifically PC1 and PC2, was computed to explore the molecular structures. Following principal component analysis (PCA), we retained two principal components (PC1 and PC2) based on their cumulative variance contribution. The selection of two principal components was guided by the goal of maximizing variance explained while minimizing model complexity, ensuring robustness and interpretability of the subsequent analysis.

The selection of PCs was based on achieving the highest cumulative variability. Molecular structures with factor loadings (FL) greater than or equal to |0.750| were considered to have a strong contribution, those with |0.500| < FL < |0.749| were considered to have a moderate contribution, and those with FL less than or equal to |0.499| were considered to have a weak contribution. Molecular structures with weak FL were excluded from further analysis.

Using the molecular structures with strong and moderate FLs, a new PCA was conducted to produce updated molecular structure plots and correlation biplots. The FL and correlations of the molecular structures were reassessed, and their contributions to differentiating between the OA and healthy groups were further evaluated and explained.

Linear Discriminant Analysis (LDA)

After completing the data pre-processing, the dataset, comprising a total of 200 spectral data, was partitioned into training, cross-validation (utilizing one leave-out cross-validation), and verification subsets. The data distribution was as follows: 70% for training, 10% for cross-validation, testing, and verification, based on a commonly reported distribution.[14-16] In chemometrics, the replication of samples for testing, training, and cross-validation can vary depending on the specific requirements of the analysis and the type of model being developed.[17] Figure 1 illustrated chemometrics plots of the infrared spectra were used in linear discriminant analysis and principal component analysis to develop a prediction model [Figure 1].

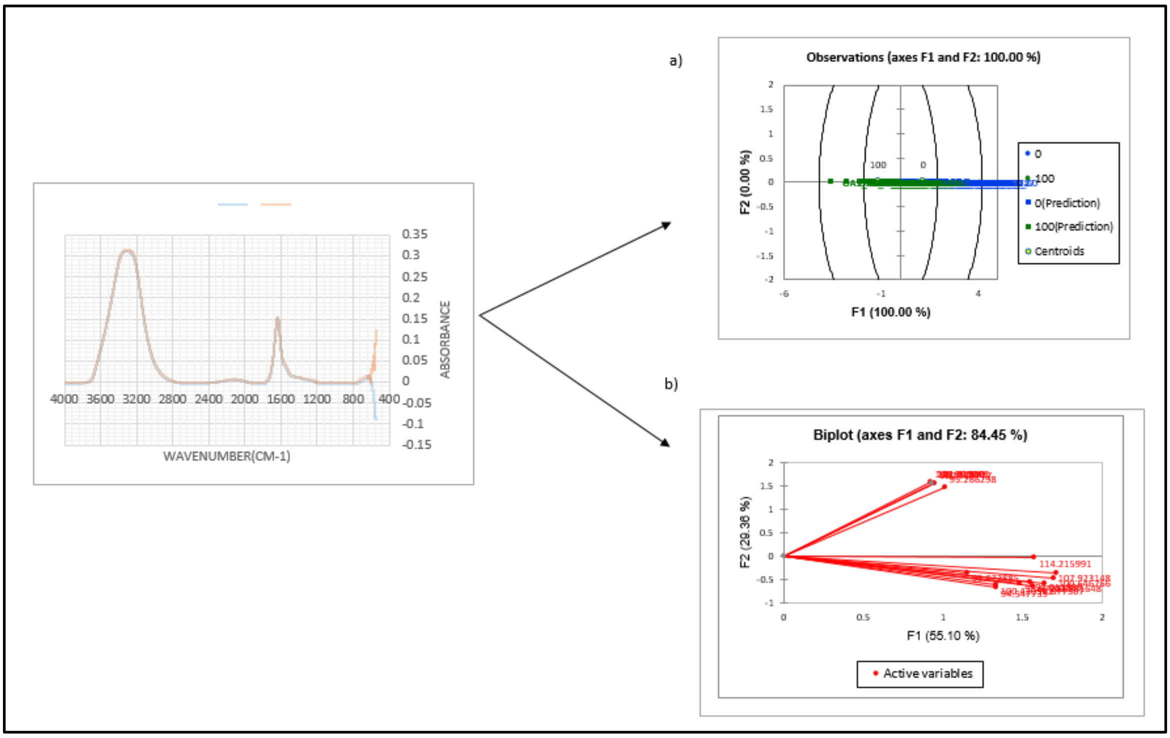

Figure 1. The chemometrics plot depicted in the infrared spectra was utilized for Linear Discriminant Analysis (a) and Principal Component Analysis (b) in developing a prediction model to differentiate between OA and non-OA categories based on human SF

Results and Discussion

Wavenumber Contributing to OA in Human Synovial Fluid

In our previous research, biological samples, specifically serum from OA patients and serum from non-OA group, were analyzed using FTIR spectroscopy as a molecular structure-based tool to discriminate between disease and normal groups based on their distinct chemical structures, particularly in the case of OA. The study demonstrated promising results, with a predictive model achieving an accuracy of 74.47%. In this study, the number of principal components (PCs) retained was two (PC1 and PC2), explaining 89.78% of the total variance observed in the. These two components, effectively summarizing the most significant spectral features differentiating between osteoarthritis (OA) and non-OA groups.

Utilizing chemometric methods, such as PCA, aided in pattern recognition, while DA facilitated differentiation based on chemical bonding presence. This approach successfully discriminated between OA and non-OA group using blood serum samples, indicating potential applicability to other biological fluids in humans. Building upon this foundation, the current study enhances the analysis of SF by incorporating variance analysis and Linear Discriminant Analysis (LDA), in addition to the techniques previously used. SF was chosen due to its representation of the actual condition in cartilage as OA progresses, offering potential for the development of a more accurate prediction model.

In this study, prior to conducting chemometrics analysis on the obtained infrared spectrum data of human SF samples, the Kaiser-Meyer-Olkin (KMO) test was performed to assess the adequacy of the dataset. The result of KMO Test with the value of 0.855 indicated a good sample size, ensuring the robustness of subsequent analyses. Subsequently, principal component analysis (PCA) was employed as an unsupervised method to explore the relationship between wavenumbers and identify significant wavenumbers contributing to OA in human SF.

The PCA analysis, as depicted in Figure 2, revealed several noteworthy findings. Notably, distinct wavenumbers were identified including 1177, 3547, 3544, 3536, 3532, 3529, 3498, 3407, 3222, and 3055 cm^{-1}, were observed to be significant contributors to the separation between OA and non-OA groups. Furthermore, Figure 2 illustrated the clear separation of these wavenumbers into two distinct clusters, with each cluster exhibiting strong correlation among its constituent wavenumbers. This spatial arrangement within the PCA plot suggests a coherent pattern in the distribution of wavenumbers associated with OA pathology, highlighting the potential utility of infrared spectroscopy coupled with PCA for diagnostic purposes in OA [Figure 2].

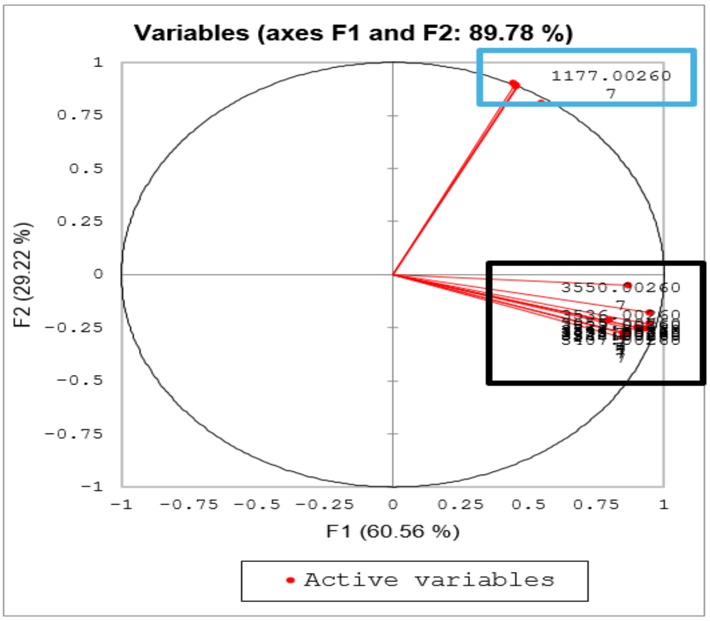

Figure 2. Principal Component Analysis illustrated two clusters of wavenumbers belonging to fingerprint wavenumbers (highlighted in blue) and functional group wavenumbers (highlighted in black

To further comprehend the spectral features indicative of OA category in SF, our study aims to identify specific spectral features associated with OA progression as shown in [Figure 2]. This endeavor involves an extensive literature review to glean insights into the wavenumbers associated with OA pathology. Typically, when scanning SF samples from OA and non-OA groups, significant differences in spectra are observed. However, these differences may be somewhat mitigated when comparing non-OA samples derived from individuals with anterior cruciate ligament (ACL) injuries, who may exhibit similar comorbidities as OA patients. Nonetheless, even subtle differences persist in synovial fluid (SF) samples across groups, owing to the varied chemical structures of the compounds present [Figure 3]. Therefore, attenuated total reflection Fourier-transform infrared spectroscopy (ATR-FTIR) was selected for this study, as it offers a powerful tool for molecular profiling.

Referring to Figure 3, the hydroxyl group is responsible for the distinctive signal at 633 cm^{-1}. These primary vibrational peaks fit the description of hyaluronic acid's distinctive peaks.[18] Repetitive disaccharide units of glucuronic acid and N-acetylglucosamine combine to form hyaluronic acid, a long, linear polymer that makes up a significant portion of SF.[19] The presence of hydroxyl groups (-OH) along the backbone of HA is one of its distinguishing characteristics.[20] Shifts in the location of the hydroxyl group absorption bands could be a sign of changes in the hydration or hydrogen bonding state of HA molecules. These alterations may be a sign of modifications to the interactions or structure of HA in the SF. According to Adachi et al. (2022),[21] the collagen structure of cartilage tissue can be structurally determined by examining the amide III spectral zone within the wavenumber interval of 1170 to 1500 cm^{-1}.

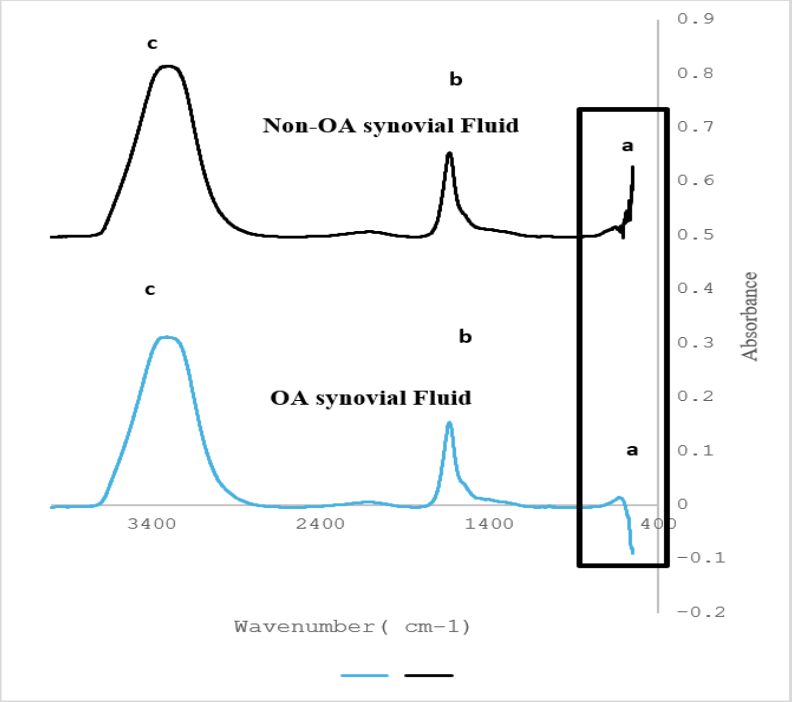

Figure 3. Comparison of FTIR spectra of SF in OA and non-OA group

Calcium phosphate accumulation inside joint tissues, particularly the SF, may be more likely in OA patients.[22] The production of calcium-containing crystals, such as hydroxyapatite or basic calcium phosphate crystals, as a result of this deposition might aggravate joint inflammation and damage and worsen OA symptoms.[23] Phosphate ions may be released from the breaking down of bone tissue during the tissue deterioration process in OA, adding to the total phosphate content in the SF.[24] The fingerprints and functional group obtained from absorption spectra in OA synovial fluid and non-OA synovial fluid were also illustrated in the [Table 1].[16,19,25-27]

Table 1. FTIR wavenumbers, as well as fingerprints and functional groups obtained from absorption spectra, were analyzed in OA SF and non-OA SF.

Peak	Wavenumber (cm^{-1})	Functional group	Compound class	Reference
OA SF				
a	633	O-H stretching	Hydroxyl	[16]
b	1170	C-H stretching	Carbonyl	[19]
c	3547	O-H stretching	Alcohol	[25]
Non-OA SF				
a	564	O-H stretching	Hydroxyl	[16]
b	1650	C=C stretching	Amide	[26]
c	3450	O-H stretching	Amine	[27]

It can occasionally be difficult to visually distinguish minute changes in infrared spectra from several samples or categories, particularly if the overall spectral pattern is similar. Variance spectra can be an effective tool in these situations to draw attention to notable distinctions between two categories or groups of spectra.[28] The statistical examination of several spectra within each category yields variance spectra. They serve as a representation of the variations or variety among the spectra in each group. A variance spectrum can be obtained by deducting one group's mean spectrum from another group's mean spectrum, or by computing the standard deviation or other measures of variability.[29] Figure 4 illustrates the analysis of variance spectra, which highlight the differences in absorbance between the synovial fluid samples from the OA and non-OA groups. The graph displays specific wavenumbers along the x-axis and their corresponding absorbance values on the y-axis. At a wavenumber of 551 cm^{-1}, a notable divergence in absorbance is observed between the two groups. This region reflects the presence of specific biomolecular changes indicative of osteoarthritis pathology. By focusing on these variance spectra, it is possible to effectively demonstrate how certain wavenumbers, particularly 551 cm^{-1}, serve as potential spectral biomarkers for distinguishing between osteoarthritis (OA) and non-OA conditions. Statistical comparison at the wavenumber 551 cm^{-1} revealed a significant difference between the OA and non-OA groups ($p < 0.05$). Furthermore, the effect size (Cohen's d) was calculated as 0.8 (large effect), reinforcing the identification of 551 cm^{-1} as a key discriminative biomarker associated with osteoarthritis progression [Figure 4].

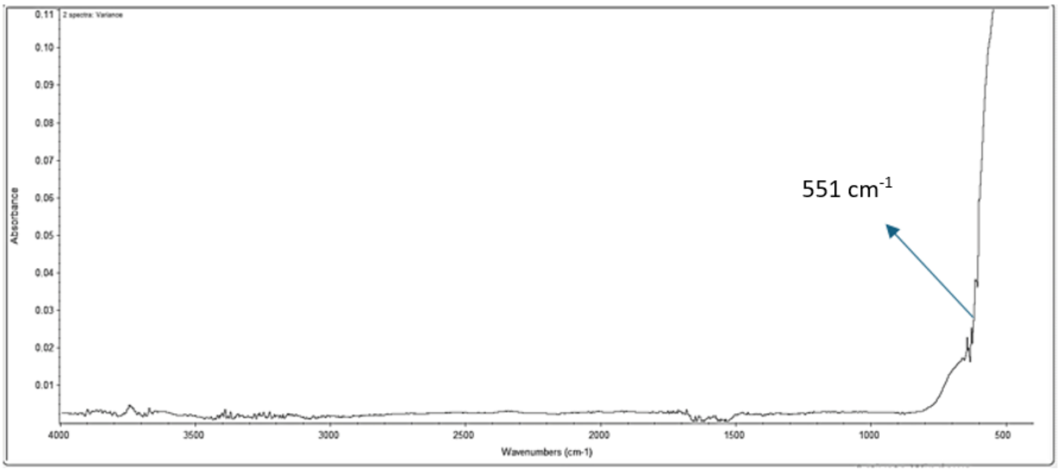

Figure 4.Variance spectra of SF in OA and non-OA

The significant wavenumber identified at 551 cm^{-1} corresponds to vibrational modes associated with hydroxyl groups, notably present in hyaluronic acid (HA), a major component of synovial fluid. In osteoarthritis (OA), HA undergoes depolymerization due to oxidative stress and enzymatic degradation, primarily mediated by reactive oxygen species (ROS)[30] and hyaluronidase enzymes. This degradation leads to a reduction in HA molecular weight and viscosity, impairing the lubricating and shock-absorbing properties of synovial fluid, which contributes to cartilage wear and joint inflammation.[31] Furthermore, fragmented HA can act as a damage-associated molecular pattern (DAMP), activating toll-like receptors (TLRs) and CD44 receptors on synovial cells, thereby triggering inflammatory pathways such as NF-κB signalling.[32] This inflammation exacerbates synovial membrane activation, matrix metalloproteinase (MMP) production, and further cartilage degradation.[33]

As PCA is an unsupervised method, it cannot be solely relied upon. Therefore, it is imperative to incorporate additional data as reference to corroborate the findings. This can be achieved by integrating FTIR spectra alongside variance spectra analysis and biplot graph. Biplot graph is useful to identify which wavenumbers contribute most to the separation between different categories or groups of samples.[34] Upon examining the biplot graph depicted in Figure 5, it is evident that the OA group exhibits higher occurrences of wavenumbers associated with the OA progression in SF compared to the non-OA group. Specifically, the biplot graph of the OA group indicates peaks at 1177, 3055, 3222, 3407, 3498, 3529, 3532, 3536, 3544, and 3547 cm^{-1}. In contrast, the biplot for the non-OA group presents different wavenumbers, denoted as 1000, 1095, 1101,1114,3107 and 3450 cm^{-1} [Figure 5].

Validation and Verification of the LDA Model between OA and Healthy Synovial Fluid Samples

Synovial fluid from OA and non-OA group were classified using Linear Discriminant Analysis (LDA). The LDA classifies these samples by calculating the distance from each class evaluated in distance units. The class of unknown samples to one of the specific classes can be predicted, after classification model is obtained.[35] In this study, LDA model have been established by highlighting wavelengths that can differentiate one source from another, which is known as supervised pattern recognition technique. LDA model as in Table 2 and Figure 6 accurately classified 100.00 % for training test, 85.00 % for cross-validation test, and 75.00 % for testing test to its classes. The developed models were subsequently utilised to predict synovial fluid sample suspected to be derived from OA patient for the purpose of model verification. Decisions were made regarding whether the unknown samples closely aligned with OA and non-OA group. For verification test, the correct classification percentage is 65.00 %. In the confusion matrix, it was noted the number of samples that were wrongly classified. This includes instances where the model incorrectly identified the presence or absence of OA in the SF samples. False positives occurred when the model incorrectly classified a sample as indicating OA when it did not, while false negatives occurred when the model failed to detect OA in samples where it was present [Table 2, Figure 6].

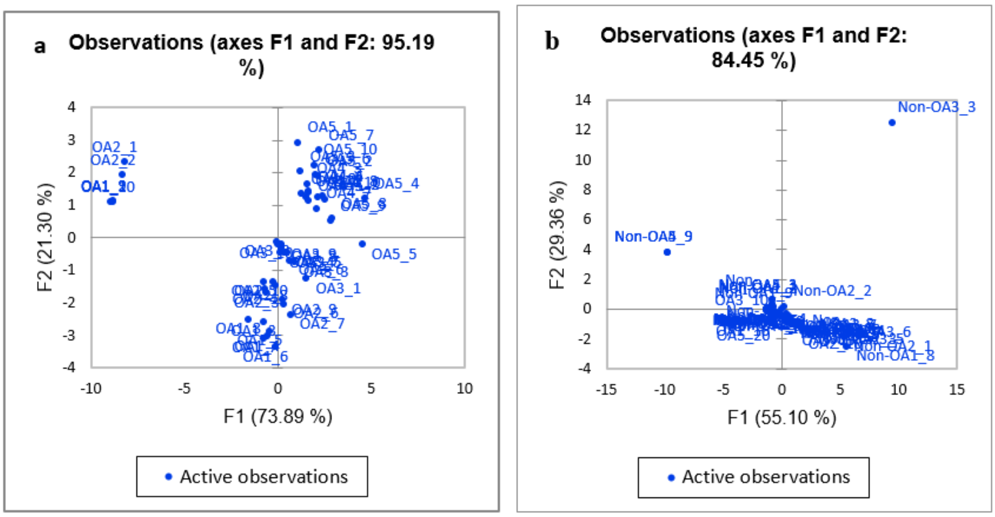

Figure 5. Biplot of OA (a) and non-OA (b) SF

Table 2. Classification matrix of training, validation, testing and verification datasets by DA

Discriminating model (DA model)	Dataset	Correct classification (100%)
DA model: Non-OA and OA SF	**Training dataset**	
	OA	100.00 %
	Non-OA	100.00 %
	Total	100.00 %
	Cross-validation dataset	
	OA	85.00 %
	Non-OA	85.00 %
	Total	85.00 %
	Testing dataset	
	OA	100.00 %
	Non-OA	50.00 %
	Total	75.00 %
	Verification dataset	
	OA	70.00 %
	Non-OA	60.00 %
	Total	65.00 %

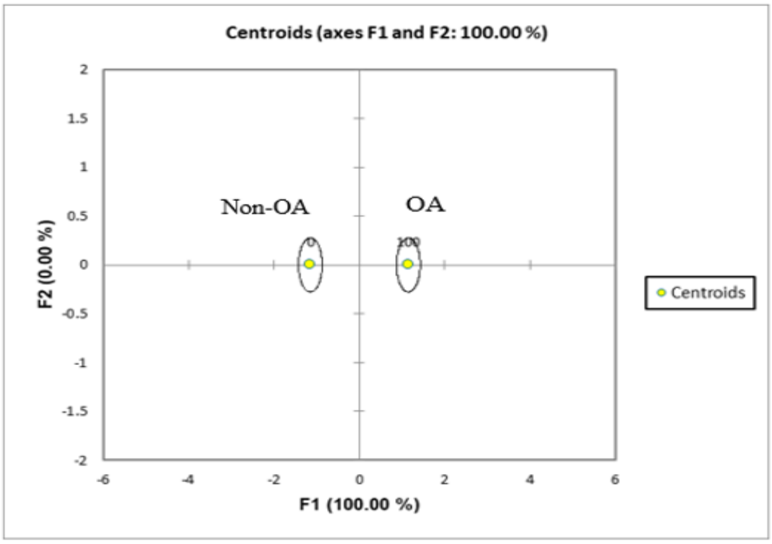

Figure 6. Linear Discriminant Analysis in predicting OA and non-OA group in SF

The Linear Discriminant Analysis (LDA) results indicated that both serum and synovial fluid exhibit distinctive patterns in classifying osteoarthritis (OA) and non-OA samples. Serum analysis achieved classification rates of 100.00% for the training dataset, 74.47% for cross-validation, and 100% for both the testing and verification datasets.[2] In contrast, synovial fluid analysis demonstrated superior classification rates, with 100.00% accuracy for the training dataset, 85.00% for cross-validation, and 75.00% for testing datasets. These findings suggest that synovial fluid may be more suitable than serum for LDA in this context, potentially enhancing diagnostic capabilities.

The cross-validation dataset systematically utilized all replicates for the same physical sample,[36] revealing that synovial fluid exhibited a higher percentage accuracy. This indicates a greater likelihood of reproducibly measuring the same physical sample, as assessing fragments of cartilage and synovial membrane in synovial fluid provides a more direct measure of tissue turnover compared to serum analysis. The cross-validation results for the synovial fluid model further demonstrated higher predictive accuracy than those for serum [2,] indicating that the synovial fluid approach may be more effective in capturing localized pathological changes associated with osteoarthritis.

For verification, the serum dataset achieved 100% accuracy, allowing clear differentiation between unidentified samples resembling osteoarthritis (OA) and those from healthy individuals. While serum alone can clearly distinguish between these groups, the verification dataset for synovial fluid displayed only 65% accuracy, indicating that the distinction between OA and non-OA samples using this model was less clear when analyzing synovial fluid. However, synovial fluid serves as a valuable tool for diagnosing and monitoring osteoarthritis. According to Solarino et al. (2022),[37] synovial fluid acts as a reservoir for disease-related proteins that could serve as potential biomarkers for various articular diseases. Unlike serum, synovial fluid is in close proximity to joint tissues, which experience significant changes during these diseases. This localization allows synovial fluid to capture more specific and relevant biochemical signals related to joint health. While serum can distinguish between OA and non-OA groups, synovial fluid provides a more direct reflection of the pathological processes occurring within the joint.

Table 2 shows that the testing dataset for synovial fluid revealed an overall accuracy of 75%, indicating some misclassification of non-OA samples during validation. In contrast, the serum testing dataset achieved 100% accuracy in its respective classes, as reported by Aziz et al. (2023).[2] This difference in accuracy may be attributed to several factors, including the complexity of the synovial fluid matrix and the presence of overlapping signals from various biomolecules that could obscure clear distinctions between OA and non-OA samples [Table 2].

The observed discrepancy between cross-validation accuracy (85%) and verification accuracy (65%) can be attributed to several factors. Firstly, the limited sample size inherent in this preliminary study may have led to model overfitting, where the model performs optimally on familiar data but less effectively on entirely new samples. Secondly, biological variability within the synovial fluid (SF) samples, particularly in the non-OA group, may introduce heterogeneity not captured during cross-validation, which used leave-one-out internal validation. Finally, ACL-injury patients were used as the non-OA controls; while radiographically confirmed as non-arthritic, subtle biochemical changes in the joint environment could resemble early OA signatures, thereby complicating classification. Despite this, the model's performance during cross-validation demonstrates promising discriminatory power, and future studies with larger and more diverse sample cohorts are planned to enhance model generalizability and stability.

Despite the lower accuracy, it is important to note that synovial fluid remains the predominant bio fluid employed in OA research due to its close localization to the pathological environment. According to Stabile et al. (2022),[38] this localization allows synovial fluid to capture breakdown products, enzymes, and signaling molecules released from surrounding tissues, making it a valuable source of biochemical information regarding the metabolic state of the affected joint.[39] Thus, while serum may provide clear distinctions in certain diagnostic contexts, synovial fluid offers insights into the localized pathological processes of osteoarthritis that serum cannot fully capture.

Moreover, Kalogera et al. (2023) [9] noted that joint deterioration in OA leads to the release of protein fragments that can serve as biomarkers for systemic (serum) or local (synovial fluid) assessment, providing insights into structural changes within the joint. A study by Hou (2016)[13] reported that the accuracy of the combined dataset for diagnosing canine OA based on serum showed 99.53% accuracy compared to 96.85% accuracy for joint fluid. Despite some limitations in the accuracy of the test set, which indicated that 50% of non-OA cases were incorrectly predicted as OA, this does not diminish the potential benefits of using synovial fluid for diagnosis. The primary advantage of analyzing synovial fluid lies in its ability to reflect localized joint conditions more accurately than serum, which primarily provides systemic information.

To enhance the predictive model utilizing synovial fluid, future experiments could focus on refining analytical techniques, such as employing more advanced spectroscopic methods or integrating multi-omics approaches to capture a broader range of biomarkers. Additionally, increasing sample size and diversity in the datasets may help improve classification accuracy and the model's robustness, ultimately leading to better diagnostic capabilities for osteoarthritis.

This study is preliminary in nature, and the use of a smaller sample size is justified as a preliminary step before scaling up to a larger cohort. Previous research has also employed small sample sizes in preliminary studies to establish foundational data before broader implementation. For instance, Hou et al. (2016)[13] conducted a chemometric analysis of canine synovial fluid for osteoarthritis detection using a small sample size of 10, justifying this approach by the need for preliminary data prior to scaling up. Similarly, a previous study by Karpiński (2022)[40] aimed to evaluate the effectiveness of selected discriminants for acoustic signals generated in the knee joint, recorded from a control group (healthy subjects) and a study group (individuals with degenerative changes), to diagnose cartilage damage using machine learning. Notably, their study was based on a small sample size, with 20 samples per group. In chemometric studies, varying numbers of scans are often used to ensure reliable results chemometric analysis, depending on the analytical requirements and this practice is standard.[41]

Lastly, while the sample size is a limitation, another challenge of this study lies in the selection of ACL injury patients as non-OA controls. ACL injuries may be associated with early subclinical joint changes, which could influence the outcomes. However, careful selection criteria, including clinical and radiographic assessments, were employed to minimize this risk. Future studies with broader healthy control groups are warranted to further validate these findings.

Beyond the validation of our diagnostic model, it is crucial to consider the clinical feasibility and translational potential of the proposed method. The application of ATR-FTIR spectroscopy for SF analysis demonstrates strong clinical feasibility The application of ATR-FTIR spectroscopy for synovial fluid SF analysis demonstrates strong clinical feasibility. The technique requires only a small volume of SF, minimizing invasiveness and sample burden. SF can be easily collected intraoperatively or via minimally invasive arthrocentesis procedures, making it suitable for both clinical and community-based screening programs with appropriate informed consent. Furthermore, ATR-FTIR equipment is relatively cost-effective compared to advanced imaging or omics platforms, and the method offers rapid analysis without the need for extensive sample preparation. In practice, SF samples can be collected from community populations, analyzed centrally in laboratory settings, and individuals whose spectral profiles indicate potential early-stage OA could be invited for follow-up clinical evaluation. Thus, this method provides a promising pathway for scalable, early OA screening and intervention programs, particularly in resource-limited or high-burden settings.

Conclusion

The DA showed the overall performance of 85% for DA model which is very good. The usage of ATR-FTIR spectroscopy supported by the chemometric method, effectively distinguished between OA and non-OA group through analysis of SF samples. As of now, there is still limited research on human SF employing ATR-FTIR spectroscopy and DA. Further investigation is warranted to better comprehend the mechanisms involved in utilizing SF between OA and non-OA groups to differentiate the capability of SF and serum in becoming the early detection tool of OA. Consequently, these preliminary findings highlight the significance of spectral features in differentiating OA and non-OA groups based on the molecular structures of synovial fluid (SF) samples. Therefore, further investigation is needed, as these molecular signatures in SF may offer unique insights into the distinct underlying processes of OA.

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V.S, writing review and editing: A.A.A, V.S and N.F.A.A.H, visualization: T.K, supervision: T.K, project administration: A.A.A and Y.F.B.A.F, funding acquisition: T.K. All authors have read and agreed to the published version of the manuscript.

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