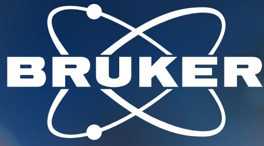

Ultra Deep Proteomic Profiling of Synovial Fluid Identifies Osteoarthritis Associated Biomarkers

Deep synovial fluid profiling with timsUltra AIP and Enrich-iST.

Abstract

Comprehensive proteomic characterization of synovial fluid is essential for understanding osteoarthritis (OA) pathophysiology and identifying disease associated biomarkers, yet remains challenging due to the extreme dynamic range of protein abundances. In this study, we applied the Enrich iST workflow in combination with the timsUltra AIP mass spectrometer to achieve deep, reproducible proteomic profiling of human synovial fluid from osteoarthritis patients and healthy controls. Synovial fluid samples were processed using the Enrich iST protocol to compress the dynamic range enabling detection of low abundance proteins, followed by robust, high throughput dia PASEF analysis and directDIA based data processing. This workflow enabled the identification of approximately 4800 protein groups and more than 45,000 peptides per sample without the need for extensive fractionation or depletion. The entire workflow proved to be highly reproducible, with median coefficients of variation at or below 10%. Multivariate analysis clearly separated OA and control samples, while statistical testing revealed numerous proteins significantly up or downregulated in OA. Functional enrichment analysis highlighted pathways related to extracellular matrix remodeling, inflammation, immune response, and cartilage degradation, including altered abundance of matrix metalloproteinases. Overall, this workflow establishes a robust and scalable platform for ultra deep synovial fluid proteomics and provides a foundation for biomarker discovery and mechanistic studies in osteoarthritis.

Keywords:
Enrich-iST, timsUltra AIP, Spectronaut, biomarkers, LFQ, synovial fluid, PASEF

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Introduction

Osteoarthritis (OA) represents a widespread and incapacitating disorder with no cure. As the most common form of arthritis, OA is defined by the progressive deterioration of articular cartilage, resulting in pain, stiffness, and reduced mobility. The most frequently affected sites include the hands, knees, hips, and spine; currently, there are no medications for curing or slowing OA progression. Synovial fluid, a viscous substance that lubricates joints, serves as a valuable liquid biopsy resource for OA disease research. Comprehensive proteomic analysis of synovial fluid is essential for elucidating the molecular mechanisms of OA, identifying potential biomarkers, and differentiating OA subtypes beyond what is possible with more limited analytical approaches.

The analysis of synovial fluid presents significant challenges due to its broad spectrum of protein concentrations, posing a dynamic range obstacle similar to other biological fluids1. Enrich-iST technology has demonstrated considerable efficacy in facilitating the detection of low-abundance proteins within samples exhibiting high dynamic range, such as plasma, serum, and cerebrospinal fluid. This study we apply this technology to synovial fluid. In combination with the sensitivity and specificity of the timsUltra AIP system, this methodology delivers rapid and comprehensive coverage and to our knowledge, this investigation achieves the most extensive proteome coverage reported for synovial fluid, providing a detailed proteomic characterization of knee joint synovial fluid from OA patients and identifying numerous OA-associated proteins, both upregulated and downregulated.

Methods

Human synovial fluid (10 µL) from a clinical cohort of healthy (n=4) and OA patients (n=4) were processed with the Enrich-iST kit protocol (Preomics, USA) (Figure 1). The resulting peptides were loaded onto Evotips according to the manufacturer's instructions and peptide separation was achieved using the 30 samples per day method on an EVOSEP Eno (Evosep, Denmark) with a PepSep column (15 cm × 150 µm × 1.5 µm). The column was connected to a Bruker timsUltra AIP mass spectrometer using a CaptiveSpray Ultra ion source with a 10 µm emitter. Data were acquired in dia-PASEF mode. The ion mobility range was set between 0.75 and 1.3 V·s/cm^2, and the mass range covered 100–1700 *m/z*. Accumulation and mobility ramp

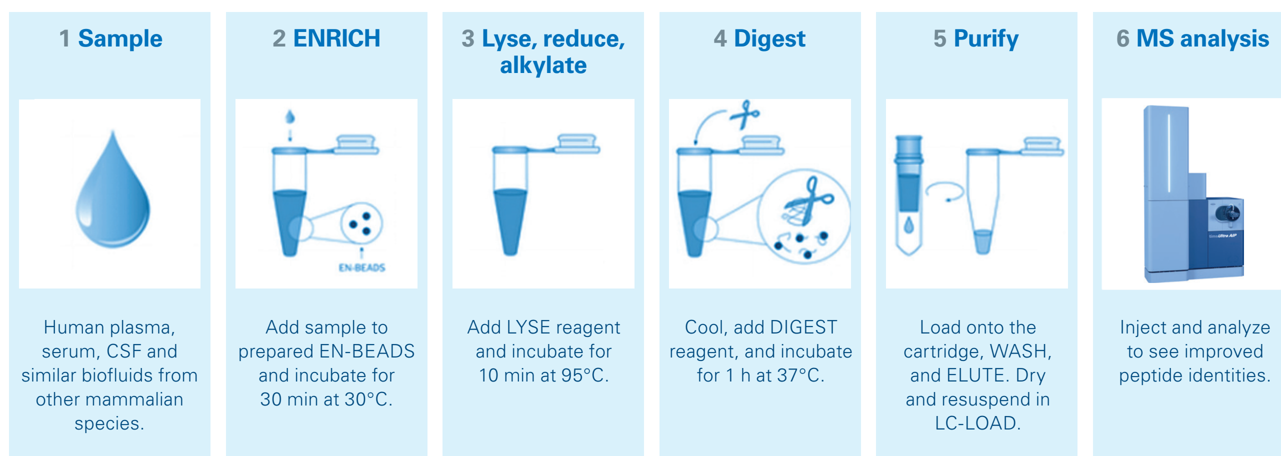

Figure 1. Sample preparation steps for the PreOmics Enrich-iST protocol.
The Enrich-iST kit offers a flexible, fast, and robust solution targeting low-abundance proteins in plasma, serum, CSF, and other high dynamic range samples.

times were set to 50 ms. dia-PASEF windows with variable isolation widths were created with py_diAID2. Samples were injected in duplicate. Data acquisition order was randomized using the “Shuffle Samples” feature in the Hystar sample table editor to minimize any batch effects in the analysis. Data were processed with Spectronaut 20 using directDIA+ mode using a Human UniprotKB reviewed database. Further statistical analysis was performed in R and Python (Figure 2).

Figure 2. Data acquisition and analysis workflow.
Peptides were separated using the Evosep Eno system and analyzed with the timsUltra AIP mass spectrometer, enabling high-throughput and sensitive proteomic profiling. Spectronaut software provided robust DIA data processing and quantification, while downstream statistical analyses were performed in R and Python to ensure comprehensive and reproducible results.

Average #protein groups

Counts: 6000, 5000, 4000, 3000, 2000, 1000, 0

Healthy_1: 4662; Healthy_2: 4242; Healthy_3: 4068; Healthy_4: 4752; OA_1: 3862; OA_2: 3238; OA_3: 4035; OA_4: 4229

Condition

Average #peptides

Counts: 60,000, 50,000, 40,000, 30,000, 20,000, 10,000, 0

Healthy_1: 40,405; Healthy_2: 32,596; Healthy_3: 31,141; Healthy_4: 44,820; OA_1: 28,064; OA_2: 21,572; OA_3: 28,450; OA_4: 32,968

Condition

Protein CVs, 95% Cutoff

CV%: 50, 40, 30, 20, 10, 0

Healthy_1, Healthy_2, Healthy_3, Healthy_4, OA_1, OA_2, OA_3, OA_4

Condition

Figure 3. Deep proteomic profiling of synovial fluid yields approximately 4800 protein groups with over 45,000 peptide identifications per sample, while achieving robust reproducibility as evidenced by median coefficients of variation (CVs) at or below 10% across technical replicates.

Results

The integration of the PreOmics Enrich-iST kit and timsTOF Ultra AIP mass spectrometer enabled a highly detailed proteomic analysis of synovial fluid from OA patients and healthy controls. This approach identified roughly 4,800 protein groups and over 45,000 peptides per sample (Figure 3A), setting a new benchmark for synovial fluid proteome coverage without requiring extensive fractionation or depletion. Technical replicates demonstrated strong reproducibility, with median coefficients of variation around 10% or lower (Figure 3B), ensuring reliable differentiation of true biological variation from background.

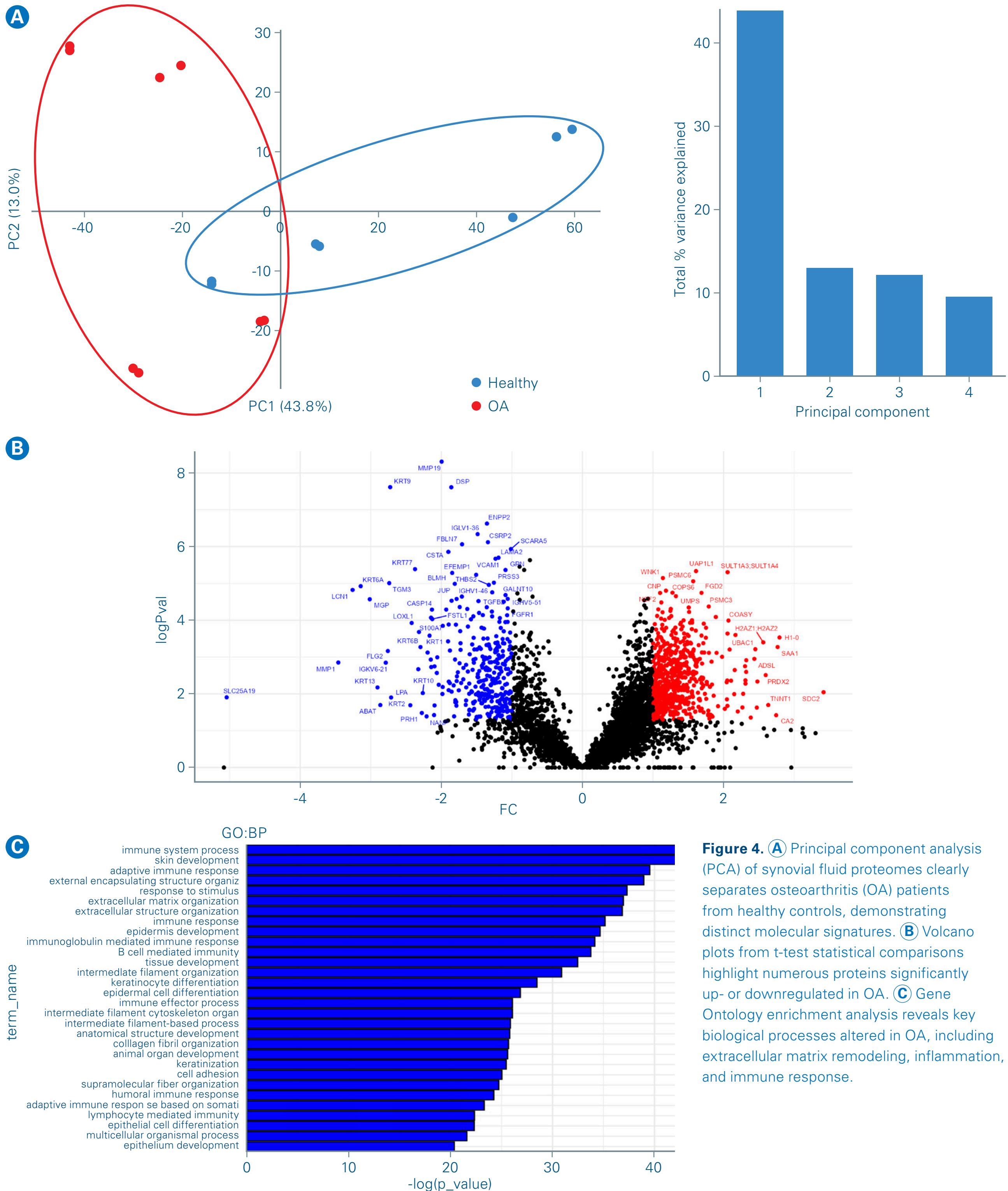

Figure 4. Ⓐ Principal component analysis (PCA) of synovial fluid proteomes clearly separates osteoarthritis (OA) patients from healthy controls, demonstrating distinct molecular signatures. Ⓑ Volcano plots from t-test statistical comparisons highlight numerous proteins significantly up- or downregulated in OA. Ⓒ Gene Ontology enrichment analysis reveals key biological processes altered in OA, including extracellular matrix remodeling, inflammation, and immune response.

Despite patient heterogeneity, principal component analysis (PCA) of the proteomic profiles clearly separated OA patients from healthy controls (Figure 4A), underscoring the method's capacity to detect disease-specific molecular signatures. Statistical comparisons by t-test revealed numerous proteins significantly upregulated or downregulated in OA samples (Figure 4B). Functional enrichment analyses pointed to key biological processes affected in OA, including extracellular matrix remodeling, cytokine production, inflammation, and immune response (Figure 4C). In particular, changes in OA-associated proteases, such as the matrix metalloproteinases (MMPs), central to cartilage degradation, were highlighted.

Overall, this comprehensive profiling of synovial fluid not only maps the molecular alterations associated with OA but also identifies specific proteins and pathways as potential biomarkers or therapeutic targets. The robust performance of this workflow provides a solid platform for advancing OA research, precision diagnostics, and the development of personalized treatments.

Conclusion

- The Enrich-iST kit and timsTOF Ultra AIP identified ~4800 protein groups and 45,000 peptides in synovial fluid, achieving record coverage without fractionation.
- The method showed strong reproducibility, with median CVs at or below 10% for proteins and peptides.
- PCA analysis distinguished OA patients from healthy controls based on proteomic profiles. Numerous OA-associated proteins were significantly up- or downregulated in OA compared to controls.
- MMPs were notably changed, implicating them in cartilage degradation and OA development.
- The approach reveals potential OA biomarkers and therapeutic targets.

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