RNA Sequencing and Machine Learning Analysis of the Synovium Reveals a Unique Disease Signature in Ankle Osteoarthritis

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INTRODUCTION: Osteoarthritis (OA) is a debilitating condition that impacts millions of people across the globe. Ankle OA, despite its significant impact on patient quality of life, has rarely been studied as a unique disease process. Currently, we do not have a clear understanding of the cellular and molecular changes driving ankle OA progression specifically, or the role of the synovium in the process. Prior histological assessments have displayed both increased fibrosis and vascularization of synovial tissue in ankle OA, but machine learning approaches, particularly unsupervised learning via clustering, might enable an unbiased assessment of synovium with cellular and spatial resolution. Thus, the primary objective of this study was to uncover distinctions between ankle and knee OA via comparison of the synovial transcriptome through RNA sequencing (RNA-seq). In addition, we performed an unbiased histological assessment of synovium to ultimately gain insight into cellular features displayed in each experimental cohort.

METHODS: Synovium samples were harvested from end stage knee and ankle OA procedures for RNA-seq and histology. Bulk 150 bp, paired-end RNA seq was performed using the Illumina NovaSeq6000 platform, and alignments were made to Human Reference genome (GRCh38/hg38) using Hisat2 (v2.0.5). Differential expression was determined using the R edgeR (3.22.5) package after adjusting via a scaling normalization factor and correcting P-values via the Benjamini Hochberg method. Paraffin-embedded synovium samples were sectioned, stained with hematoxylin and eosin (H&E), and imaged at 20x magnification. QuPath software with built-in functions and scripting was used to i) load and deconvolve images of H&E sections, ii) manually trace the synovium, iii) detect cell nuclei, iv) expand (0.5µm) nuclei detection to define cell boundary, and v) quantify cellular morphological and staining features. Exported nucleus and cell measurements (e.g., areas and intensity of H&E) were used to cluster cells via scripting written in Python using standard libraries and packages. Data were first reduced using principal component analysis (PCA) or uniform manifold approximation and projection (UMAP) techniques. PCA and UMAP reduced data were clustered via K-means, fuzzy c-means, and gaussian mixture model. The technique with the highest silhouette score was chosen for subsequent analysis. The number of principal components and clusters were determined via skree plot and elbow method, respectively. Each cell was assigned to a cluster and then visualized qualitatively with density plots and quantified in Qupath. Differences for each histological metric were determined using Mann Whitney U tests. Statistical and trending significance set at $p < 0.05$ and $0.05<p<0.10$, respectively.

RESULTS SECTION: RNA-seq data uncovered distinct clustering of ankle and knee OA synovium. Functional enrichment analyses revealed upregulation in Gene Ontology terms "Extracellular Matrix Organization", "Extracellular Structural Organization", "Skeletal Development", and "Ossification" in synovium from ankle compared to knee. Reactome terms with significant enrichment in ankle samples included "Extracellular Matrix Organization", "Collagen Formation", "Collagen Biosynthesis and Modifying Enzymes", and "Collagen Chain Trimerization". Synovium cells in histological sections were assigned to 14 cluster types for analysis using the best silhouette score. Ankle synovium displayed a modest decrease in the total number of cells compared to the knee ($p = 0.08$; Fig. 1A). Cluster 2 cells were decreased ($p = 0.08$; Fig. 1B), and cluster 13 cells were significantly decreased in the ankle compared to knee ($p = 0.05$; Fig. 1C). Qualitatively, cluster 2 cells appeared cuboidal, larger, deeply stained with hematoxylin, and concentrated near vascular appearing structures (Fig. 2); cluster 13 cells were weakly stained with hematoxylin, smaller, and irregularly elongated (Fig. 2).

DISCUSSION: Synovium from knee and ankle end-stage OA are found to be distinct from each other based on RNA-seq and machine learning-based analyses of quantitative histology. We identified an upregulation in multiple genes and pathways related to cell communication and extracellular matrix organization via RNA-seq. Literature regarding RNA-seq of knee OA synovium reveals a more inflammatory signature, with a focus on ECM and protein degradation. Our machine learning histological analysis revealed significant differences in clusters of cells, particularly nuclear area and cellular staining, indicative of an altered biological process that largely corroborate the RNA-seq findings. The spatial concentrations of specific cell clusters to vascular-like structures might indicate a relationship to chemotaxis and the inflammatory process. This work suggests it will be helpful to (i) characterize the identified cluster cell types in pathological contexts via immunohistochemistry, and (ii) confirm these findings using higher magnification (e.g., 40x). Ultimately, an unbiased machine learning approach to evaluate synovium from OA knees and ankles might complement genomic data and accelerate understanding disease pathology to identify therapeutic targets.

SIGNIFICANCE/CLINICAL RELEVANCE: We revealed profound differences in the transcriptomic and histological landscape of the synovium in ankle versus knee OA using machine learning. Clinically this provides rationale for joint specific study of OA to approach development of effective therapies that are specific to joint context.

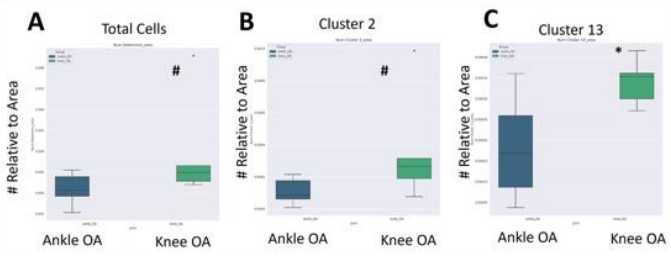

Fig. 1: Key quantitative histological features relative to synovium area (A) Total cells detected. (B) Cluster 2 cells (C) Cluster 3 cells. * Indicates significant difference between knee and ankle; # indicates trending significance.

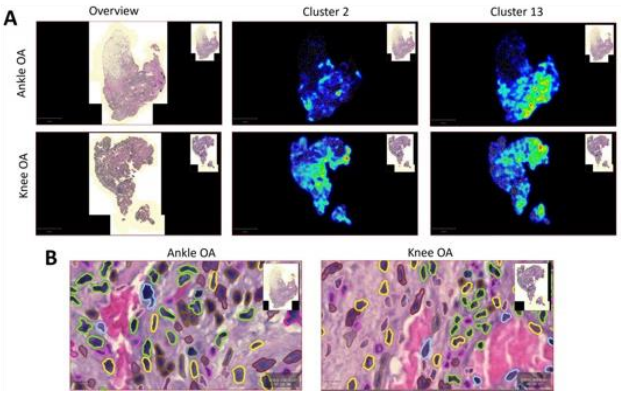

Fig. 2: (A) Density plots highlight cluster 2 and cluster 13 cells spatially within ankle and knee OA. (B) Zoomed up images highlighting cluster 2 (green color) and cluster 13 (pale color) cells, with other cluster types.