

Assessment of Clinical, Tissue, and Cell-Level Metrics Identify Four Biologically Distinct Knee Osteoarthritis Patient Phenotypes

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Abstract

Objective. Clinical heterogeneity of primary osteoarthritis (OA) is a major challenge in understanding pathogenesis and development of targeted therapeutic strategies. This study aims to (1) identify OA patient subgroups phenotypes and (2) determine predictors of OA severity and cartilage-derived stem/progenitor concentration using clinical-, tissue-, and cell-level metrics. **Design.** Cartilage, synovium (SYN) and infrapatellar fatpad (IPFP) were collected from 90 total knee arthroplasty patients. Clinical metrics (patient demographics, radiograph-based joint space width (JSW), Kellgren and Lawrence score (KL)), tissue metrics (cartilage histopathology grade, glycosaminoglycans (GAGs)) and cell-based metrics (cartilage-, SYN-, and IPFP-derived cell concentration ([Cell], cells/mg), connective tissue progenitor (CTP) prevalence (P_{CTP} , CTPs/million cells plated), CTP concentration, [CTP], CTPs/mg)) were assessed using *k*-mean clustering and linear regression model. **Results.** Four patient subgroups were identified. Clusters 1 and 2 comprised of younger, high body mass index (BMI) patients with healthier cartilage, where Cluster 1 had high CTP in cartilage, SYN, and IPFP, and Cluster 2 had low [CTP] in cartilage, SYN, and IPFP. Clusters 3 and 4 comprised of older, low BMI patients with diseased cartilage where Cluster 3 had low [CTP] in SYN, IPFP but high [CTP] in cartilage, and Cluster 4 had high [CTP] in SYN, IPFP but low [CTP] in cartilage. Age ($r = 0.23$, $P = 0.026$), JSW ($r = 0.28$, $P = 0.007$), KL ($r = 0.26$, $P = 0.012$), GAG/mg cartilage tissue ($r = -0.31$, $P = 0.007$), and SYN-derived [Cell] ($r = 0.25$, $P = 0.049$) were weak but significant predictors of OA severity. Cartilage-derived [Cell] ($r = 0.38$, $P < 0.001$) and P_{CTP} ($r = 0.9$, $P < 0.001$) were moderate/strong predictors of cartilage-derived [CTP]. **Conclusion.** Initial findings suggests the presence of OA patient subgroups that could define opportunities for more targeted patient-specific approaches to prevention and treatment.

Keywords

cartilage, patient phenotypes, osteoarthritis, cluster analysis, clinical-tissue-cell level metrics

Introduction

Osteoarthritis (OA) is a leading cause of pain and disability, currently affecting about 7% global population (500 million people).¹ The number of people affected globally rose by 48% between 1990 to 2019.¹ Of the various joints impacted, knee OA is the most prevalent with about 10% to 38% of the elderly population suffering with severe symptoms. Osteoarthritis presents an enormous public health challenge in the coming decades.²

Primary OA is a complex heterogeneous disease of the whole joint with multiple aetiologies.³ Several factors (eg. genetic, metabolic, and biomechanical) and mechanisms

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can contribute to OA.^{4,5} It has thus been proposed that OA comprises of multiple distinct phenotypes rather than a single disease, where each phenotype can be described as a collection of characteristics. Studies in the literature have grouped knee OA patients into distinct phenotypes from different perspectives.⁶⁻¹¹ Different set of characteristics have been used in diverse studies to determine OA phenotype, including imaging, biochemical profiles, clinical characteristics, and more recently metabolomics, proteomic, and genomic profiles.⁶⁻¹¹ Although the field is emerging with rich data and analytical methods, there is lack of clarity over the phenotypes that comprise OA. Importantly, the diversity of studies emphasizes the need to identify key factors that delineate OA phenotypes to better understand OA heterogeneity and classify knee OA phenotypes.

Study of cartilage tissue to identify OA phenotypes primarily tend to rely on end-stage human cartilage obtained at the time of joint replacement surgery.^{7,10} In such cases, it potentially is challenging to obtain the characteristics of the joint and tissues in the early or mild disease progression. In order to initiate early interventions and therapeutic approaches that could prevent progression and severe structural alterations in the joint associated with later stages of OA, there is a great interest and demand in studying the biological processes involved in the initiation and early stages of this joint disease in humans.

Our group has extensively reported that the cartilage on lateral femoral condyle (LFC) in varus malalignment total knee arthroplasty (TKA) patients is relatively preserved, and these patients predominantly suffer from destruction of the medial compartment.¹²⁻¹⁴ The cartilage from LFC exhibited mild or moderate OA without exhibiting severe OA as determined using Mankin's Histological Histochemical Grading System (HHGS), advanced Osteoarthritis Research Society International (OARSI), and Polarized Light Microscopy (PLM) scoring systems for primary OA cartilage.¹²⁻¹⁴ Comprehensive reporting of histopathological, biochemical, and cellular changes in these mild OA cartilage specimens along with patient demographics and clinically measured knee joint metrics might help with providing further insight into the disease-state of the joint and identifying factors important to delineate OA phenotypes. Furthermore, no study in the literature has investigated the role of stem and progenitor cells (connective tissue progenitors [CTPs]) resident in various primary tissues around the knee to determine their potential contribution to OA disease and phenotypes.

Using a novel-set of characteristics including clinical-level factors (patient demographics, joint metrics), tissue-level factors (cartilage ECM-GAG content) and cell-level metrics (cell and CTP concentrations in cartilage, synovium (SYN) and infrapatellar fatpad (IPFP)), this study aims to (1) identify potential knee OA patient phenotypes; (2) identify

predictors of OA severity as determined by HHGS and PLM histological scoring systems, and (3) identify predictors of cartilage-derived CTP concentrations. This study will help advance our understanding of cartilage pathobiology, identify potential characteristics to delineate OA phenotypes, and inform clinical cell sourcing decisions for targeted patient populations.

Methods

Patients

This study was approved by the Institutional Review Board committee of the Cleveland Clinic (Protocol: 13641). Ninety patients (female = 48.9%, median age = 64 years, median body mass index [BMI] = 30.1 kg/m²) scheduled for TKA were recruited. Study cohort: 83.7% patients were non-Hispanic whites, 10.9% were non-Hispanic blacks with the remaining 5.4% being non-Hispanic Asians, Hispanic, and Multiracial; 17.4% of the patient population were current tobacco smokers, and 15.2% of the patient population had diabetes. Inclusion criteria: idiopathic OA, primarily medial compartment, and/or patellofemoral disease ($n = 6$) exhibiting a relatively preserved lateral compartment based on preoperative weight-bearing, anterior-posterior radiographs taken in full extension and 30° of fixed flexion. Exclusion criteria: secondary arthritis related to systemic inflammatory arthritis; history of autoimmune disorders, gout or pseudogout, previous surgery to the index knee, current or previous treatment with systemic glucocorticoids or osteotropic medication; cancer within previous 2 years; known or suspected infection; and osteonecrosis.

Joint Health

Weight-bearing anteroposterior (AP) radiographs were taken with the knee in 30° of flexion (Rosenberg radiograph).¹ Joint space width (JSW) in the lateral compartment was determined in a systematic manner following the midpoint technique described by Ravaut *et al.*¹⁵ A digital calibrated scale was used to measure the distance in millimeters. The radiographs were graded independent by at least two scorers in blinded fashion using the Kellgren and Lawrence (KL) grading system.¹³

Tissue Procurement

Cartilage, SYN, and IPFP were harvested during the TKA procedure (**Fig. 1**). Cartilage was harvested from LFC, SYN (0.5-1.5cc) was harvested from the medial suprapatellar area, and IPFP (~2.5cc) was harvested after removal of overlying SYN.^{16,17} Tissues were immediately transported to the laboratory for same day processing.

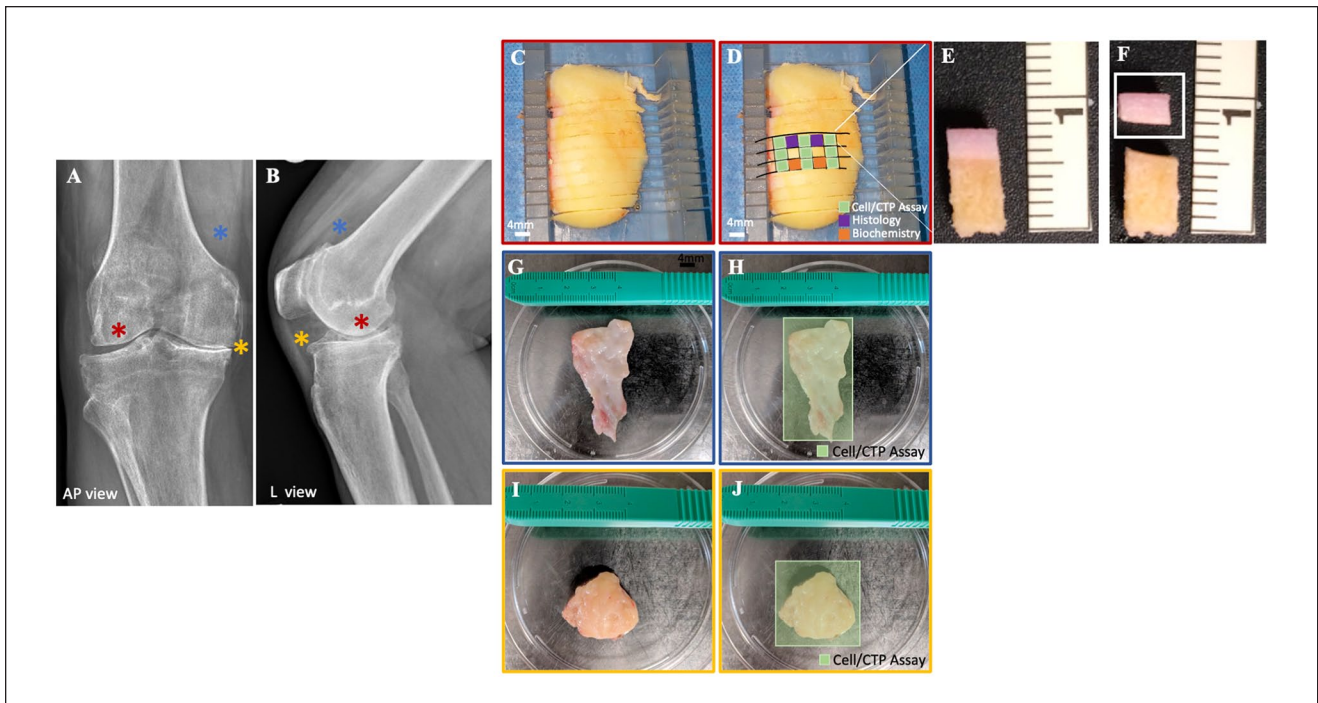


Figure 1. Specimen procurement from total knee arthroplasty (TKA) patients. (A, B) Anterior-posterior view (AP) radiograph and lateral view (L) radiograph indicating the location of harvest of cartilage (red asterisk), synovium (blue asterisk), and infrapatellar fatpad (IPFP; yellow asterisk) were obtained from each TKA patient during surgery. (C) Cartilage was harvested from the lateral femoral condyle (LFC) with the initial distal femoral cut. (D) Osteochondral specimens ($4 \times 4 \times 8$ mm) were systematically cut from the weight-bearing portion of the LFC and allocated for cell/CTP assay (green box), histology (purple box) and biochemistry (orange box). (E) Intact osteochondral specimen was used for histology. (F) Cartilage was separated from the subchondral bone for biochemistry and cell/CTP analysis. (G) Synovium (SYN) was harvested from the medial suprapatellar area, assuring that fat tissue was not present. (H) The entire SYN specimen was used for cell/CTP assay (green box). (I) IPFP was harvested after removal of overlying SYN. (J) The entire IPFP specimen was used for cell/CTP assay (green box). CTP = connective tissue progenitor.

Cartilage Tissue Allocation

Osteochondral specimens ($4 \times 4 \times 8$ mm) were cut from the weight-bearing portion of the LFC as shown in **Figure 1B-D** and allocated for histological (purple), biochemical (orange) and cell and CTP analysis (green).

Cartilage Histopathology

Using established techniques, cartilage was prepared for staining and imaging.^{12-14,18} Briefly, specimens were fixed for 48 h at 4°C , subsequently decalcified, paraffin embedded with consistent spatial orientation, and sectioned ($5 \mu\text{m}$) in the coronal plane. Digital images of unstained sections were acquired under polarized light microscope for PLM scoring and hematoxylin and eosin. Safranin O and fast green-stained sections were acquired under brightfield mode for HHGS.^{14,19}

Cartilage Extracellular Matrix Analysis

Cartilage was precisely separated from the subchondral bone by one experienced professional (VPM) (**Fig. 1E**).

Every 100 mg wet weight of tissue was digested using 250 μL of 1 mg/mL proteinase K in 0.1 M ammonium acetate, 0.01% (w/v) SDS at pH 7.0, at 60°C overnight. Postdigestion, 100% prechilled ethanol was added at 4:1 ratio for ethanol precipitation overnight, followed by centrifugation (14,000 g for 15 minutes) and washing to recover pellets. Samples were analyzed for dsDNA using the Quant-iT PicoGreen dsDNA assay (Invitrogen) and sulfated GAGs using the dimethylmethylene blue colorimetric assay.²⁰ Both assays were performed in 96-well plates (ThermoFisher, Lot#SJ2555598) using a Cytation 5 plate reader (BioTek). For each sample, triplicates were used to measure total DNA and GAG based on standard curves, respectively. Total cells were calculated using a literature value of 7 pg dsDNA/cell.²¹ GAG per cell (ng/cell) was calculated.

Cell and CTP Analysis

Cartilage, SYN, and IPFP. Cartilage tissue was separated from the subchondral bone (**Fig. 1E**), wet weight measured, minced, and enzymatically digested to obtain cells using previously established techniques.^{16,17,22,23} Briefly, the cartilage tissue was digested using Collagenase II (6,000 U/mL) and

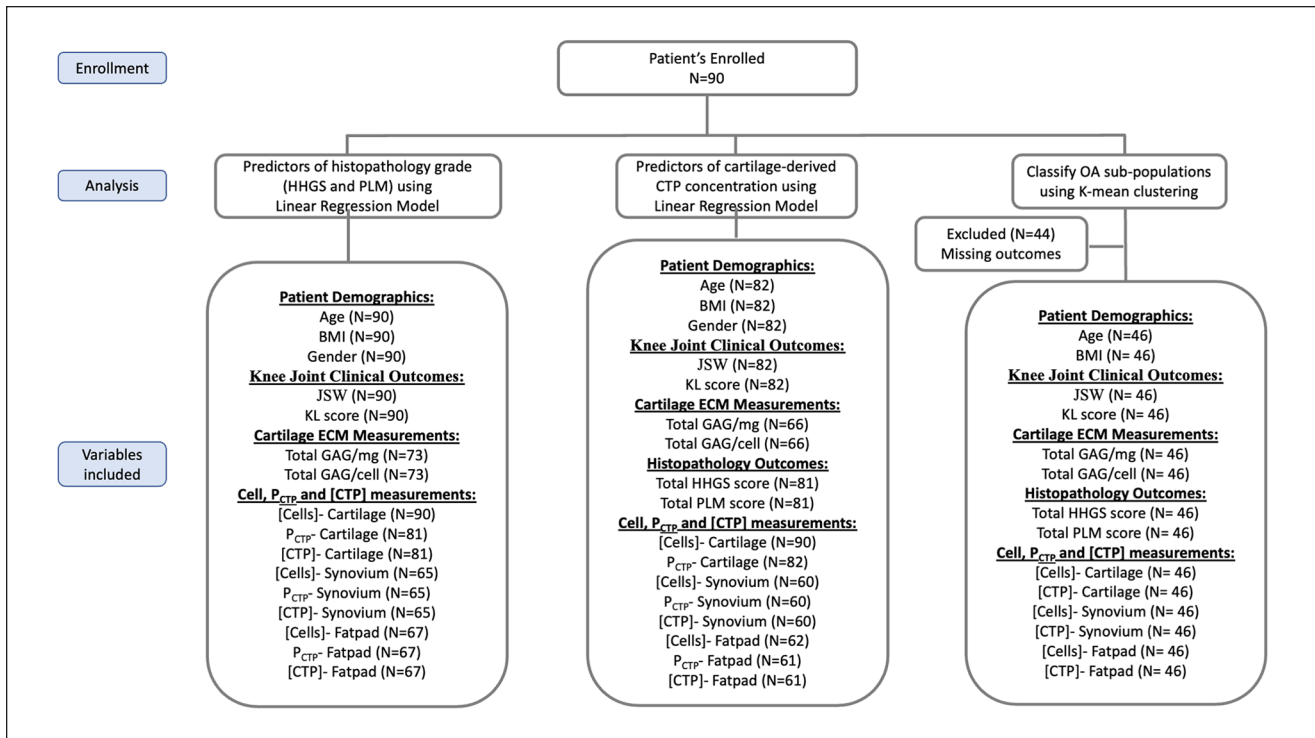


Figure 2. Overview of the study workflow and analysis. Flow diagram to illustrate the number of patients and the variables included in the three different analysis performed in the study. HHLS = histological histochemical grading system (Mankin's system); PLM = polarized light microscopy scoring system; P_{CTP} = CTP prevalence (CTPs/million cells plated); [Cell] = cell concentration (cells/mg); [CTP] = CTP concentration (CTPs/mg); BMI = body mass index (kg/m^2); ECM = extracellular matrix; JSW = joint space width (mm); GAG = glycosaminoglycan; KL = Kellgren and Lawrence Score; CTP = connective tissue progenitors.

disperse (3 U/mL) at 37°C for 3 h. SYN (**Fig. 1F**) and IPFP (**Fig. 1H**) were weighed, minced, and enzymatically digested using Collagenase I (111 U/mL) and dispase (24 U/mL) at 37°C for 2 h as per established technique.^{16,17} Postdigestion, cell counts were calculated to determine cell concentration ([Cell], cells/mg). Cells from each tissue source were plated in Lab-tek™ chambers, at a plating density of 24,000 cells/ cm^2 , and cultured at 37°C , 80% to 90% humidity, 20% O_2 and 5% CO_2 in chondrogenic media for 6 days for colony-forming unit (CFU) assay.^{16,17,22-24} On Day 6, cells were fixed and stained with bisbenzimidazole (nuclei stain) and imaged for automated CFU analysis using Colonyze™ image analysis software using previously established protocols.^{16,17,22-24}

Statistical analysis. For the 46 patients without any missing values, *k*-mean clustering analysis was performed to identify OA patient sub-groups in the cohort (**Fig. 2**). Elbow method was used to explore the number of clusters, and *k* = 4 was identified for this data set. Simple linear regression models were used to individually assess potential predictors of (1) OA disease state (as measured by total HHGS score and PLM score) and (2) Cartilage-derived [CTP], **Figure 2**. A log transformation was applied to all cell and CTP measures. In the event that a cell or CTP measure was 0, the 0 was

replaced with one half the minimum non-zero value before applying the log transformation to establish a non-zero low baseline value. Pearson coefficient (*r*) and coefficient of determination (R^2) were reported for each model. A significance level of 0.05 was applied. As this was an exploratory study, no adjustment was made for multiple comparisons.

Results

Table 1 summarizes the percent distribution of categorical variables and median (interquartile range [IQR]) for the continuous variables measured in this study.

Knee OA Patient Phenotypes

For the 46 patients with complete data set, four patient phenotypes were identified (**Fig. 3**). Both **Cluster 1** (*n* = 16) and **Cluster 2** (*n* = 1) comprised of younger patients with elevated BMI and healthier cartilage (low KL score, high JSW and low HHGS, PLM scores). However, **Cluster 1** tended to have high [CTP] in all of the three knee-resident tissue sources assessed that include cartilage, SYN, and IPFP, whereas Cluster 2 had patient with low [CTP] in all the three knee-resident tissue sources. On the contrary, both

Table 1. Summary of Parameters Measured in This Study Cohort ($n = 90$).

Parameters Assessed in the Study Cohort	
Patient demographics	
Age (years)	64.0 [54.0, 70.0]
BMI (kg/m ²)	30.1 [27.4, 35.1]
Gender (female)	48.9%
Race	
Non-Hispanic white	83.7%
Non-Hispanic black	10.9%
Others (Hispanic, non-Hispanic Asian, Multiracial)	5.6%
Tobacco use	
Current	17.4%
Former	46.7%
Diabetes (yes %)	15.2%
Knee joint clinical outcome measures	
JSW (mm)	6.0 [5.0, 7.0]
KL score	4.0 [3.0, 4.0]
Cartilage ECM content	
Total GAG per mg (μg/mg)	49.7 [42.2, 60.6]
Total GAG per cell (ng/cell)	12.9 [8.7, 20.1]
OA severity as measured by cartilage histology	
Total HHGS score	5.0 [4.0, 6.0]
Total PLM score	5.0 [3.0, 5.0]
Cell and CTP prevalence, concentration in knee-derived tissues	
Cartilage-derived [Cells] (cells/mg)	4,590 [3,045, 7,200]
Cartilage-derived P_{CTP} (CTPs/10 ⁶ cells plated)	87 [16, 535]
Cartilage-derived [CTP] (CTPs/g)	705 [56, 3,339]
SYN-derived [Cells] (cells/mg)	6412 [2,734, 11,015]
SYN-derived P_{CTP} (CTPs/10 ⁶ cells plated)	430 [186, 828]
SYN-derived [CTP] (CTPs/g)	1917 [735, 6,703]
IPFP-derived [Cells] (cells/mg)	4248 [3,172, 6,646]
IPFP-derived P_{CTP} (CTPs/10 ⁶ cells plated)	430 [198, 993]
IPFP-derived [CTP] (CTPs/g)	2,259 [685, 4,454]

Categorical variables are presented as percent distribution and continuous variables are presented as median (IQR).

BMI = body mass index; JSW = joint space width; KL score = Kellgren and Lawrence score; ECM = extracellular matrix; GAG = glycosaminoglycan; OA = osteoarthritis; HHGS = histologicalistochemical grading system; PLM = polarized light microscopy scores; CTP = connective tissue progenitors; [Cell] = cell concentration; P_{CTP} = CTP prevalence (CTPs/million cells plated); [CTP] = CTP concentration; SYN = synovium; IPFP = infrapatellar fatpad; IQR = interquartile range.

Clusters 3 ($n = 22$) and **Cluster 4** ($n = 7$) comprised of older patients with lower BMI and degenerated cartilage (high HHGS and PLM scores, low JSW and high KL score). However, the two clusters showed inverse outcomes in terms of total [CTP] between cartilage and SYN, IPFP. **Cluster 3** had higher [CTP] in cartilage and lower [CTP] in SYN and IPFP, whereas **Cluster 4** had higher [CTP] in SYN and IPFP and lower [CTP] in cartilage.

Predictors of OA Severity as Determined by Total HHGS Score

As shown in **Figure 4A-D**, age ($r = 0.23$, $P = 0.026$), JSW ($r = 0.28$, $P = 0.007$), KL score ($r = 0.26$, $P = 0.012$) and

total sulphated GAGs per milligram wet weight of cartilage ($r = -0.31$, $P = 0.007$) were found to be weak or moderate but significant predictors of OA severity as determined by HHGS score. Gender, race, tobacco use, presence of diabetes, total sulphated GAGs per cell, cell and CTP concentrations in cartilage, SYN, and IPFP did not help predict OA severity. **Table 2** summarizes the correlations observed between total HHGS score and the various factors included in this study.

Predictors of OA Severity as Determined by Total PLM Score

As shown in **Figure 4E, F**, KL score ($r = 0.28$, $P = 0.007$) and SYN-derived [Cell] ($r = 0.25$, $P = 0.049$) were found to be weak but significant predictors of OA severity as determined by PLM score. Age, gender, race, tobacco use, presence of diabetes, total sulphated GAGs per cell or mg wet weight cartilage tissue, cell and CTP concentrations in cartilage, and IPFP and SYN [Cell] did not help predict OA severity as determined by PLM scores. **Table 3** summarizes the correlations observed between total PLM score and the various factors included in this study.

Predictors of CTP Concentration in Cartilage

Cartilage-derived [Cell] ($r = 0.38$, $P < 0.001$) and cartilage-derived P_{CTP} ($r = 0.9$, $P < 0.001$) were moderate and significant predictors of [CTP] in cartilage (**Fig. 4G, H**). Age, gender, race, tobacco use, presence of diabetes, total sulphated GAGs per cell or mg wet weight cartilage tissue, and cell and CTP concentrations in IPFP and SYN did not help predict cartilage-derived [CTP]. **Table 4** summarizes the correlations observed between cartilage-derived [CTP] and the various factors included in this study.

Discussion

This exploratory study focused on using a novel-set of characteristics to identify knee OA patient phenotypes as well as independent correlate these variables with OA disease severity and cartilage-derived [CTP]. The characteristics include clinical-level factors (age, gender, BMI, JSW, KL score), tissue-level factors (total sulphated GAGs per mg cartilage and total sulphated GAGs per cell) and cell-level factors ([Cell], P_{CTP} , and [CTP] in knee-resident tissues including cartilage, IPFP, and SYN).

Cluster analysis identified four patient phenotypes. Along with patient demographics and the disease severity in cartilage, we also found the progenitors (CTP) concentration in the knee-resident tissues maybe a potential new factor to delineate knee OA patient phenotypes. The results informed us that (1) patients with healthy or diseased cartilage can have high or low [CTP] and (2) while in a healthier cartilage,

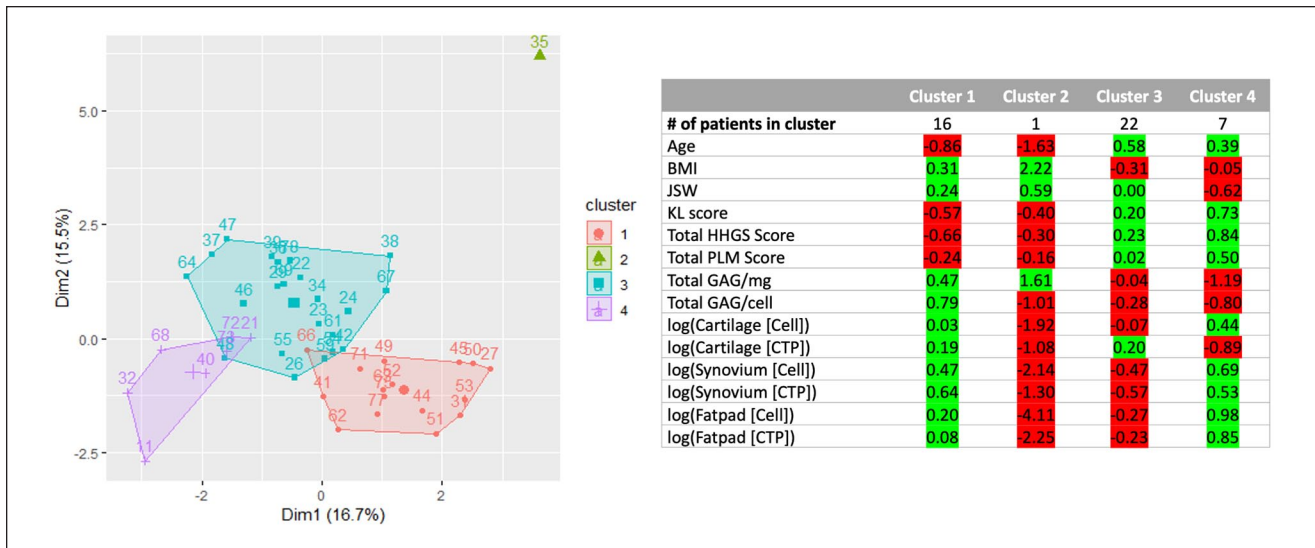


Figure 3. K-mean cluster analysis to identify knee OA patient subgroups. For the 46 patients with complete data set, four patient sub-populations were identified as indicated by the plot on the left. The table on the right shows the mean for each variable (after standardization) for each cluster. Positive values (highlighted in green) indicate cluster means that are higher than the global mean, negative values (highlighted in red) indicate cluster means that are lower than the global mean. Using this, the results can be interpreted as: Cluster 1 ($n = 16$) comprised of younger patients with elevated BMI and healthier cartilage (low KL score, high JSW, and high histology score) tended to have high [CTP] in the all the three knee-resident tissue sources assessed that include cartilage, SYN, and IPFP. Cluster 2 ($n = 1$, this patient appears to be an outlier) comprised of younger patient with elevated BMI and healthier cartilage (low KL score, high JSW, and high histology score) with low [CTP] in the knee-resident tissue sources. Both Cluster 3 ($n = 22$) and Cluster 4 ($n = 7$) comprised of older patients with lower BMI with more degenerated cartilage (high HHGS, low JSW, and high KL score). Cluster 3 had higher [CTP] in cartilage and lower [CTP] in SYN and IPFP, whereas cluster 4 had higher [CTP] in SYN and IPFP and lower [CTP] in cartilage. Note: the numbers in the plot indicate patient ID in our data set. OA = osteoarthritis; BMI = body mass index; KL score = Kellgren and Lawrence score; JSW = joint space width; [CTP] = CTP concentration; SYN = synovium; IPFP = infrapatellar fatpad; HHGS = histological histochemical grading system; PLM = polarized light microscopy; GAG = glycosaminoglycan; [Cells] = cell concentration.

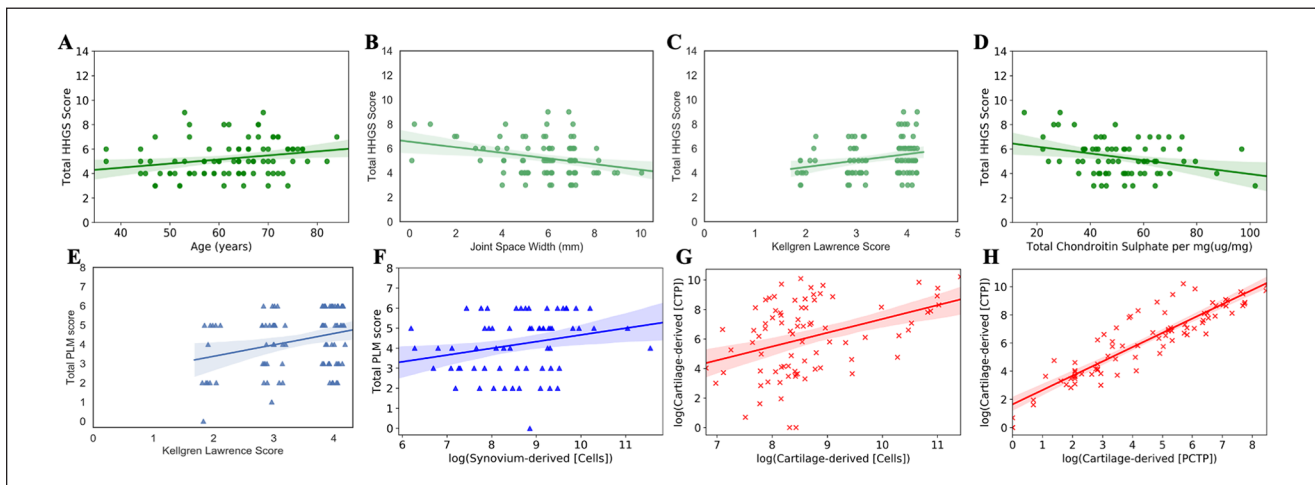


Figure 4. Significant predictors of OA severity as measured using Histopathological Histochemical Grading system (HHGS) and Polarized Light Microscopy (PLM) scoring system, as well as cartilage-derived CTP concentration [CTP]. Higher HHGS score which will be indicative of more severe OA was found to (A) increase with increasing age of patient, (B) decrease with increasing joint space width (JSW), (C) increase with increasing Kellgren Lawrence (KL) radiological score, (D) decrease with increasing chondroitin sulfate (CS4) content. Higher PLM score which will be indicative of more severe OA was found to (E) increase with increasing KL radiological score and (F) increase with increasing synovium-derived cell concentration [Cell]. Cartilage-derived CTP concentration [CTP] was found to (G) increase with increasing cartilage-derived [Cell] and (H) increase with increasing cartilage-derived CTP prevalence (P_{CTP}). OA = osteoarthritis.

Table 2. Predictors of OA Severity as Determined by Total HHGS Score.

Predictors	Histopathology Grade—Total HHGS Score		
	N	R ²	P Value
Patient demographics			
Age (years)	90	0.055	0.026
BMI (kg/m ²)	90	0.020	0.185
Gender	90	0.001	0.763
Knee joint clinical outcome measures			
JSW (mm)	90	0.081	0.007
KL score	90	0.069	0.012
Cartilage ECM content			
Total GAG per mg (μg/mg)	73	0.097	0.007
Total GAG per cell (ng/cell)	73	0.003	0.668
Cell and CTP prevalence, concentration in knee-derived tissues			
Cartilage-derived [Cell] (cells/mg)	90	0.019	0.197
Cartilage-derived P _{CTP} (CTPs/10 ⁶ cells plated)	81	0.001	0.82
Cartilage-derived [CTP] (CTPs/g)	81	0.002	0.692
SYN-derived [Cell] (cells/mg)	65	0.001	0.831
SYN-derived P _{CTP} (CTPs/10 ⁶ cells plated)	65	<0.001	0.886
SYN-derived [CTP] (CTPs/g)	65	0.000	0.884
IPFP-derived [Cell] (cells/mg)	67	0.036	0.123
IPFP-derived P _{CTP} (CTPs/10 ⁶ cells plated)	67	<0.001	0.95
IPFP-derived [CTP] (CTPs/g)	67	0.006	0.554

Simple linear regression models were used to individually assess potential predictors of OA disease that include patient demographics, knee joint clinical outcome measures, cartilage ECM content and cell, and CTP concentration in knee-derived tissues including cartilage, synovium (SYN), and infrapatellar fatpad (IPFP). The bold text indicates factors that were significant and impacted the outcome being measured.

OA = osteoarthritis; HHGS = histological histochemical grading system; BMI = body mass index; JSW = joint space width; KL score = Kellgren and Lawrence score; ECM = extracellular matrix; GAG = glycosaminoglycans; CTP = connective tissue progenitors; [Cell] = cell concentration; P_{CTP} = CTP prevalence (CTPs/million cells plated); [CTP] = CTP concentration.

all the three knee-resident tissue (cartilage, SYN, and IPFP) sources had either high or low [CTP] synchronously, the synchronicity was disturbed in diseased cartilage. We observed that in diseased cartilage, [CTP] in cartilage was inversely associated with [CTP] in SYN and IPFP.

To our knowledge, no study has simultaneously evaluated the clinical-level, tissue-level, and cell-level variables to classify OA patient phenotypes as well as access its impact on OA severity and stem/progenitor concentrations in cartilage. A complete data set on all these metrics can help us better understand the factors that affect the occurrence and development of OA as well as identify potential targets for diagnosis and therapeutic strategies for OA.

Four Biologically Distinct Patient Subgroups Identified May Define Opportunities for More Targeted Patient-Specific Approaches to Prevention and Treatment

Osteoarthritis is a heterogeneous disease making it very challenging to develop a one-size-fits-all therapy to such a diverse patient population. Identification of well-defined phenotypes or subgroups of OA patients could help us

better understand the driving factors in the development and progression of OA and to define subgroup-specific treatments to improve therapeutic effectiveness. To group patients into distinct phenotypes in this study, we used the following set of characteristics—clinical characteristics of patients, radiological image-based characteristics of the knee joint, extracellular matrix in cartilage, *ex vivo* histopathological degradation characteristics of cartilage and *in vitro* characteristics of cell and CTP population from SYN, IPFP, and cartilage tissue obtained from knee joint. The ultimate goal of this study was to identify phenotypic characteristics that distinguish OA patient subgroups with the intent to use this information to customize cell-based therapeutic approaches for improved clinical outcomes. Clinical studies have reported good correlation between *in vitro* cell-based outcome measures like [Cell] and P_{CTP} in primary tissue sources and tissue repair outcome, suggesting the importance of these metrics.^{25,26} Cluster analysis on a subset of patient population with no missing data ($n = 46$) identified four subgroups of patients. These preliminary data suggest that younger patients with elevated BMI (16 patients, Cluster 1) tended to have increased [Cell] and [CTP] in cartilage, IPFP, and SYN. However, in older patients with lower BMI, [CTP] in SYN and fatpad tended

Table 3. Predictors of OA Severity as Determined by Total PLM Score.

Predictors	Histopathology Grade—Total PLM Score		
	N	R ²	P Value
Patient demographics			
Age (years)	90	0.038	0.067
BMI (kg/m ²)	90	0.003	0.606
Gender	90	0.017	0.214
Knee joint clinical outcome measures			
JSW (mm)	90	<0.001	0.980
KL score	90	0.080	0.007
Cartilage ECM content			
Total GAG per mg (μg/mg)	73	0.005	0.552
Total GAG per cell (ng/cell)	73	0.024	0.189
Cell and CTP prevalence, concentration in knee-derived tissues			
Cartilage-derived [Cell] (cells/mg)	90	0.026	0.126
Cartilage-derived P _{CTP} (CTPs/10 ⁶ cells plated)	81	<0.001	0.983
Cartilage-derived [CTP] (CTPs/g)	81	0.003	0.598
SYN-derived [Cell] (cells/mg)	65	0.060	0.049
SYN-derived P _{CTP} (CTPs/10 ⁶ cells plated)	65	<0.001	0.886
SYN-derived [CTP] (CTPs/g)	65	0.004	0.638
IPFP-derived [Cell] (cells/mg)	67	<0.001	0.887
IPFP-derived P _{CTP} (CTPs/10 ⁶ cells plated)	66	<0.001	0.952
IPFP-derived [CTP] (CTPs/g)	66	0.003	0.645

Simple linear regression models were used to individually assess potential predictors of OA disease that include patient demographics, knee joint clinical outcome measures, cartilage ECM content and cell and CTP concentration in knee-derived tissues including cartilage, synovium (SYN) and infrapatellar fatpad (IPFP). The bold text indicates factors that were significant and impacted the outcome being measured.

OA = osteoarthritis; PLM = polarized light microscopy; BMI = body mass index; JSW = joint space width; KL score = Kellgren and Lawrence score; ECM = extracellular matrix; GAG = glycosaminoglycans; CTP = connective tissue progenitors; [Cell] = cell concentration; P_{CTP} = CTP prevalence (CTPs/million cells plated); [CTP] = CTP concentration.

to be inversely related to [CTP] in cartilage, defining two subgroups (29 patients, Cluster 3 and 4). Clinical cell sourcing decisions for cell therapy approaches can be targeted. For example, depending on whether the patient falls into Cluster 2 or 3, SYN or IPFP maybe preferred tissue source for harvesting cells compared to cartilage. Note, Cluster 2 only has one patient and could be a potential outlier. In addition, we performed a sensitivity analysis to compare the baseline characteristics of the 46 patients included in the analysis versus 44 patients nonincluded in the analysis (data not shown). We found that only one factor (SYN-derived [Cell]) differed significantly between the groups. Although no studies to our knowledge considered cell and CTP concentration as characteristics in identifying OA subgroups, there are studies reporting OA phenotypes using other characteristics. Many of these studies considered various combination of clinical characteristics only including but not limited to BMI, radiographic scores, pain metrics, bone mineral density, magnetic resonance imaging (MRI) score, Hb-A1C, cholesterol, depression symptoms, serum, and urine biomarkers.^{8,9,27-29} Similar to our findings, some of these studies also found KL radiographic score and BMI as characteristics that can be used to discriminate OA subgroups.^{8,27,28} Few other studies focused on the RNA

sequencing and metabolic profiling of cartilage and SYN to identify OA subgroups.^{6,7,10,11,30} These studies have reported cartilage extracellular matrix genes (GAG) and collagen-associated genes to discriminate OA subgroups. Wyatt *et al.* identified three subgroups using the cartilage and SYN OA histopathological features.³¹ Patients vary widely, in addition to inherent genetic differences, several external factors including but not limited to comorbidities, occupational factors, physical activity, dietary exposures, and medication history could individually or in combination impact the various factors considered for OA subgroup classification in this study. These characteristics need to be systematically assessed to investigate the relationships and their impact on OA subgroups. Given the multidimensionality of the OA disease, depending on the dimensions (characteristics) considered in the study, different phenotypes or subgroups can be identified. Currently, there is no generally accepted classification system for OA phenotypes. Future studies will need to focus on identifying the minimal set of characteristics important to identify the OA phenotypes or subgroups, such that they are relevant to diagnosis, prognosis, and therapy. To validate our initial findings on the existence of these subgroups, future follow-up studies of larger cohort size need to be completed.

Table 4. Predictors of Cartilage-Derived Connective Tissue Progenitor (CTP) Concentration.

Predictor	Cartilage-Derived [CTP] (CTPs/mg)		
	N	R ²	P Value
Patient demographics			
Age (years)	82	0.013	0.306
BMI (kg/m ²)	82	<0.001	0.859
Gender	82	0.038	0.080
Knee joint clinical outcome measures			
JSW (mm)	82	0.012	0.325
KL score	82	0.024	0.167
Cartilage ECM content			
Total GAG per mg (µg/mg)	66	0.004	0.616
Total GAG per cell (ng/cell)	66	0.001	0.776
OA severity as determined by cartilage histopathological assessment			
Total HHGS score	81	0.002	0.692
Total PLM score	81	0.004	0.599
Cell and CTP prevalence, concentration in knee-derived tissues			
Cartilage-derived [Cell] (cells/mg)	90	0.148	<0.001
Cartilage-derived P _{CTP} (CTPs/10 ⁶ cells plated)	82	0.82	<0.001
SYN-derived [Cell] (cells/mg)	60	0.003	0.695
SYN-derived P _{CTP} (CTPs/10 ⁶ cells plated)	60	0.013	0.377
SYN-derived [CTP] (CTPs/g)	60	0.013	0.381
IPFP-derived [Cell] (cells/mg)	62	0.017	0.311
IPFP-derived P _{CTP} (CTPs/10 ⁶ cells plated)	61	0.003	0.688
IPFP-derived [CTP] (CTPs/g)	61	0.020	0.283

Simple linear regression models were used to individually assess potential predictors of cartilage-derived [CTP] that include patient demographics, knee joint clinical outcome measures, cartilage ECM content, OA severity as measured by cartilage histology scores and cell and CTP concentration in knee-derived tissues including cartilage, synovium (SYN), and infrapatellar fatpad (IPFP). The bold text indicates factors that were significant and impacted the outcome being measured.

BMI = body mass index; JSW = joint space width; KL score = Kellgren and Lawrence score; ECM = extracellular matrix; GAG = glycosaminoglycans; OA = osteoarthritis; HHGS = histological histochemical grading system; PLM = polarized light microscopy scores; [Cell] = cell concentration; P_{CTP} = CTP prevalence (CTPs/million cells plated); [CTP] = CTP concentration.

If true, these findings may define opportunities for more targeted patient-specific approaches to prevention and cell-therapy as well as other treatments.

Age, JSW, KL Score, Sulphated GAGs Per Milligram Cartilage, and SYN-Derived [Cell] Influence OA Severity as Determined by Histopathological Grades

Our data indicated that as age and KL score increase while JSW and total sulphated GAGs per mg cartilage decrease, total HHGS grade increases (OA worsens). For OA severity as measured by PLM grade, our data suggested that as KL score and SYN-derived [Cell] increases, total PLM grade increases. Studying these independent correlations helped us identify parameters that could potentially be influencing OA progression, especially in these early OA specimens that showed minimal surface degradation.

Histological evaluation is one of the most reliable methods to semiquantitatively measure cartilage degeneration

and OA severity. HHGS and OARSI are the two established scoring systems used for grading primary human OA cartilage.^{19,32} Both these scoring systems were established using late-stage OA specimens, and lacks features that could be present in early and mild OA specimens as previously demonstrated by our group.^{12,14} We therefore established a PLM scoring system for primary OA specimens to take into account some of the early and mild OA features particularly observed in the extra-cellular matrix organization and composition as well as changes seen near the calcified cartilage region (tidemark).^{14,18} Although we had OARSI scores for all the cartilage specimens, we did not perform separate correlation analysis because we previously found strong correlation between HHGS and OARSI scores ($r = 0.75$).¹⁴ Using the different clinical-level, tissue-level, and cell-level factors recorded in this study, we assessed their impact on HHGS and PLM scores.

In general, elderly, female, overweight and obese patients are considered more prone to OA.³³ However, several other factors including education-level, race, diet, genetic, other comorbidities, use of joints, bone density, muscle weakness,

joint laxity, previous injuries and more have been shown to play a role in the development of joint OA, making the assessment of patient demographics impacting OA complex.^{34,35} Some of these factors are modifiable and others are nonmodifiable. Identifying modifiable risk factors may help with connecting appropriate interventions to help treat or prevent OA. It should be noted that all the patients in this study were undergoing TKA for knee OA primarily in the medial compartment. Herein, cartilage and other tissues were obtained from a location (LFC) in these OA joints that was less disease impacted and not the primary reason for the patient undergoing TKA. Thus, our analysis of patient demographic factors will not be addressing the question of risk factors (as we did not have non-OA patient cohort in this study) but rather analyzing the OA patient cohort to determine if age, gender, or BMI was associated with OA severity in the less-impacted OA joint cartilage. Our data indicated that as age increases, OA severity increases. However, only 5.5% of variance in the HHGS scores could be predicted by patient's age.

Radiological examination is the most employed clinical technique for evaluating knee OA. Typically, the critical measures using the radiograph include the KL score, JSW, and Hip-Knee-Ankle angle. Studies in literature have reported poor or no correlation between radiological grades and histopathological grades.^{13,36,37} The data collected in this study suggests that as KL score increases or as JSW decreases, OA severity increases. However, both these variables could only predict for ~7% to 8% of variance in the HHGS and PLM scores. Larger study cohorts may be required to confirm these findings due to large patient variability.

The composition and organization of cartilage, primarily GAG and collagen have been reported to change with OA progression. This change has been reported to impact mechanical properties of cartilage that can eventually lead to irreversible structural damage of cartilage.³⁸ Some studies have reported a decrease in GAG content with increase in OA severity,³⁹ some have reported no difference between normal and OA cartilage with possible changes in content and distribution,⁴⁰ while others have reported no significant difference in GAG content between intact cartilage and early OA cartilage, with a decrease seen only in very severe OA specimens.⁴¹ Our data suggests that in mild to moderate OA specimens, GAG/mg cartilage decrease with increase in HHGS scores. However, due to patient heterogeneity, only 10% of the variance in HHGS scores could be predicted by sulphated GAG/mg. Quantitative changes in GAGs are important to measure as changes in the extracellular matrix composition.

It is now well established that in an adult knee, the disruption of physiological relationship and cross-talk between SYN, IPFP, and cartilage plays a critical role in OA pathogenesis.⁴²⁻⁴⁴ There are reports in literature which

indicate that CTPs from all the three tissue sources are upregulated in OA versus normal tissues.⁴⁵⁻⁴⁷ A previous study by our group found that cartilage-resident [Cell] increased and P_{CTP} decreased in late-stage (Outerbridge grade 3) cartilage in comparison to mild-moderate stage (Outerbridge grade 1,2) cartilage specimens from TKA patients.²³ Surprisingly the biological performance of CTPs from both late-stage and mild-stage OA cartilage were comparable, suggesting that even late-stage OA cartilage may serve as a source for cell-based approaches with appropriate selection strategies.²³ Another study by our group reported that in mild-moderate stage OA specimens (Outerbridge grade 1,2), [Cell] was higher in superficial-zone cartilage in comparison to deep-zone cartilage, but the P_{CTP} in the two zones was comparable.²² To our knowledge, there is no report in literature that performed paired assessment of alterations to [Cell] and [CTP] in cartilage, SYN and IPFP in the knee with OA severity in cartilage. Our results suggest that [Cell] and [CTP] in cartilage and IPFP did not change significantly with increasing OA severity in LFC. Interestingly, SYN-derived [Cell] increased with increasing OA severity as measured by PLM scores. Since PLM scoring system was designed specifically focusing on ECM and collagen alterations in mild/moderate OA specimens, these results could suggest that in specimens with intact cartilage surface (early OA), SYN may be playing a role in initiating cartilage ECM degradation.

Cartilage-Derived [CTP] Is Influenced by Cartilage-Derived [Cell] and P_{CTP}

Cartilage-derived cells are the first obvious choice for use in engineered articular cartilage. Given the limited access of cartilage tissue in patients, these often result in inadequate numbers of primary cells/CTPs for therapeutic treatment thereby demanding cell expansion *in vitro* to generate MSCs for use in clinical strategies like autologous chondrocyte implantation (ACI) and matrix-induced autologous chondrocyte implantation (MACI).^{48,49} Irrespective of whether the clinical strategy use primary cells or culture-expanded cells, it is necessary to study the prevalence and concentration of primary tissue resident CTPs and the various factors impacting them. Our results suggests that cartilage-derived [CTP] in our cohort of donors is independent of age, (range of 37 to 84 years of age; mean \pm SD = 62.4 ± 10.2 years), gender and BMI (range of 18.1 to 51.4 kg/m²; mean \pm SD = 31.8 ± 7.03 kg/m²). As discussed before, cartilage-derived [CTP] did not decrease with increasing OA severity in these mild-moderate OA cartilage specimens, suggesting that non-eroded OA cartilage can serve as a cell-source for therapeutic applications.⁵⁰ Although there are studies in literature referring to decline of cells resident in cartilage with age and

progressing OA, these studies are not directly analyzing the CTPs and thereby cannot be compared.⁵¹⁻⁵³ Cartilage-derived [Cell] and P_{CTP} had moderate and strong correlation with cartilage-derived [CTP] respectively. [Cell] is measured on the day of harvest and may be used as a first order assessment of cartilage tissue quality. Unlike the assay of P_{CTP} and [CTP], which requires several days of in vitro culture, [Cell] can be measured early to determine the patients likely to possess higher CTPs and probably benefit more from cell-based therapy.

The study is not without limitations. Due to large patient variability, larger study cohorts might be necessary to confirm these preliminary findings. Several other factors at clinical-level and tissue-level that have not been recorded in this study can influence the observations. A systematic database comprising of all the potential variables that can influence outcomes will need to be assembled to get superior understanding of OA patient phenotypes along with improved clinical applicability of these patient classifications. Osteoarthritis Initiative (OAI) is one such resource and can be further strengthened to include variables at cell-level like [Cell], P_{CTP} and [CTP] in joint tissues to begin understanding the role of stem and progenitor cells in OA development and progression as well as cell-based cartilage repair strategies.

Conclusion

Cluster analysis including clinical, cell and tissue level analysis suggests that patients undergoing TKA may be partitioned into four distinct biological subgroups. These preliminary data suggest that younger patients with elevated BMI (16 patients, Cluster 1) tend to have increased cell and CTP concentration in cartilage, IPFP and SYN tissues. However, in older patients with lower BMI (29 patients, Cluster 3 and 4), CTP prevalence in SYN and IPFP tended to be inversely related to CTP concentration in cartilage, defining two groups. The consistent presence of these subgroups will need to be tested in future cohorts. If true, these cohorts may define opportunities for more targeted patient-specific approaches to prevention and treatment.

Authors' Note

All the work was performed at Lerner Research Institute, Cleveland Clinic.

Author Contributions

1. Conception of study: VPM and GFM.
2. Study design, acquisition of data, data analyses: VPM, AC, NSP, CB, WB, JB, RJM, and GFM.
3. First draft preparation: VPM.
4. Critical revision of manuscript: AC, NSP, JB, RJM, CB, WB, and GFM.
5. Final approval of version to be submitted: VPM, AC, NSP, RJM, CB, WB, and GFM.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

This study was approved by the Institutional Review Board committee of the Cleveland Clinic (Protocol: 13641).

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