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The influence of chondrocyte source on the manufacturing reproducibility of human tissue engineered cartilage

Jill M. Middendorf[a], Nicole Diamantides[b], Byumsu Kim[a], Caroline Dugopolski[c], Stephen Kennedy[c], Eric Blahut[c], Itai Cohen[d], Lawrence J. Bonassar[a,b,*]

[a] Sibley School of Mechanical Engineering, Cornell University, Ithaca, NY, United States
[b] Meinig School of Biomedical Engineering Cornell University, Ithaca, NY, United States
[c] Histogenics Corporation, Waltham, MA, United States
[d] Department of Physics, Cornell University, Ithaca, NY, United States

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ABSTRACT

Multiple human tissue engineered cartilage constructs are showing promise in advanced clinical trials but identifying important measures of manufacturing reproducibility remains a challenge. FDA guidance suggests measuring multiple mechanical properties prior to implantation, because these properties could affect the long term success of the implant. Additionally, these engineered cartilage mechanics could be sensitive to the autologous chondrocyte source, an inherently irregular manufacturing starting material. If any mechanical properties are sensitive to changes in the autologous chondrocyte source, these properties may need to be measured prior to implantation to ensure manufacturing reproducibility and quality. Therefore, this study identified variability in the compressive, friction, and shear properties of a human tissue engineered cartilage constructs due to the chondrocyte source. Over 200 constructs were created from 7 different chondrocyte sources and tested using 3 distinct mechanical experiments. Under confined compression, the compressive properties (aggregate modulus and hydraulic permeability) varied by orders of magnitude due to the chondrocyte source. The friction coefficient changed by a factor of 5 due to the chondrocyte source and high intrapatient variability was noted. In contrast, the shear modulus was not affected by changes in the chondrocyte source. Finally, measurements on the local compressive and shear mechanics revealed variability in the depth dependent strain fields based on chondrocyte source. Since the chondrocyte source causes large amounts of variability in the compression and local mechanical properties of engineered cartilage, these mechanical properties may be important measures of manufacturing reproducibility.

Statement of significance

Although the FDA recommends measuring mechanical properties of human tissue engineered cartilage constructs during manufacturing, the effect of manufacturing variability on construct mechanics is unknown. As one of the first studies to measure multiple mechanical properties on hundreds of human tissue engineered cartilage constructs, we found the compressive properties are most sensitive to changes in the autologous chondrocyte source, an inherently irregular manufacturing variable. This sensitivity to the autologous chondrocyte source reveals the compressive properties should be measured prior to implantation to assess manufacturing reproducibility.

1. Introduction

Clinical trials of human tissue engineered cartilage have shown promising results in the repair of focal cartilage defects [1,2], but accurately identifying manufacturing variability remains a challenge. Assessment of manufacturing variability requires identifi-

* Corresponding author at: Meining School of Biomedical Engineering 149 Weill Hall, Cornell University, Ithaca, NY 14853, United States.
E-mail address: lb244@cornell.edu (L.J. Bonassar).

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cation of critical quality attributes that are appropriate for tissue implants. In guidance documentation, the FDA recommends that multiple mechanical properties (compression, friction, and shear) be evaluated prior to implantation [3]. These mechanical properties could serve as critical quality attributes of human tissue engineered cartilage. However, these mechanical properties are rarely measured by manufacturers. As a result, manufacturers do not know if any mechanical property has large variations due to the manufacturing process. Large variability in any mechanical property of engineered cartilage could cause differences in the in vivo function and may need to be measured prior to implantation.

The autologous chondrocyte source, an inherently irregular manufacturing variable, could be a major source of manufacturing variability and could contribute to changes in engineered cartilage mechanics. Studies that isolate human chondrocytes have shown that patient variables such as age [4–6], BMI [7], and presence of arthritis [6,8] can affect gene expression and a chondrocyte's ability to produce matrix. In studies on engineered cartilage with animal chondrocytes, differences in the chondrocyte source such as age and anatomic location influenced production and concentration of collagen and proteoglycans [9–12]. These concentrations of collagen and proteoglycans are usually correlated with the mechanical properties of engineered constructs [13–19]. Although evidence suggests that the autologous chondrocyte source will affect construct mechanics, the mechanical property (compression, friction, or shear) that will be most sensitive to chondrocyte source is unknown.

Previous work has shown evidence that the mechanical properties of human tissue engineered cartilage are sensitive to other manufacturing variables, specifically the culture period. During the manufacturing process of most human tissue engineered cartilage, autologous chondrocytes are seeded into porous scaffolds [2,20–22]. Once seeded some constructs are implanted directly into the joint, while others are grown in vitro for approximately 5 weeks. The construct compressive properties can improve 2-3-fold and approached values similar to native tissue in this 5 week culture period [18,23,24]. During this culture period, the friction coefficients reached values similar to native articular cartilage early (between 1 and 3 weeks) and remained similar to native tissue throughout the remaining growth period [18]. The shear modulus was the mechanical property least likely to change and typically remained an order of magnitude less than native cartilage even after 5 weeks of culture. Finally, local mechanical properties, such as depth dependent tissue modulus, can change by a factor of 10 with depth in a single sample and between constructs [25]. These differences were related to the growth period and local concentrations of new matrix deposition [26]. These previous results show some mechanical properties (compression and local mechanics) appear to be very sensitive to variability in the manufacturing and culture process. This subset of mechanical properties may also be the most sensitive to differences in the autologous chondrocyte source.

The main goal of this study was to assess the sensitivity of construct mechanical properties due to one inherently variable part of the manufacturing process – the autologous chondrocyte source. Specifically, we determined how the global compressive properties, friction coefficients, global shear modulus, depth dependent compressive strain, and depth dependent shear strain varied with changes in the human chondrocyte source. These results will help manufacturers identify the range of mechanical properties that are currently implanted into patients and establish which mechanical properties better define the manufacturing reliability. Based on previous work [18], each construct used in this study is expected to produce a unique quantity and spatial pattern of new matrix deposition. These spatial patterns of new matrix deposition may produce a wide range of mechanical properties. Here we test the hypothesis that the mechanical properties most sensitive to increased growth (compressive properties and local mechanics) will also exhibit the largest changes due to the chondrocyte source.

2. Methods

2.1. Construct preparation

All constructs were cultured in a manner similar to a clinically relevant human tissue engineered cartilage construct in a good manufacturing practices (GMP) compliant facility (Histogenics Inc, Waltham, MA) as previously described [18,21,25,27,28]. Briefly, human chondrocytes from 7 different cadavers were obtained from NDRI (Philadelphia, PA) as per policy of the Cornell University Institutional Review Board. Donor ages ranged from 28 to 42 years old with BMIs ranging from 23.7 to 34.9. Once obtained, all chondrocytes were expanded in monolayer culture and seeded onto scaffold (passage 1) or reseeded onto culture flasks and harvested after a second monolayer culture (passage 2). Since the total number of passages was small for all constructs tested, no changes due to passaging cells were expected. After being passaged, cells were seeded at a concentration of 5×10^6 cells/ml into a 3 mg/ml collagen gel. The cell seeded gel was then pipetted into a 6 mm diameter by 1.5 mm deep type I collagen honeycomb scaffold (Koken Co, Tokyo, JP). The pore size for all scaffolds was verified to be within the manufacturing release criteria. All constructs were then incubated under low oxygen conditions (2%) at 37 °C and 5% CO_2 with media changes (DMEM/ F12 with 10% FBS, Gibco, Thermo Fisher Scientific, Waltham, MA) at regular intervals for approximately 5 weeks. A total of 4 chondrocyte sources underwent dynamic growth conditions for the first 7 days followed by static culture conditions. The remaining sources underwent static culture conditions only. Data obtained from all culture conditions were pooled for subsequent analysis. Once incubation was complete, constructs were removed from culture then stored prior to testing.

The final depth of each construct was measured prior to testing. The depth of some scaffolds increased from the initial scaffold depth of 1.5 mm indicating that new extracellular matrix was deposited beyond the scaffold surfaces in some constructs. Consistent with how manufacturers would most likely implement testing, sample depths were not modified prior to mechanical testing.

Towards this end, chondrocytes from 7 human cadavers were used to make 240 constructs, which were then used in 3 distinct mechanical experiments and 2 biochemical assays (Fig. 1). Each mechanical experiment or biochemical assay was performed using at least 51 constructs and at most 96 constructs.

2.2. Compressive properties

A total of 93 samples were tested using confined compression to determine the aggregate modulus and hydraulic permeability of each engineered cartilage construct. All chondrocyte sources had at least 8 samples with a maximum of 16 samples (Fig. 1). All constructs were evaluated using a previously described technique [18,25,29,30]. Briefly, constructs were cut to a diameter of 4 mm using a biopsy punch. Then constructs were placed inside a confining chamber, covered with a porous platen, and mounted into an EnduraTEC ELF 3200 (Eden Prairie, MN) for stress relaxation testing. Constructs were compressed using a series of 8 step functions with each step being 5% strain, reaching a maximum strain of 40%. A poro-elastic model was then fit to the linear portion of this stress strain curve and used to calculate the aggregate modulus and the hydraulic permeability.

		Mechanical Samples per Test				Biochemical Samples per Test	
Chondrocyte Source		Aggregate Modulus (Ha)	Hydraulic Permeability (k)	Friction Coefficient (μ_b)	Shear Modulus (G*)	sGAG Content (µg/construct)	DNA Content (µg/construct)
A	Age 38 BMI 31.1	16	16	16	8	16	16
B	Age 37 BMI 25.9	16	16	16	8	16	16
C	Age 36 BMI 27.4	16	16	16	8	16	15
D	Age 34 BMI 34.1	15	15	16	8	16	16
E	Age 28 BMI?	8	8	8	7	8	8
F	Age 42 BMI 23.8	15	15	16	8	16	15
G	Age 28 BMI ?	7	7	8	4	8	7
Total		93	93	96	51	96	93
		Total Mechanical		333	Total Biochemical		189

Fig. 1. Number of samples for a given combination of global mechanical property and the chondrocyte source or combination of biochemical property and the chondrocyte source. (BMI was unknown for sample E and G).

2.3. Friction coefficient

A total of 96 samples were tested to measure the friction coefficient (Fig. 1). Each chondrocyte source had at least 8 samples, with most sources having 16 samples. Constructs were tested using a custom cartilage-on-glass tribometer as previously described [15,18,28,31,32]. Engineered cartilage constructs were mounted onto a plastic plate then glued into the tribometer, such that the surface of the sample was in contact with a glass slide. Constructs were then compressed and allowed to relax for at least 30 min prior to reaching an equilibrium load of approximately 100 g. By allowing samples to reach an equilibrium load, the boundary mode friction coefficient could be obtained. Once samples reached this equilibrium load, they were slid at 0.1 mm/s in both the forward and reverse directions for 3 cycles. A biaxial load cell measured both shear and normal loads, which were used to calculate the boundary mode friction coefficient. The friction coefficient was averaged in both the forward and reverse direction for all 3 cycles.

2.4. Shear modulus

A total of 51 samples were used to measure the shear modulus and depth dependent strain in constructs (Fig. 1). Each chondrocyte source contained at least 4 samples with a maximum of 8. The construct shear modulus, depth dependent axial strain, and depth dependent shear strain were obtained using a previously reported technique [18,26,28,30,33]. Briefly, constructs were cut in half, then stained with 14 µg/ml 5-dichlorotriazinyl-aminofluorescein (5-DTAF) (Molecular Probes1, Grand Island, NY) for 30 min followed by a rinse in phosphate buffer saline (PBS, Corning Cellgro, Manassas, VA) for at least 20 min. Constructs were then mounted between 2 plates and placed on an inverted confocal microscope. All constructs were submerged in PBS throughout testing. Constructs were imaged while being compressed to 10% axial strain then allowed to relax for at least 10 min. After relaxing, constructs were imaged using a 488 nm laser while being subjected to a 1% oscillatory shear strain at a frequency of 0.5 Hz. To verify there was minimal slippage between the oscillating plate and the construct, the input shear strain amplitude of the sample surface and oscillating plate was measured and compared. The displacements of the two tissue surfaces were measured and subtracted to calculate the global shear strain amplitude. The global shear stress was calculated by dividing the measured shear force on the sample by the cross-sectional area. Global shear modulus was then calculated by dividing the shear stress by the shear strain for each sample.

2.5. Depth dependent strains

Depth dependent Green-Lagrangian shear strain (E_{xy}) and Green-Lagrangian axial strain (E_{xx}) were calculated for shear loading and compressive loading respectively. To obtain these values, videos of the sample being compressed and the sample being sheared were analyzed using an open source DIC software as previously reported [26,28,34–36] in MATLAB (Mathworks, Natick, MA). Image resolution and size were 512 × 512 pixels and 2.6 µm/pixel respectively. For large samples undergoing shear analysis, images were stacked to obtain shear strain throughout the entire tissue depth. After DIC analysis, the average strain at each construct depth was calculated by averaging all strain values reported for that depth. The average strain values for each chondrocyte source were then plotted versus depth and compared.

2.6. Proteoglycan and DNA content

The biochemical content of each sample was measured using a biochemical assay. A total of 96 constructs were measured for sulfated glycosaminoglycans (sGAG) content and 93 constructs were measured for DNA content (Fig. 1). A Hoechst dye assay was used to measure DNA content. Prior to testing, constructs were lyophilized, then digested in 0.125 mg/ml papain solution at 60 °C for up to 16 h [32]. A Dimethylmethylene Blue (DMMB) assay was used to measure sGAG content [37]. Construct sGAG content was normalized to the DNA content to observe how efficient chondrocytes were at producing sGAGs.

2.7. Statistics

All global mechanical and biochemical data were analyzed using R Studio (RStudio, Boston, MA). The chondrocyte source was tested for significant differences using a linear model and a Kruskal Wallis post hoc test with Bonferroni correction. All linear models were checked for normality (friction coefficient and sGAG content). Mechanical and biochemical outcomes with non-normal distributions (shear modulus, aggregate modulus, hydraulic permeability, DNA content, and sGAG per DNA) were log transformed prior to running the linear model. To verify additional variables had no effect on the mechanical results, t-tests were run for growth parameters such as passage number and culture conditions (static versus dynamic). Results were considered significant for $p < 0.05$. The power analysis for the chondrocyte source was completed using powerSim in RStudio.

3. Results

3.1. Compressive properties

The compressive properties (aggregate modulus and hydraulic permeability) of constructs spanned a large range of values primarily arising from the chondrocyte source rather than intrapatient variability. The aggregate modulus ranged from 1.6 kPa to 593 kPa with a single broad peak (median) at 110 kPa (mean = 131 kPa and standard deviation = 125 kPa) and a right skewed histogram

Table 1
Result (p-values) from sttistical liner model determining the effect of chondrocytesource on the measured global mechanical or global biochemical property.

	Aggregate Modulus (Ha)	Hydraulic Permeability (k)	Friction Coefficient (μ_b)	Shear Modulus (G*)	sGAG Content (µg/construct)	DNA Content (µg/construct)
p-value	2.20E-16	1.30E-15	9.90E-07	4.94E-01	2.20E-16	2.20E-15

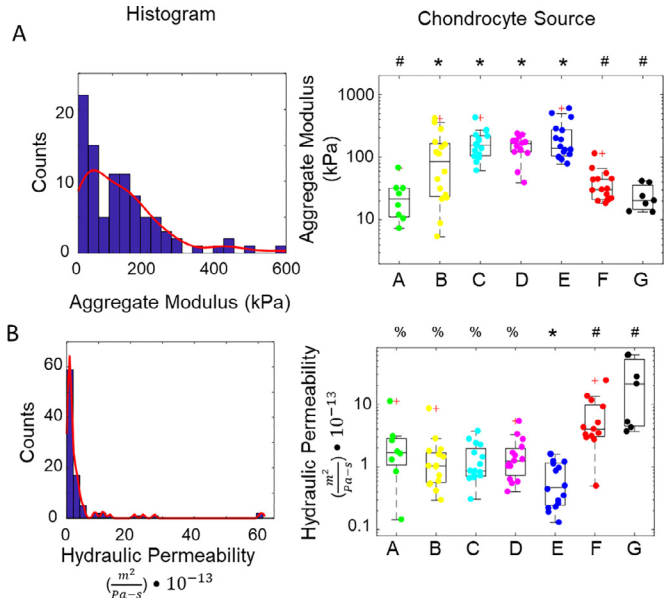

Fig. 2. Compressive properties summary: A) Aggregate modulus histogram shows a broad peak with right skewed data and a large range of values. B) Hydraulic Permeability histograms show a narrow peak with right skewed data and a large range of values. The chondrocyte source had a large effect on the hydraulic permeability and aggregate modulus. Different symbols indicate statistical significance ($p < 0.05$).

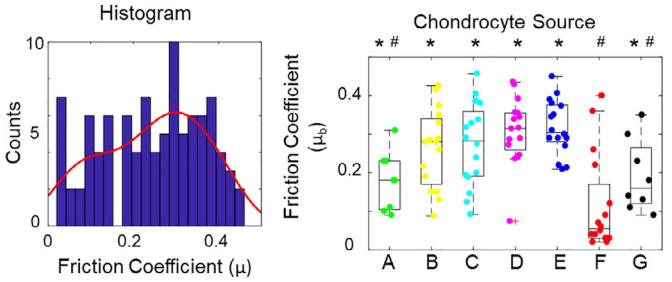

Fig. 3. Friction coefficient summary: The histogram shows a broad peak and a large range of values. The chondrocyte source has a large effect on the friction coefficient. Different symbols indicate statistical significance ($p < 0.05$).

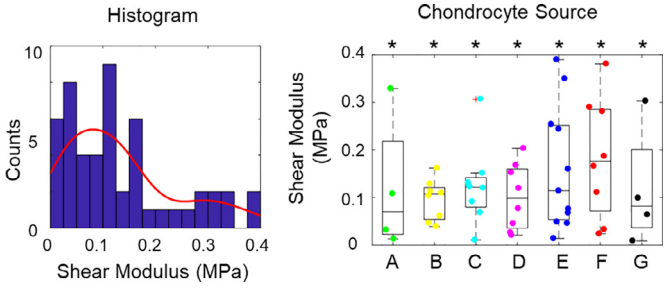

Fig. 4. Shear modulus summary: The histogram shows a broad peak, a large range of values, and right skewed data. The chondrocyte source had no effect on the shear modulus. Different symbols indicate statistical significance ($p < 0.05$).

(Fig. 2A). The hydraulic permeability of constructs spanned from 0.11×10^{-13} to 13.5×10^{-13} m^2/Pa-s with a single sharp peak (median) at 1.28×10^{-13} m^2/mPa-s (mean = 2.3×10^{-13} m^2/mPa-s and standard deviation = 3.5×10^{-13} m^2/mPa-s, Fig. 2B) and a right skewed histogram (Fig. 2B). With the exception of source B, intrapatient variability for the compressive properties was relatively low (standard deviations ≈ 50%) compared to differences between the chondrocyte source (orders of magnitude). Source A, F, and G had the lowest aggregate modulus (Ha ranged from 23 to 30 kPa, Fig. 2A) and the highest permeabilities (k ranged from 1.6×10^{-13} to 4.0×10^{-13} and m^2/Pa-s respectively, Fig. 2B). Chondrocyte source C had an aggregate modulus (Ha = 166 kPa) that was 5 times larger than sources A, F, and G. Source E had the lowest permeability (k = 0.25×10^{-13} m^2/Pa-s; $p < 0.001$; power = 1.0, Table 1), which was over an order magnitude less than sources A, F, and G. The order of magnitude differences observed in the compressive properties due to the chondrocyte source were larger than all other observed differences in the mechanical properties.

3.2. Friction coefficients

The friction coefficient of constructs spanned a large range of values due to the chondrocyte source and intrapatient variability. A histogram of all friction coefficients from all experiments, showed a left skewed distribution with a median at µ ~ 0.26, a mean of µ ~ 0.25, and a standard deviation of µ ~ 0.13 (Fig. 3). Intrapatient variability was comparable (standard deviations ≈ 41%) to interpatient variability. The chondrocyte source had a large effect on the friction coefficient measured in tissue engineered cartilage constructs ($p < 0.05$, Table 1). Chondrocyte source F had the lowest friction coefficients measured ($\mu_{mean} = 0.06$), while source D had the highest friction coefficient ($\mu_{mean} = 0.32$). All other chondrocyte sources had similar friction coefficients with averages that ranged from 0.21 to 0.30. These results show that friction coefficients for human tissue engineered cartilage constructs can vary by a factor of 5 both within and across chondrocyte sources.

3.3. Shear modulus

The shear modulus of constructs also spanned a large range of values due to a high degree of intrapatient variability. A histogram of all the shear modulus values showed a median at G = 0.11 MPa, a mean of G = 0.13 MPa, and a standard deviation of G = 0.11 MPa (Fig. 4). The data were right skewed and ranged from G ~ 0.01 to 0.38 MPa. The chondrocyte source did not cause significant differences in the shear modulus ($p = 0.494$, power = 0.60; Table 1), despite the range of shear modulus magnitudes (ranged from 0.09 to 0.18 MPa). Overall, the shear modulus showed little dependence on chondrocyte source and showed the greatest variations (standard deviations ≈ 76%) within the same patients.

3.4. Depth dependent mechanics

Under compressive loading, the depth dependent pattern of the axial strain varied per chondrocyte source indicating some chondrocyte sources may respond differently to local scaffold proper-

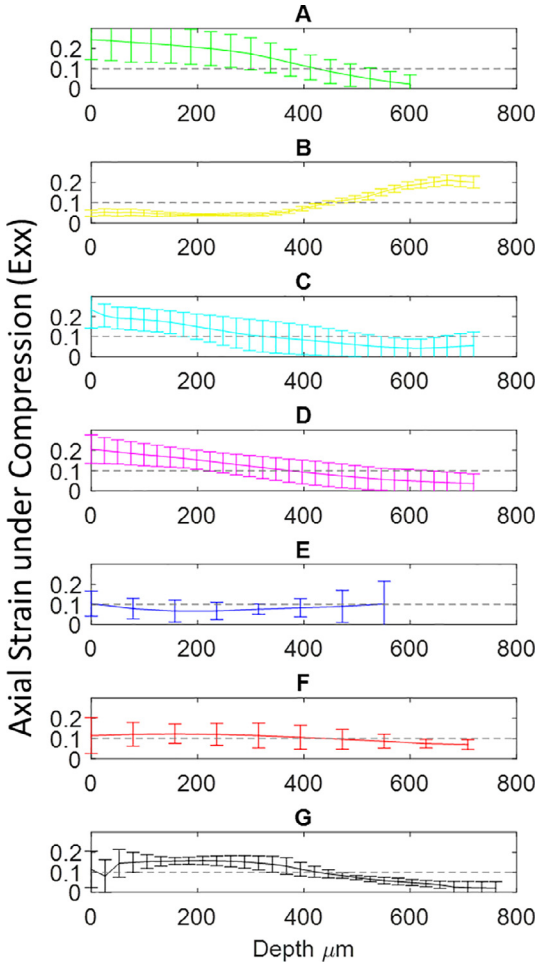

Fig. 5. Depth dependent axial strain for each chondrocyte source. Some chondrocyte sources produced uniform strain fields across the sample while other sources produced very heterogeneous strain. Gray dashed line is the input strain (10%).

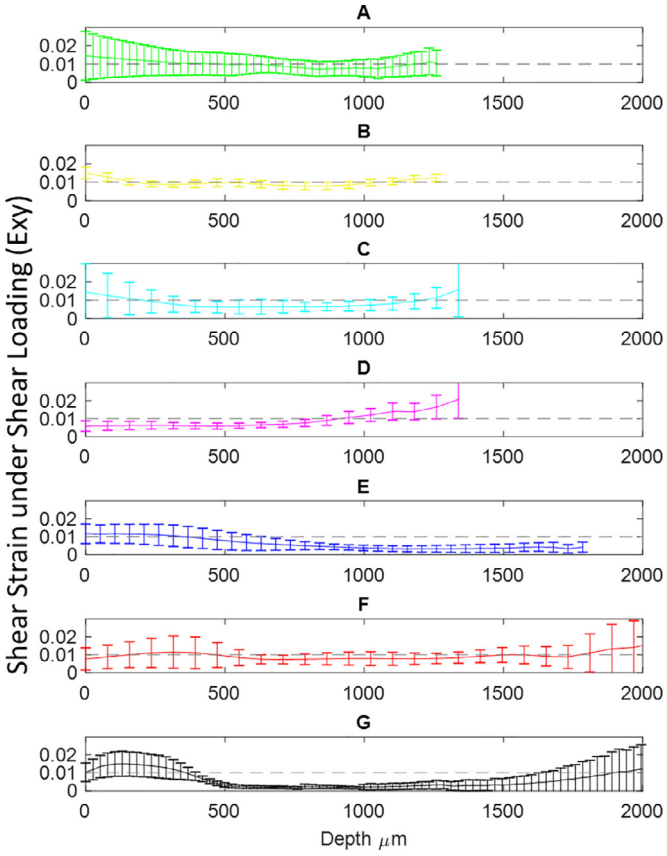

Fig. 6. Depth dependent shear strain for each chondrocyte source. Some chondrocyte sources produced uniform strain fields across the sample while other sources produced very heterogeneous strain. Additionally, the total length of each construct could vary based on the location of new matrix deposition. Gray dashed line is the input shear strain (1%).

ties, local concentrations of nutrients, and/or nearby chondrocytes (Fig. 5). Sources A, B, C, D, and G exhibited highly heterogeneous axial strain fields that varied by up to a factor of 10 through the tissue depth. In contrast, sources E and F exhibited relatively uniform axial strain across the sample. These data suggest the depth dependent axial strain patterns do change based on the chondrocyte source and its response to the local environment.

Similarly, depth dependent patterns of shear strain varied per chondrocyte source indicating differences in the local deposition of new matrix by chondrocytes. Sources C, D, E, and G exhibited non-uniform shear strain across the construct with depth dependent strains varying by at most a factor of 7 through a tissue depth (Fig. 6). In contrast, source A, B, and F experienced relatively uniform shear strains across each construct (ΔExy $<$ 0.009, Fig. 6). These data suggest the depth dependent shear strain patterns also change based on the chondrocyte source and the chondrocyte response to its local environment.

3.5. Biochemical composition

The synthesis of biochemical content in human tissue engineered cartilage constructs varied based on the chondrocyte source. The histogram of sGAG content was bimodal with a maximum (median) at 60.2 µg/construct and a secondary peak near 129.2 µg/construct (mean = 67.5 µg/construct, standard deviation = 11.2 µg/construct, Fig. 7A). This bimodal histogram appears to be caused by distinct differences between chondrocyte sources

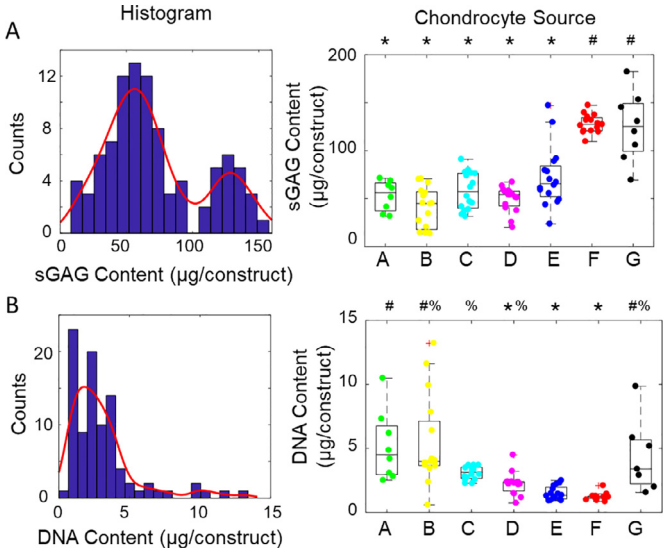

Fig. 7. Biochemical properties summary: A) The sGAG content histogram shows a bimodal distribution with a large range of values. B) The DNA content histogram shows a broad peak with right skewed data and a large range of values. The chondrocyte source had a large effect on the sGAG and DNA content. Different symbols indicate statistical significance ($p < 0.05$).

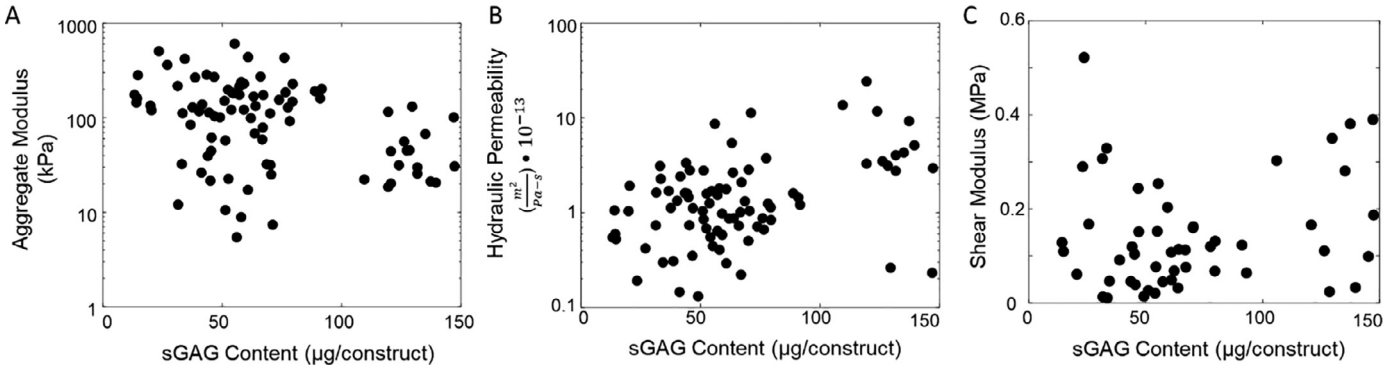

Fig. 8. The global structure function relationship of human tissue engineered cartilage showed no strong correlations between the global sGAG content and the A) aggregate modulus, B) hydraulic permeability, or C) the shear modulus. This lack of correlation may be caused by heterogeneity in new matrix deposition.

A through E, which have low biochemical content when compared to sources F and G, which have high biochemical content. The total sGAG content per construct varied from 1.1 to 147 µg/construct. Chondrocyte source F and G produced the most sGAG (~ 126. 9 µg/construct). All other sources produced at least 50% less sGAG per construct (~46 to 64 µg/construct, $p < 0.001$; power = 1, Table 1). The total sGAG content per construct was affected by the chondrocyte source, but the observed differences were much less than the order of magnitude differences observed in the compressive properties.

Although constructs were initially seeded with the same number of cells, the DNA content was also affected by the chondrocyte source. The histogram of DNA content was right skewed with a median (peak) at 2.42 µg/construct, a mean of 2.93 µg/construct, and a standard deviation of 0.73 µg/construct (Fig. 7B). DNA content ranged from 2.8 to 13.2 µg/construct. Chondrocyte source A had the largest amount of DNA content (5.5 µg/construct), while sources E and F had the least amount of DNA content and about 5 times less than source A (~1.1 µg/construct, $p < 0.001$; power = 1, Table 1). After normalizing the sGAG content by the DNA content, source F had produced the largest amount of sGAG per DNA (supplemental Figure 1). Similar to the compressive properties and sGAG content, the chondrocyte source can influence the number of chondrocytes in these engineered cartilage constructs.

To understand the relationship between global mechanical properties and tissue function we plotted the aggregate modulus, hydraulic permeability, and shear modulus versus the sGAG content. From these plots we note little to no correlation between the aggregate modulus and sGAG content and little to no correlation between the hydraulic permeability and sGAG content (Fig. 8A-8B; $R^2 = 0.16$ for both hydraulic permeability and aggregate modulus). The shear modulus did not correlate with sGAG content (Fig. 8C; $R^2 = 0.01$). Although surprising, these results show that global sGAG content is not a strong predictor of a construct's aggregate modulus and hydraulic permeability.

4. Discussion

This study analyzed hundreds of human tissue engineered cartilage constructs and discovered that the autologous chondrocyte source, an inherently irregular manufacturing variable, has a large effect on multiple mechanical properties. The global compressive properties showed large sensitivity to changes in the chondrocyte source with less intrapatient variability than both the friction coefficient and the shear modulus. Additionally, both depth dependent compressive and shear mechanical properties of constructs showed a variety of behaviors associated with tissue heterogeneity and the chondrocyte source. These findings indicate the aggregate modulus, hydraulic permeability, and depth dependent mechanical properties are sensitive to changes in the chondrocyte source and could be important measures of manufacturing reproducibility.

The sensitivity of compression to slight changes in the manufacturing process is consistent with previous work showing large changes in the compressive properties but smaller changes in both friction and shear properties after 5 weeks of construct growth [18,28,38]. Compressive properties continuously improve from the initial scaffold mechanics during the 5 week culture period [18]. This improvement in compressive properties may be caused by high collagen concentration in the scaffold and the heterogeneous deposition of proteoglycan content (new matrix) [18,38]. Friction coefficients reach values similar to native tissue early during the in vitro culture process (~3 weeks), then the coefficients stop improving [18]. These friction coefficients have been associated with decreases in surface roughness [39]. In contrast, the shear modulus remains relatively constant with increased growth, which has been linked to the relatively small changes in collagen and collagen fiber formation [26,40]. In this study we note proteoglycan production by 2 of the 7 chondrocyte sources used in this study was very large compared to all other chondrocytes. A larger cohort of chondrocytes should be used to determine if this ratio of overperforming chondrocytes to average chondrocytes is to be expected for a general sample of the population. Nevertheless, the sensitivity of the compressive properties to two aspects of the construct manufacturing process (growth period [18] and chondrocyte source) show compressive properties could be an important measure of manufacturing reproducibility.

Despite FDA guidance, manufacturers rarely measure mechanics prior to implantation. Instead, the global biochemical content is measured because mechanical properties typically correlate well with biochemical composition [13,14,17–19,38,41]. However, this study did not observe a correlation between global compressive or shear properties and global sGAG content (Fig. 8). Although surprising, we believe this low correlation ($R^2 = 0.16$, $R^2 = 0.01$) may be caused by heterogeneity in the deposition of the sGAG content. Heterogeneity in sGAG content has previously been shown to have distinct effects on the construct mechanics based on the location of new matrix deposition [26]. Matrix deposited in pores results in a much larger improvement in compressive properties than matrix deposited outside pores on the scaffold surface [26]. This finding suggests that measurements of global sGAG content may not be a suitable surrogate for compressive properties. Notably, the confined compression technique is most affected by the chondrocyte source and is one of the simplest and most common mechanical measurements performed by researchers on engineered cartilage [9,10,23,41–43]. Therefore, measuring the global compressive properties of engineered cartilage could be a straightforward and simple method to satisfy FDA guidance.

The presence of intrapatient variability in some mechanical properties but not others provides an opportunity to identify the mechanical parameters that could be most easily implemented during manufacturing. Implementing mechanical testing on the manufacturing line will most likely require more surrogates, which are representative tissue samples that can increase complexity of the manufacturing process. To implement mechanical tests, the minimum number of additional surrogates necessary for this evaluation can be calculated using a power analysis [44], which uses multiple factors including the intrapatient variability. Although it's not clear what causes intrapatient variability, we believe slight variations in chondrocyte distribution within the construct and nutrient transport, as well as variability in chondrocyte behavior may cause local construct heterogeneity [45–50] that will lead to the observed intrapatient variability. Since the intrapatient variability of the friction coefficient and shear modulus in this study was very high compared to interpatient variability, many surrogates would be necessary to determine differences between groups. In contrast, confined compression resulted in low intrapatient variability compared to interpatient variability. As such, measuring the compressive properties on constructs prior to implantation should require the fewest surrogates compared to other mechanical tests, and may be a good first step towards constraining the variability due to the autologous chondrocyte source.

The depth dependent mechanical properties may also be an important quality control attribute of human tissue engineered cartilage [51]. This study observed large variabilities in the depth dependent strain fields of constructs based on the chondrocyte source. Since all scaffolds were made using the same process and the relative pore size was verified in each batch, we expect depth dependent scaffold variability to have a small effect on the local strain. Instead, these differences in depth dependent mechanics may be associated with local sGAG content that varied due to the response of chondrocytes to their local scaffold properties, local nutrient concentrations, and/or local chondrocyte concentration [48,49]. This heterogeneity in new matrix deposition can cause localized strengthening that leads to heterogeneous mechanical properties under compression [25,26]. Because native cartilage tissue also has large depth dependent variations in mechanical properties, such heterogeneity in construct local mechanics may be a desirable feature. If such mechanical heterogeneity is critical to the success of implanted constructs, our data suggest that the autologous chondrocyte source could cause depth dependent variability. Consequently, mechanical heterogeneity may be an important measure to improve manufacturing repeatability of implanted constructs.

Heterogeneous depth dependent mechanical properties are present in a large number of tissue engineered cartilage constructs [11,16,18,19,52–55], but measuring these depth dependent mechanics is destructive in nature and may require high sample numbers to implement. Alternatively, quantitative measures of local biochemical composition (i.e. FTIR and Raman spectroscopy) [26,56–61] may be able to non-destructively infer the depth dependent mechanical properties. The benefit of spectroscopy techniques includes the ability to directly quantify the relative concentration of collagen and proteoglycans on a local scale. Some techniques can be implemented in real time and are non-destructive. Thus, implementing spectroscopy techniques that can be correlated with measurements of local mechanical properties would be advantageous. This correlation allows manufacturers to more readily characterize acceptable ranges of manufacturing variability due to autologous chondrocyte source.

Many studies show engineered cartilage has inferior mechanical properties compared to native cartilage [15,18,19,41,62–64], these differences may be caused by local structural features in tissue engineered cartilage that are distinct from native cartilage. Specifically, the engineered cartilage used in this study has a stiff scaffold with very large pores made from collagen type I, while native tissue has a collagen type II network with much smaller pores. The difference in pore sizes also affects how new matrix deposition (proteoglycans) change the tissue mechanical properties. A previously proposed percolation theory that states the connectivity of collagen is critical to understanding when small increases in collagen content will greatly increase the shear modulus [40,65] may explain the observed differences between native and engineered tissues. The high interconnectivity of collagen in native tissue allows for small increases in collagen to greatly increase the shear modulus. In contrast, our constructs have shear moduli that are orders of magnitude less than native tissue and have a much less interconnected collagen network than native tissue [18,26], implying that large amounts of collagen content may be needed to improve construct mechanics. This percolation theory also shows that the effects of proteoglycan content on the shear modulus of constructs is dependent on the connectivity of the local collagen network. A similar theory may be used to understand why permeability of tissue engineered constructs remains so much larger than native tissue, but the aggregate modulus can approach values similar to native tissue. In engineered cartilage, the large pore size greatly increases the permeability and decreases the aggregate modulus compared to native cartilage [18]. Achieving mechanical properties in engineered cartilage that approach native tissue, likely requires highly levels of matrix deposition in the scaffold pores [26]. These levels of matrix deposition may take more than 5 weeks of in vitro growth, as was used in this study. The vast differences in the collagen network structure of native versus engineered tissues may contribute to the large differences seen in their mechanical properties.

In this study, a large number of human and chondrocyte specific factors may have affected the differences observed in the global and depth dependent mechanical properties of constructs. Human factors such as age [4,5,8,66], BMI [7], and degree of osteoarthritis [8] could contribute to the effectiveness of the chondrocytes in producing new matrix and resulting desirable mechanical properties. These factors were not controlled, and this study does not have the statistical power necessary to make any conclusions about which chondrocyte factor (age, BMI, etc.) caused the differences in the observed mechanical properties. Previous work has shown increased BMI may be associated with increased sGAG production per chondrocyte [7]. However, our study shows different results, with the smallest BMI (patient source F) producing the most sGAG content. These differences could indicate another patient variable is more predictive of sGAG production by chondrocytes. Future experiments may be designed to specifically identify which human and chondrocyte specific factors cause the observed changes in the global compressive and depth dependent mechanical properties. Until future studies identify the many complex and interacting factors associated with the observed mechanical differences, measuring construct mechanics may be necessary to verify the repeatability of the manufacturing process.

In addition to the chondrocyte source, other manufacturing parameters could affect constructs mechanics. In this study, both the passage number and the presence of dynamic culture conditions were varied. Previous research has shown these parameters can change the production of new matrix in engineered cartilage [63,67–73]. However, this study did not observe any differences caused by either of these culture conditions (supplemental Figure 2). The differences in the mechanical properties caused by the chondrocyte source are magnitudes greater than any differences caused by either passage number or dynamic culture. These culture conditions do not change the overall results of this study showing that compressive properties and local mechanical properties of human tissue engineered cartilage should be measured prior to implantation.

Since a relationship between construct mechanics and the in vivo success of tissue engineered cartilage is unknown, some researchers believe engineered cartilage properties need to match native articular cartilage [46,51,74]. However, native tissue properties may not be necessary to ensure success of a construct after implantation. For example, the integration of native and engineered tissues may be stronger if constructs are implanted prior to the constructs reaching native tissue properties [75]. In the current study, many of the mechanical properties measured were inferior to those of native articular cartilage [18], yet this manufacturing process and implantation procedure has produced favorable clinical outcomes [21,27]. These favorable outcomes may be because the constructs continue to mature after implantation. The mechanical variability seen in the current study identifies the range of mechanical parameters that should be studied relative to clinical outcomes. Once a relationship between pre-implantation mechanics and clinical outcomes has been identified, constructs with inferior compressive properties (e.g. group A's aggregate modulus and hydraulic permeability) could be allowed to grow for a longer period of time to reach the desired mechanical threshold. Conversely, constructs containing autologous chondrocytes that are able to produce large quantities of proteoglycans (group F and G) or high compressive properties (group C, D, and E for aggregate modulus) may not require the full 5 week growth process. This study laid the groundwork for future studies to tune the manufacturing process to optimize cost, mechanical function, and in vivo success by identifying the mechanical variability that should be measured by manufacturers.

In conclusion, the chondrocyte source can cause large variability in the compressive and local mechanical properties of engineered cartilage. By identifying the mechanical properties that are most sensitive to changes in the manufacturing process, this work helps manufacturers focus on a few mechanical properties listed in the recommendations from the FDA guidance [3]. Future work should consider determining acceptable ranges for these mechanical properties of engineered constructs by measuring the compressive and local mechanical properties of human tissue engineered cartilage constructs prior to implantation.

Declaration of Competing Interest

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CRediT authorship contribution statement

Jill M. Middendorf: Conceptualization, Data curation, Formal analysis, Writing – review & editing. **Nicole Diamantides:** Data curation, Visualization, Writing – review & editing. **Byumsu Kim:** Data curation, Visualization, Writing – review & editing. **Caroline Dugopolski:** Conceptualization, Funding acquisition, Project administration, Writing – review & editing. **Stephen Kennedy:** Conceptualization, Funding acquisition, Project administration, Writing – review & editing. **Eric Blahut:** Conceptualization, Funding acquisition, Project administration, Writing – review & editing. **Itai Cohen:** Conceptualization, Formal analysis, Writing – review & editing. **Lawrence J. Bonassar:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actbio.2021.07.003.

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