

Full length article

The maturation state and density of human cartilage microtissues influence their fusion and development into scaled-up grafts

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ABSTRACT

Functional cartilaginous tissues can potentially be engineered by bringing together numerous microtissues (μ Ts) and allowing them to fuse and re-organize into larger, structurally organized grafts. The maturation level of individual microtissues is known to influence their capacity to fuse, however its impact on the long-term development of the resulting tissue remains unclear. The first objective of this study was to investigate the influence of the maturation state of human bone-marrow mesenchymal stem/stromal cells (hBM-MSCs) derived microtissues on their fusion capacity and the phenotype of the final engineered tissue. Less mature (day 2) cartilage microtissues were found to fuse faster, supporting the development of a matrix that was richer in sulphated glycosaminoglycans (sGAG) and collagen, while low in calcium deposits. This enhanced fusion in less mature microtissues correlated with enhanced expression of N-cadherin, followed by a progressive increase in markers associated with cell-extracellular matrix (ECM) interactions. We then engineered larger constructs with varying initial numbers (50, 150 or 300 μ Ts per well) of less mature microtissues, observing enhanced sGAG synthesis with increased microtissue density. We finally sought to engineer a scaled-up cartilage graft by fusing 4,000 microtissues and maintaining the resulting constructs under either dynamic or static culture conditions. Robust and reliable fusion was observed between microtissues at this scale, with no clear benefit of dynamic culture on the levels of matrix accumulation or the tensile modulus of the resulting construct. These results support the use of BM-MSCs derived microtissues for the development of large-scale, engineered functional cartilaginous grafts.

Statement of significance: Microtissues are gaining attention for their use as biological building blocks in the field of tissue engineering. The fusion of multiple microtissues is crucial for achieving a cohesive engineered tissue of scale, however the impact of their maturation level on the long-term properties of the engineered graft is poorly understood. This paper emphasizes the importance of using less mature cartilage microtissues for supporting appropriate cell-cell interactions and robust chondrogenesis *in vitro*. We demonstrate that tissue development is not negatively impacted by increasing the initial numbers of microtissues within the graft. This biofabrication strategy has significant translation potential, as it enables the engineering of scaled-up cartilage grafts of clinically relevant sizes using bone marrow derived MSCs.

1. Introduction

Cartilage tissue engineering (TE) aims to partially or completely regenerate tissue that has had its function impaired by trauma or disease. Current clinical strategies such as matrix-induced autologous chondrocyte implantation (MACI) fail to consistently promote

regeneration of functional hyaline cartilage [1–3] and is an unsuitable treatment modality for large scale joint degeneration. Therefore, there is an urgent need to develop scalable cartilage TE strategies capable of directing hyaline cartilage regeneration.

Limitations associated with the use of scaffolds for tissue regeneration has motivated increased interest in scaffold-free TE strategies [4].

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Such approaches leverage the inherent ability of cells to self-organize in response to interactions with neighbouring cells and the extracellular matrix (ECM) [5]. Modular scaffold-free TE strategies often involve the use of numerous microtissues, defined as highly cellular aggregates that secrete their own ECM, which are subsequently combined to bio-fabricate a more complex, macro-scale tissue [6–9]. The goal of such approaches is that the final engineered graft will exhibit a structure, morphological composition and function that closely resembles the native tissue. Microtissues have found diverse applications in the field of tissue TE [10–12], including their use as biological building blocks in the biofabrication and 3D bioprinting of complex tissues and organs [9,13,14]. Despite their promise for cartilage TE, it remains unclear whether such modular scaffold-free approaches can be used to engineer scaled-up, functional articular cartilage (AC) grafts using clinically relevant cell sources.

Modulating the process and kinetics of microtissue fusion and (re)modelling is integral to their use in TE [15,16]. Successful fusion of multiple microtissues involves the complete integration of the cells and their ECM components to create a more cohesive tissue structure [17]. Fusion is known to be strongly dependent on the maturation levels of individual microtissues (i.e., how long microtissues are kept in culture in isolation before they are combined with other microtissues), with superior fusion observed for less mature microtissues. Bhumiratana *et al.* (2014) [17] explored the kinetics of microtissue fusion and how it can influence the final cartilaginous phenotype. They showed that microtissues set tenascin boundaries after 7 days of growing independently before fusion. The presence of these boundaries could impair cohesive tissue fusion [17]. De Moor *et al.* (2020) [18] observed fusion of cartilage microtissues after they were independently matured for 7 days, however the resulting tissue was not fully compact after a 21-day culture period. Although previous studies have generally observed superior fusion using less mature microtissues, the impact of such microtissue maturation levels on extended tissue development post-fusion remains unclear. Therefore, in the context of cartilage TE, further work is required to identify the specific microtissue maturation levels most suited to the development of scaled-up grafts. In addition, most existing strategies have focused on the fusion kinetics of relatively small numbers of individual microtissues [19–21], providing limited insight into the cellular and molecular mechanisms that regulate the fusion process in larger engineered tissues.

The overall aim of this study was to assess the capacity of cartilage microtissues generated from human bone marrow derived MSCs to fuse and generate a functional, scaled-up cartilage graft. To this end, we first sought to investigate the kinetics of microtissue fusion and subsequent chondrogenesis for microtissues of differing maturation levels. During the initial stages of fusion, we assessed changes in cell-cell and cell-ECM interactions in less and more mature microtissues. We then explored how microtissue density would impact chondrogenesis in longer term culture. We finally sought to engineer a scaled-up cartilaginous tissue (10 mm in diameter) by fusing a large number of immature microtissues.

2. Materials and Methods

2.1. Isolation and expansion of hBM-MSCs

Human bone marrow mesenchymal stem cells (MSCs) were isolated from fresh human bone marrow samples obtained from 21-year-old female donors (Lonza, Switzerland). A total of 30 ml of fresh human bone marrow was collected under sterile conditions. High Glucose Dulbecco's Modified Eagle's Medium GlutaMAX, (hgDMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 1% penicillin-streptomycin (Gibco), and human Fibroblast Growth Factor (FGF-2) at 5ng/mL, was used as the culture medium (termed 'XPAN' media). Cells were seeded at a density of 40–60 million cells per T-175 flask (Corning) in XPAN. The T-175 flasks were incubated at 37°C in a humidified atmosphere containing 5% CO₂ and 5% O₂. After 4 days of initial seeding, when the

media was changed, 10 mL of the media volume containing non-adherent cells was collected and re-seeded in another T-175 flask to promote adherence of slower-adhering cells. The cells were cultured for 12–14 days under 5% CO₂ and 5% O₂, and the media was changed every 3 days. After 12–14 days, hBM-MSCs formed colonies, and the cells were trypsinized using 0.25% (v/v) Trypsin ethylenediaminetetraacetic acid (EDTA) and frozen at passage one. For all experiments, hBM-MSCs at passage 3 (P3) were used. All studies in this paper used human MSCs, except for the scaled-up grafts (section 3.4) where caprine MSCs were used.

2.1.1. Isolation and expansion of caprine BM-MSCs

Caprine bone marrow mesenchymal stem cells (MSCs) were isolated from the sternum of skeletally mature, female goats. The bone marrow was cut into 2mm³ pieces, vortexed for 2 minutes in XPAN to aid liberate cellular components and then seeded at a density of 5.7×10^4 cells/cm² and maintained in a humidified atmosphere under physiologic conditions (5% CO₂, 5% O₂). After 72 hours, the flasks were washed twice with sterile PBS to remove non-adherent cells and debris. Cell culture media was exchanged to fresh XPAN every 2 days. When the MSCs colonies were 80% confluent, cells were trypsinized as described in 2.1. For further expansion of cells, flasks were seeded at a density of 5×10^3 cells/cm² at 5% CO₂, 5% O₂ and expanded up P3 for all experiments.

2.2. Fabrication of MSC microtissues

The microtissues were fabricated as previously described [22]. The 3D printed mould stamps containing either 401 or 1,889 micro resections and 4% (w/v) ultrapure agarose were sterilised in autoclave at 120°C for 20 minutes. Next, 6 mL of ultrapure agarose was gently poured into each well of 6-well plates and allowed to cool down for 15 min after moulding it. Agarose moulds were then soaked overnight in Endothelial growth media (EGM), composed of Endothelial Cell Basal Medium-2 (EBM) (Lonza) supplemented with MV Microvascular Endothelial Cell Growth Medium-2 BulletKit™ (Lonza), at 37°C in a humidified atmosphere with 5 % CO₂ and 5 % O₂ to enhance cartilage formation [23]. MSCs were seeded in a density of 5.35×10^5 into the microwells in a final volume of cell suspension of 3 mL, to obtain 401 or 1889 microtissues with 4,000 cells/microtissue. The MSCs were maintained in chondrogenic media (CDM) consisting of hgDMEM GlutaMAX supplemented with 100U/mL penicillin, 100 µg/mL streptomycin (both Gibco), 100 µg/mL sodium pyruvate, 40 µg/mL L-proline, 50 µg/mL L-ascorbic acid-2-phosphate, 4.7 µg/mL linoleic acid, 1.5 mg/mL bovine serum albumin, 1 × insulin–transferrin–selenium (ITS), 100 nM dexamethasone (all from Sigma), 2.5 µg/mL amphotericin B, and 10 ng/mL of human transforming growth factor-beta 3 (TGF-β) (Peprotech, UK). 20 minutes after seeding, the plates were centrifuged for 5 minutes at 700 × g to collect cells at the bottom of each well. Due to the smaller area available for each microtissue in the 1,889 micro resections mould system, cells were seeded at a density of 7.5×10^5 /mL into the microwells for a final volume of 5 mL of CDM and centrifuged as described above. Albeit the high density of cells in a low media amount, after 2 days the cells showed high viability (Appendix).

2.3. Fusion of microtissues

To ensure that the exposure of the microtissues in CDM was equal for all the groups, we cultured each group in CDM for a total amount of 28 days as follows. The 'F-D2' microtissues was cultured in CDM for 2 days in their individual microwells prior to fusion, followed by a further 26 days of fusion in CDM. The 'F-D4' microtissues was cultured in CDM for 4 days in their individual microwells prior to fusion, followed by a further 24 days of fusion in CDM. The 'F-D7' microtissues was cultured in CDM for 7 days in their individual microwells prior to fusion, followed by a further 21 days of fusion in CDM. After respectively each maturation time, the microtissues were harvested, counted, and seeded onto 2%

(w/v) agarose coated wells in a 48 well plate. To harvest the microtissues, media was first flushed over the agarose surface using a micropipette, and subsequently collected into a 50 mL tube. The agarose mould was then removed, inverted into a new 6-well plate, pre-filled with 3 mL of fresh media, and centrifuged at 750 g for 5 min to collect the microtissues at the bottom of the well. The resulting microtissue suspension was then strained through a pluriStrainer® (pluriSelect®, Leipzig, Germany) to retain any agarose fragments. The density of the microtissue suspension was quantified, and the appropriate volume was used to seed a precise number of microtissues into each construct. The number of the microtissues seeded into each well was 40 ± 3 to ensure that each construct had the same size. Immediately after seeding the microtissues, 2 mL of CDM was gently added into each well and the plate was swirled to favour microtissues fusion. The constructs were cultured for 21 days in CDM in physiologic conditions and media was exchanged thrice a week. This experiment was performed twice with two different human female donors.

To obtain different constructs by varying the microtissues density, day 2 microtissues were harvested and fused as previously described into agarose coated 24 well plates. Next, the number of microtissues seeded for the fusion was respectively 50, 150 and 300 per construct. Immediately after seeding the microtissues, CDM was gently poured into each well and the plate was swirled to favour microtissues fusion. The developing constructs were cultured for 42 days in CDM, in physiologic conditions and media exchanged thrice a week. The experiment was performed once with 4 replicates, with one female human donor.

2.3.1. Fabrication of scaled-up cartilage constructs using caprine MSCs

The microtissues were fabricated according to the method described in 2.2 (using caprine as opposed to human MSCs) and cultured in CDM for 2 days in physiologic conditions prior to harvesting. At day 2, microtissues were harvested, counted, and seeded into non-adherent agarose wells. In this study, a cylindrical stamp with a diameter of 10 mm and a height of 1 mm was designed to cast the microtissues. This stamp was used to create a positive mould under sterile conditions using a hot 2% (w/v) agarose solution. The 3D printed stamp was designed using Autodesk® Fusion 360™ software. 4,000 microtissues were added in each agarose moulds to fill the entire mould. This was done to ensure that the appropriate number of microtissues was seeded to achieve the desired construct size and volume. After seeding the microtissues, 80 mL of CDM was gently added, and the constructs were maintained at 20% O₂ for 42 days. The fused microtissues were cultured under two distinct conditions: the static groups were cultured in a 150 mm x 25 mm Petri dish, while the dynamic groups were cultured in a spinner flask (Gibco, Fisher Scientific, UK) rotating at a speed of 110 rpm. Media was changed thrice weekly. The experiment was performed once, with 3 replicates each group, with one donor.

2.4. Histological evaluation

Briefly, samples were fixed with 4% paraformaldehyde (PFA) solution overnight at 4°C. Following fixation, the samples underwent dehydration using a series of ethanol solutions (from 50% to 100% v/v), followed by clearing in xylene and embedding in paraffin wax (Sigma). The samples were sliced into 5µm tissue sections and rehydrated. Haematoxylin and eosin (H&E) staining (Sigma) was used to assess the tissue morphology, the extracellular matrix, and the nuclei of the cells. 1% (w/v) solution of Alcian Blue (AB) 8GX in 0.1 M hydrochloric acid (HCl) was used to stain sGAG content and counter-stained with 0.1% (w/v) nuclear fast red to determine cellular distribution. A 0.1% (w/v) Picrosirius red (PSR) solution was used to visualize collagen deposition (all from Sigma). Stained sections were mounted using DPX solution and imaged using an Aperio ScanScope slide scanner (Leica, Germany). Immunohistochemistry was performed for collagen type I (Abcam ab90395 1:400), collagen type II (Santa Cruz sc52658 1:400), collagen type X (Col X) (Abcam 49945, 1:200) as previously described [24].

Cell apoptosis within the core of the scaled-up graft was evaluated using TUNEL staining. In brief, slides were rehydrated following previously established protocols [42], then treated with proteinase K (20 µg/mL) (Sigma) for 5 minutes at room temperature. After washing with 1X PBS, the slides were incubated with a DNA labelling solution (TdT Reaction Buffer, TdT Enzyme, Br-dUTP, deionised water) for 60 minutes at 37°C. Following several washing steps, an anti-BrdU-Red antibody was applied for 30 minutes at room temperature in the dark. The slides were washed again and 7-AAD/RNase A staining buffer was applied as a counterstaining, for 30 minutes at RT in the dark, in a humidified atmosphere. The slides were then mounted with gold antifade mounting medium (Sigma), and sealed using clear nail polish. Images were captured within 3 hours of staining using a Leica SP8 scanning confocal microscope. Representative images are presented as maximum projections of z-stacks.

2.5. Immunofluorescence evaluation

Briefly, after fixation in a 4% paraformaldehyde (PFA) solution overnight, the samples were resuspended in a 20% sucrose (w/v) (Sigma) solution. After 1 hour, the solution was replaced with a 30% (w/v) sucrose solution and allowed to incubate for 24 hours or until the sample settled to the bottom of the vial. The following day, the samples were embedded in OCT embedding matrix (VWR, IE) and flash-frozen using liquid nitrogen. The frozen samples were then stored at -80°C until slicing. The samples were sliced with a thickness of 20 µm. Prior to antigen retrieval, the slides were rehydrated using PBS 1X (Gibco). Antigen retrieval was performed in a sodium citrate buffer made with 10mM Sodium Citrate, 0.05% Tween 20 (all from Sigma) pH 6, in a humid environment at 80°C for 20 minutes. The slides were washed with PBS 1X and incubated with 10% (v/v) donkey serum (Sigma) for 30 minutes to allow the blocking of nonspecific sites. Samples were incubated with primary antibody in 2% BSA in a humidified chamber overnight at 4°C. Samples were washed 3X with 1X PBS and incubated with a secondary antibody in 2% BSA for 1h at RT in the dark. Samples were washed 3X with PBS 1X and counterstained with a Hoechst staining solution 1 µg/mL (Sigma) for 15 minutes in the dark. Samples were washed 3X with PBS 1X and mounted with mounting media (Sigma). Prior to imaging the slides were sealed with nail polish and stored in the dark at 4°C. Immunofluorescence was performed for Vimentin (Abcam ab92547 1:200) to assess the cell-ECM interactions and Neural Cadherin (N-Cadherin) (Abcam ab98952 1:200) to visualize cell-cell interactions. Images were taken using a Leica SP8 scanning confocal microscope. Representative images are presented as maximum projections of z-stacks.

2.6. Live dead assay

Cell viability was assessed using a live/dead assay kit (Invitrogen, Bioscience). The constructs were first rinsed in PBS 1X and then incubated for 1 hour in a solution containing 2 µM of calcein and 4 µM ethidium homodimer-1 (EthD-1). Following the incubation period, the constructs were rinsed again to remove any excess of the dyes and were imaged using a Leica SP8 scanning confocal microscope. Excitation wavelengths of 485 nm and 530 nm were used for calcein, while emission wavelengths of 530 nm and 645 nm were used for calcein and EthD-1. Maximum projection z-stack reconstructions were generated to analyse cell viability throughout the depth of the tissue, with the view from the top.

2.7. Biochemical analysis

After retrieval, the samples were washed twice with 1X PBS and dried. Papain enzyme solution created with 3.88 U/mL of papain enzyme, 100 mM sodium phosphate buffer, 5 mM Na₂EDTA, and 10 mM L-cysteine at pH 6.5 (all from Sigma), was then added to digest the

samples at 60°C for 18 hours. Immediately after digestion, DNA content was quantified using the Quant-iT™ PicoGreen® dsDNA Reagent and Kit (Molecular Probes, Biosciences). The amount of sulphated glycosaminoglycan (sGAG) was determined using a dimethylmethylene blue dye (DMMB, Sigma) binding assay. To exclude any background absorbance, the pH of the DMMB was adjusted to 1.5. A chondroitin sulphate standard (1 mg/mL, Sigma) was used, and the absorbance was measured at 530 and 590 nm using a Synergy HT multi-detection microplate reader (BioTek Instruments, Inc). The 530/590 absorbance ratio was used to generate the standard curve and determine the sGAG concentration from the digested sample. Total collagen content was determined by measuring the hydroxyproline content using a chloramine-T assay. The samples were mixed with 38% HCl (Sigma) (1:1 ratio) and incubated at 110°C for 18 hours for hydrolysis. After cooling, samples were centrifuged at 5000 x g for 5 minutes, and let dry at 60°C for 48 h. The sediment was reconstituted in 200 µL of ultra-pure H₂O. Chloramine T (2.82% w/v) and 4-(Dimethylamino) benzaldehyde (0.05% w/v) were added and incubated for 20 minutes at RT in the dark. The hydroxyproline content was quantified using a trans-4-Hydroxy-L-proline standard at a wavelength of 570 nm with a Synergy HT multi-detection microplate reader (BioTek Instruments, Inc). Hydroxyproline levels were estimated from the standard curve at a wavelength of 570 nm. The hydroxyproline-to-collagen ratio of 1:7.69 was used to calculate the collagen content.

2.8. RNA isolation and quantitative Real-Time PCR

At 24h and 7 days after fusion of the human MSC derived microtissues at different maturation levels (F-D2, F-D4, F-D7) the samples were washed 3 times with 1X PBS and then snap frozen for further processing. RNA was isolated from the samples using the Trizol method (Sigma-Aldrich), following the manufacturer's instructions. Chloroform extraction was performed, and the RNA was re-suspended in RNase-free water. The RNA samples were then stored at -80°C. For cDNA synthesis, a HighCapacity RNA-to-cDNA™ Kit (Applied Biosystems™), was used to transcribe 500 ng of RNA from each sample into cDNA (20 µL) via polymerase chain reaction (PCR). Real-time PCR was performed using an Applied Biosystems instrument, and Taqman PCR universal master mix (ThermoFisher) was used for the PCR reaction. Human-specific TaqMan probes (ThermoFisher) were used for gene amplification. The qPCR amplification followed the following cycles: 50°C for 2 minutes, 95°C for 10 minutes and 40 cycles at 60°C for 1 minute each. The levels of gene expression were quantified using the real-time PCR data and analyzed with the $2^{-\Delta\Delta Ct}$ method. The expression levels were normalized to the average expression of the 18S rRNA housekeeping gene.

2.9. Diameter measurements and Fluorescence quantification

Diameter measurement of single microtissues and growing engineered cartilage fused microtissues, were conducted using a 4x objective of an inverted microscope (Primo Vert, Zeiss, USA), and calculated using ImageJ software (Wayne Rasband and Contributors, USA). Images were captured at hourly intervals until 8 hours post-fusion, daily until day 7, and weekly until week 3 post-fusion. Four independent measurements of each construct were obtained from each experimental replicate. For immunofluorescence quantification, the intensity of 3 specific region of interests (ROI) within each sample was measured and quantified through ImageJ software. The intensity was then normalized by the number of nuclei in the ROI.

2.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software, CA, USA). For diameter measurements a two-way ANOVA was performed followed by Tukey's multiple comparison test to assess the differences in diameter between groups over time. For

biochemical analysis, a one-way ANOVA was performed followed by Tukey's multiple comparison test to assess the differences between groups. For qPCR analysis a two-way ANOVA was performed followed by Tukey's multiple comparison test to assess the difference in the gene expression between groups. For Young's Modulus analysis an unpaired t test with Welch's correction was performed to assess the differences between the two groups. Significance was accepted when $p < 0.05$.

3. Results

3.1. Human MSC derived microtissues secrete a cartilage-like extracellular matrix over time under chondrogenic culture conditions

The ability of human MSCs to form microtissues was assessed (Fig. 1A) and histological and biochemical evaluation of the single microtissues were performed at different maturation levels (2, 4, 7 and 28 days) to quantify the level of chondrogenesis and extracellular matrix production (Fig. 1B, C). At days 2, 4, and 7 of culture, no strong indication of chondrogenesis was observed (Fig. 1B). However, at day 28, the microtissues stained positively for GAGs and collagen deposition (Fig. 1B). The biochemical analysis showed a significant increase in collagen and sGAG production over time in culture (Fig. 1C). By day 28, there was a 2.5-fold increase in sGAG/microtissue ($p = 0.01$) and a 6-fold increase in collagen/microtissue compared to day 2 ($p = 0.0032$) (Fig. 1C). The sGAG/DNA ratio ($p=0.0108$) and collagen/DNA ratio ($p=0.0085$) also significantly increased with time in culture (Fig. 1C).

3.2. Cell-cell and cell-ECM interactions during fusion depend on microtissue maturity

To assess the capacity of microtissues at different stages of maturation (day 2, day 4 and day 7) to form self-organized cartilaginous constructs by fusion, 40 microtissues from each maturation group were combined and allowed to fuse (Fig. 2). The fusion of microtissues was initially assessed using brightfield microscopy, capturing images at different time points to evaluate the fusion process over 21 days of chondrogenic induction (Fig. 2A).

All microtissues successfully fused in culture, although the fusion kinetics varied between the groups (Fig. 2A). For the day 2 maturation level (F-D2), the microtissues fused into a compact tissue unit within 48 hours. For day 7 maturation level (F-D7), the microtissues achieved compact fusion only after 7 days of culture (Fig. 1B). Additionally, the final size of the construct depended on the maturation levels of the individual microtissues, with the F-D2 microtissues producing larger (1 mm) constructs than the F-D7 (0.5 mm) microtissues (Fig. 2A).

To investigate collagen deposition during microtissue fusion, PR staining was used to monitor spatial changes in collagen accumulation for the different maturation levels (Fig. 2B). Clear collagen-rich boundaries were evident between the more mature (F-D4 and F-D7) microtissues, especially at early timepoints (Fig. 2C, black arrows). As time elapsed, the boundaries between the individual microtissues were less obvious in day 2 microtissues (F-D2) (Fig. 2B, black arrows). Evidence of residual boundaries were still clear after 7 days of fusion for the more mature microtissues (Fig. 2B, black arrows).

We next examined the expression (qPCR) and spatial localization (immunohistochemistry) of vimentin and N-cadherin, markers of cell-ECM and cell-cell interactions respectively, at different timepoints post-fusion (Fig. 3). 8 hours post fusion, more intensive staining for N-Cadherin was evident in the less mature F-D2 microtissues compared to their more mature F-D4 and F-D7 microtissues (Fig. 3A). Conversely, less intense vimentin staining was observed in the less mature F-D2 microtissues at this early timepoint (Fig. 3B). Vimentin staining increased over time during the fusion of the less mature F-D2 microtissues, with an opposite trend observed in the more mature F-D7 microtissues over time in culture (Fig. 3B). After 7 days of fusion, more homogeneous vimentin staining was observed in the F-D2 group compared to F-D7 group (white

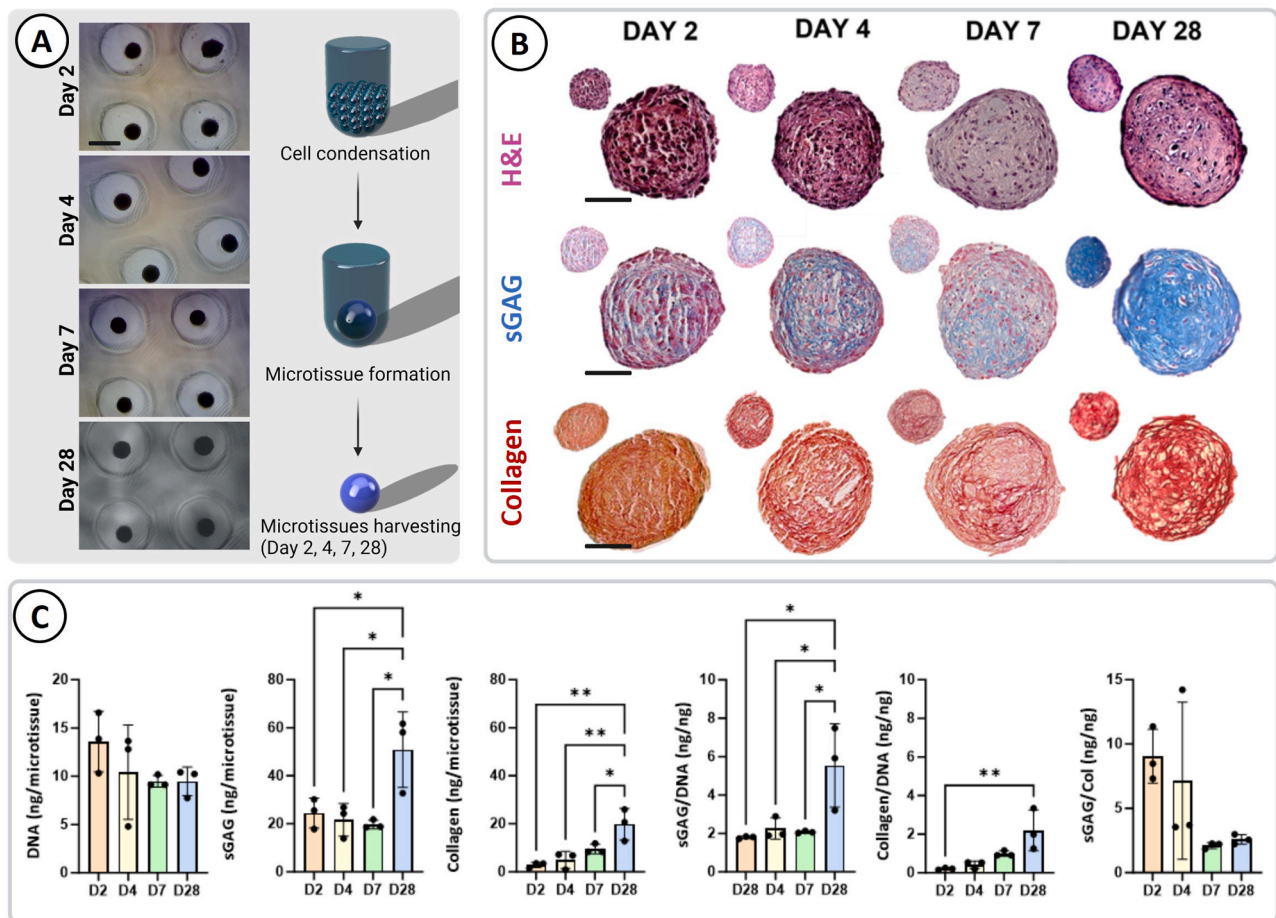


Fig. 1. MSC derived microtissues secrete a cartilaginous matrix over 28 days of culture in chondrogenic media. (A) Experimental settings: microtissues are cultured in agarose micro-moulds (B) Histological panel for matrix components after 2, 4, 7 and 28 days in chondrogenic media. (C) Biochemical quantification of the microtissues; levels of DNA, sGAG, collagen are normalized to number of microtissues and to the DNA content. Significant differences are denoted as: * $p < 0.05$, ** $p < 0.01$, when tested using a one-way ANOVA ($n = 3$). Scale bar is 60 μm . Fusion 360™ was used to design the graphical abstract, and BioRender was used for the layout of the final figure.

boxes, Fig. 3B). Gene expression quantification confirmed a significant increased vimentin expression at 7 days post-fusion within the less mature F-D2 microtissues (Fig. 3B).

3.3. Fusion of less mature microtissues supports the development of a more hyaline cartilage phenotype

We next examined the expression of a range of cartilage-specific genes 24 hours and 7 days post-fusion (Fig. 4). After 24 hours, the more mature F-D4 and F-D7 microtissues expressed significantly higher levels of the cartilage-matrix genes ACAN and COL2A1 (Fig. 4). Notably, the expression of COL1A1 was highest in the mature F-D7 microtissues at this early stage. 7 days after fusion, a significant increase in the expression of ACAN and COL2A1 was observed in the less mature F-D2 microtissues. By this later timepoint, the expression levels of ACAN, COL2A1, and SOX9 were higher in the F-D2 microtissues compared to the F-D7 microtissues (Fig. 4).

We next examined how microtissue maturation levels could influence cartilage specific ECM components after 28 days of chondrogenic induction (Fig. 5). As a control we added the single microtissues cultured for 28 days in isolation within microwells to assess if there was a change in the chondrogenic markers between fused and non-fused microtissues. Positive staining for sGAGs (alcian blue) and Col II was observed in all groups, with the most intense staining for sGAGs observed in the less mature F-D2 group (Fig. 5A). The DNA level quantification shows no statistical changes between groups ($p = 0.1686$). Biochemical analysis

further confirmed significantly higher ($p < 0.0001$) sGAG/DNA and collagen/DNA levels in the constructs generated with the less mature F-D2 microtissues (Fig. 5B). Fused microtissues (F-D2 and F-D4) exhibited a greater ability to produce sGAG ($p < 0.001$) compared to individual microtissues cultured for 28 days in the microwells, indicating differences between the fused and non-fused microtissues (Fig. 5B). Due to their superior capacity for chondrogenesis, the F-D2 microtissues were used for subsequent studies.

3.4. Increasing the density of microtissues per construct facilitates the engineering of mechanically functional grafts of a clinically relevant size

We next sought to assess how the number of microtissues used to generate a scaled-up construct would impact subsequent tissue development. To this end 50, 150 or 300 microtissues (F-D2) were fused and tissue morphology was assessed after 42 days of culture (Fig. 6). The diameter of each construct increased over 42 days of chondrogenic induction, with no statistical differences among the groups (Fig. 6A). Increasing the number of microtissues per construct led to a significant increase ($p < 0.0001$) in sGAG production (Fig. 6C), while collagen synthesis appeared less dependent on microtissue density (Fig. 6C). Collagen deposition appeared homogeneous in the 50 microtissue group, while a core region staining less intensely for collagen appeared to develop in the 300 microtissue group (Fig. 6A, arrow). sGAG deposition appeared equally homogenous in all the groups (Fig. 6B). After 42 days, a stable cartilage phenotype was achieved in all groups without

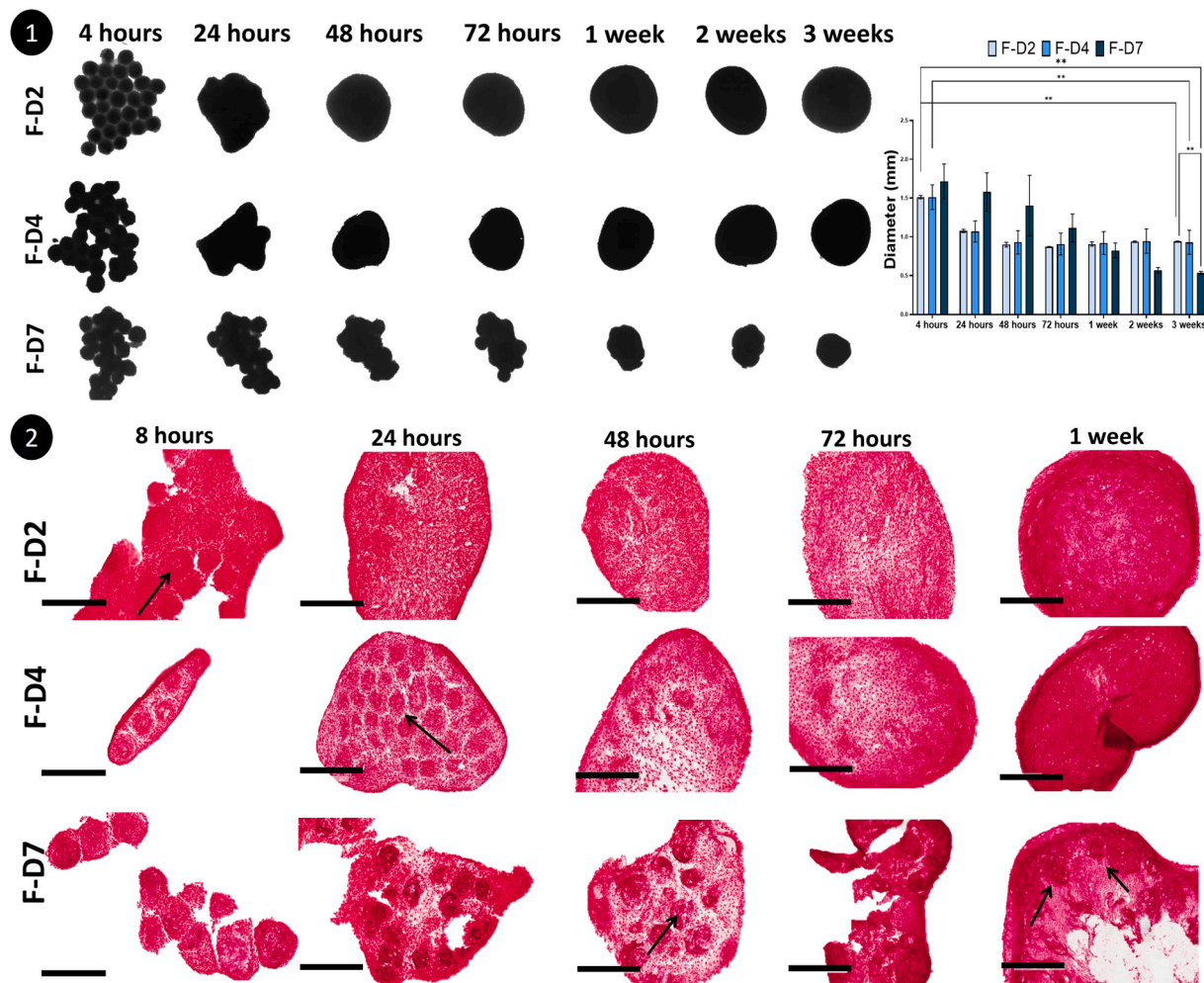


Fig. 2. Microtissues of differing maturation levels show differences in fusion kinetics, final size and collagen deposition over 28 days of culture in chondrogenic media. A) Brightfield microscope images depict fused microtissues observed over a 21-day period in culture in chondrogenic media, followed by diameter measurements. Scale bar: 500 μ m. B) Samples were subjected to picrosirius red staining to visualise the collagen present within the tissue. The arrows point to remnants of the structure of the individual microtissues. Scale bar: 200 μ m. Significant differences are denoted as: ** $p < 0.01$, when tested using a two-way ANOVA test ($n = 20$). F-D2: microtissues fused at day 2 maturation level. F-D4: microtissues fused at day 4 maturation level. F-D7: microtissues fused at day 7 maturation level.

signs of hypertrophy, as indicated by negative alizarin red and collagen X staining (Fig. 6B and D).

To create a larger construct of a clinically relevant size, a cylindrical stamp with a diameter of 10 mm and a height of 1 mm was designed to cast approximately 4,000 FD-2 microtissues (generated using caprine MSCs). The constructs were cultured in dynamic and static conditions to assess how such culture conditions would impact tissue development and biomechanical function (Fig. 7). Apoptosis staining (TUNEL) at the end of the cell culture suggests little or no cellular apoptosis in both groups (Fig. S2). Histological analysis showed positive staining for sGAG in both static and dynamic culture conditions, with less intense staining observed in the centre of the graft (Fig. 7B, arrow). The formation of this 'core' occurred at approximately 1 mm into the static constructs and 1.3–1.4 mm into the dynamic constructs (Fig. 7B, arrow). The AR staining suggested some calcium deposition in the centre of both groups (Fig. 7, arrow). The biochemical analysis showed no significant difference between both groups for sGAG and collagen deposition, while tensile testing showed no statistical difference in stiffness (Fig. 7). In addition, both groups stained positive for COL I and COL II (Fig. 7), suggesting the development of a more fibrocartilaginous tissue.

4. Discussion

In this study, we explored the fusion of microtissues at varying stages of maturation to engineer scaled-up cartilage grafts. Our findings indicate that fusing microtissues at lower maturity levels promotes enhanced chondrogenesis in the resultant grafts. Moreover, we investigated the role of cell-cell and cell-ECM interactions during microtissue fusion by assessing temporal changes in N-cadherin and vimentin expression and localization during the initial stages of fusion. We found that increased N-cadherin expression in less mature microtissues was associated with stronger cellular interactions, as evidenced by accelerated and more robust fusion. We next explored how microtissue density impacted subsequent chondrogenesis, observing that sGAG synthesis increased with increases in microtissue number. We finally explored the scaling-up of the tissue engineered graft by dramatically increasing the number of fusing microtissues. Our results demonstrate that increasing the number of microtissues does not hinder fusion, validating the feasibility of using human MSC derived microtissues for the engineering of cartilaginous constructs of a clinically relevant size.

We initially evaluated the composition of individual cartilage microtissues after 2, 4, 7 and 28 days of culture within single microwells. Despite there being little changes in ECM deposition during the first 7 days of microtissue maturation, the capacity of these cellular structures

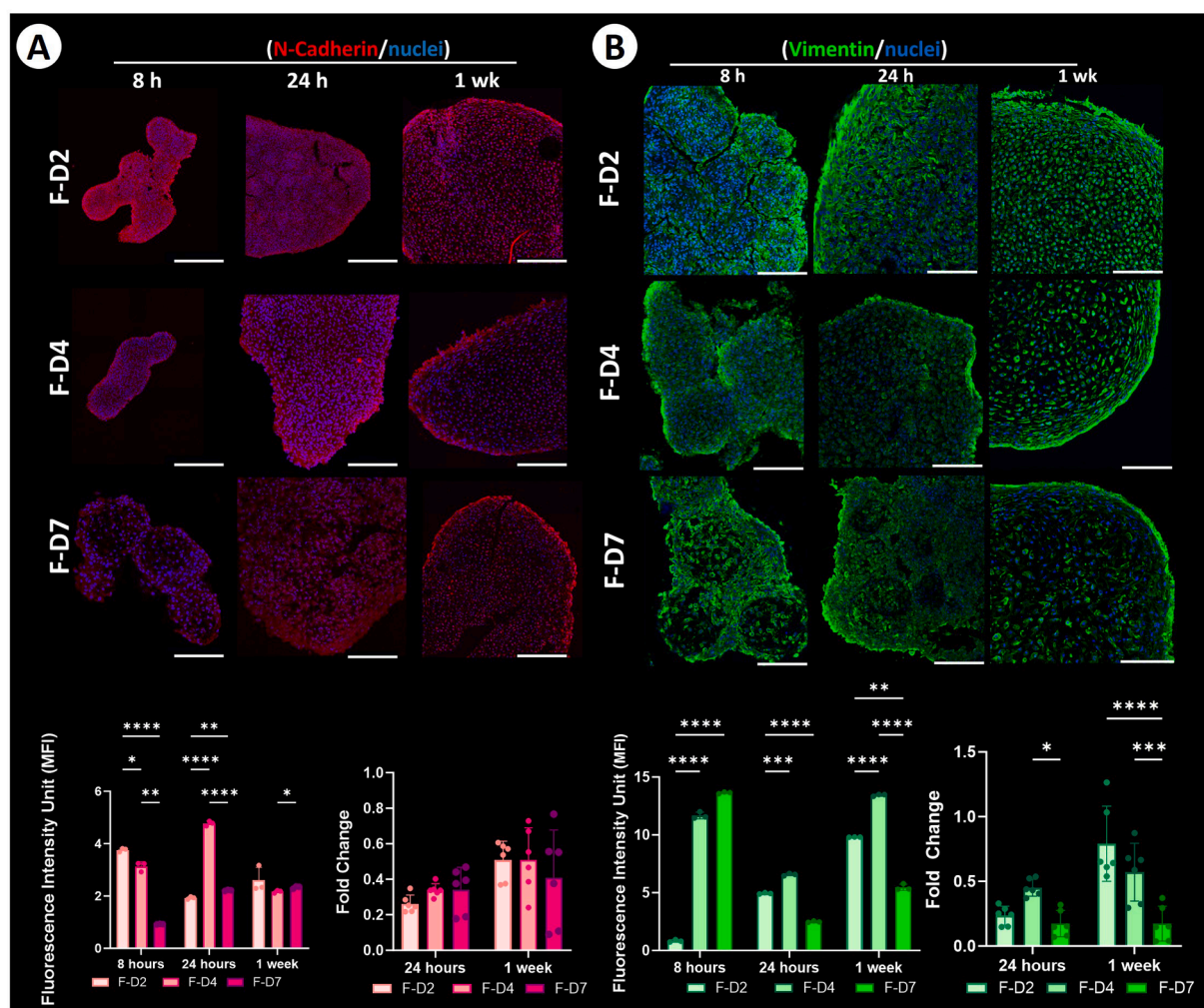


Fig. 3. Cell-cell and cell-ECM interactions during microtissue fusion are dependent on their maturation levels. N-Cadherin and Vimentin are stained with respective monoclonal antibodies, nuclei are stained with Hoechst staining. Immunofluorescence quantification was performed by normalizing the region of interest (ROI) with the number of nuclei in the same ROI. mRNA expression of the cell-cell and cell-ECM markers, vimentin (VIM) and N-Cadherin (CDH2), were analysed by real-time PCR. Samples were normalized with MSCs in monolayer. A) N-Cadherin staining, immunofluorescence quantification (left graph) and mRNA expression (right graph) for fused microtissues at different maturation levels over 7 days. B) Vimentin staining, immunofluorescence quantification (left graph) and mRNA expression (right graph) for fused microtissues at different maturation levels over 7 days. Significant differences are denoted as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ when tested using a two-way ANOVA test ($n = 6$). F-D2: microtissues fused at day 2 maturation level. F-D4: microtissues fused at day 4 maturation level. F-D7: microtissues fused at day 7 maturation level. Scale bar: 200 μm .

to fuse did noticeably change during this first week of culture. Previous studies have explored the fusion of microtissues [15,17,18,21], demonstrating that increasing microtissue maturation periods pre-fusion negatively impacts the subsequent fusion process [17,25], although the underlying mechanisms responsible for this remain unclear. In our study we investigated fusion for microtissues at early, intermediate, and later stages of maturation (day 2, 4, and 7, respectively). We observed that microtissues fused at day 2 (F-D2) compacted much faster compared to more mature microtissues (F-D4 and F-D7). This superior fusion was also associated with enhanced chondrogenesis at the end of a 28-day culture period, as evident by higher levels of sGAG and collagen deposition. These results point to the importance of ensuring robust and early fusion for supporting long-term cartilage tissue development.

As outlined in our study and corroborated by previous research [25], the presence of ECM plays a significant role in microtissue fusion. Single microtissues were typically characterized by the presence of circumferentially aligned collagen fibres. These collagen fibres became more evident with increasing microtissue maturation. Interestingly, when microtissues fused earlier, greater reorganization of the collagen fibre

network was observed. In the F-D7 group intense collagen staining persisted around the individual microtissues. These findings suggest that the spatial arrangement of collagen prior to fusion may influence the ultimate tissue architecture, microtissue fusion, and consequently, the mechanical properties of the resulting graft [17,26].

N-cadherin and vimentin are proteins intricately involved in cartilage development. N-cadherin is a transmembrane protein which functions to mediate cell-cell interactions (it is commonly used as a marker of cell-cell interactions) and is highly expressed during chondrogenesis [27–30]. In this study we observed that accelerated fusion of less mature microtissues was associated with greater N-cadherin expression in the early stages of fusion. Vimentin is a structural protein that plays a key role in cell-ECM interactions; it regulates cell adhesion strength by contributing to the development of complexes that control the formation of focal adhesion structures connecting cells to the matrix [31–34]. Vimentin staining has previously been used as a marker to assess fusion among neighbouring spheroids [35]. In our study, no discernible differences were observed in vimentin expression 8 hours after fusion across the different maturation groups. However, at 7 days post fusion, the F-D2 group exhibited notably stronger positive staining for vimentin,

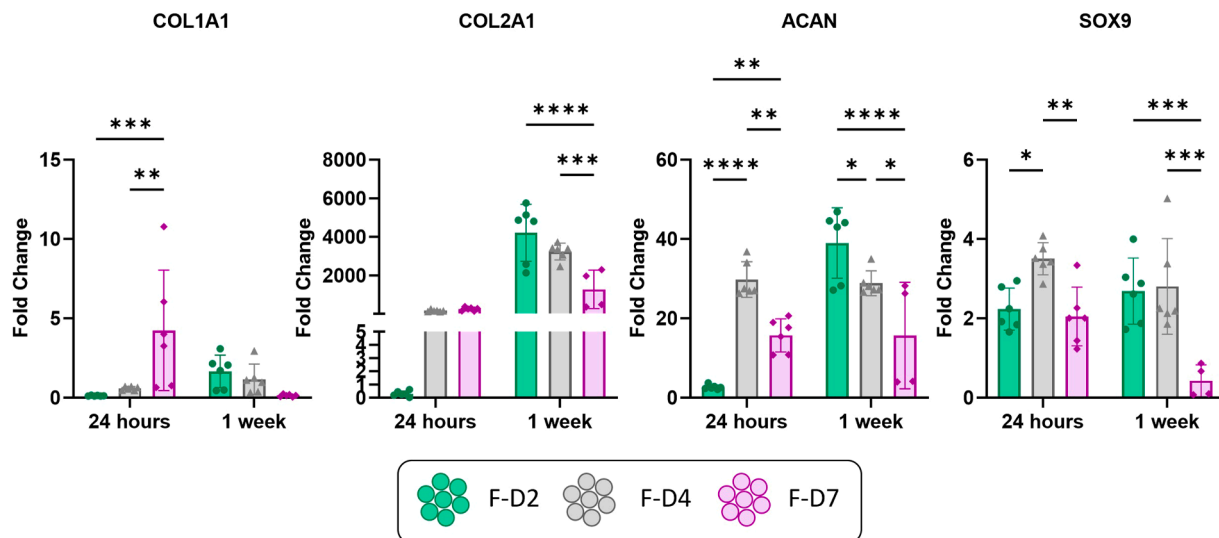


Fig. 4. Chondrogenic gene expression following microtissue fusion. mRNA expression of chondrogenic markers from left to right: COL1A1, COL2A1, ACAN and SOX9 analysed by real-time PCR and normalized against the housekeeping gene 18s rRNA. Each sample was normalized against the negative control of monolayer cells cultured for 2 days. F-D2: microtissues fused at day 2. F-D4: microtissues fused at day 4. F-D7: microtissues fused at day 7. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ when tested using a two-way ANOVA test ($n = 6$).

while the F-D7 group displayed weaker staining. This suggests that successful fusion is associated with increased vimentin localization around cells at later timepoints as they mature and form a more developed cartilage matrix.

After investigating the mechanisms involved in early fusion, we investigated the ultimate tissue phenotype. Nilsson et al. (2021) [26] demonstrated that the final tissue exhibits less intense sGAG staining when microtissues are fused after 21 days compared to microtissues fused after 1 day. Additionally, the overall biofabricated tissue appears more compact with higher sGAG intensity and more homogeneous collagen distribution. Our study aligns with these previous findings, despite exploring a narrower range of microtissue maturation levels (between day 2 and day 7). Specifically, the F-D2 group showed more intense staining for sGAGs compared to the F-D7 group, which exhibited a smaller final size. To confirm the change in the tissue type, we next explored changes in gene expression during fusion for microtissues at different stages of maturation. As expected, the expression of chondrogenic genes were generally higher in the more mature F-D4 and F-D7 microtissues after 24 hours of fusion. By 7 days of culture, ACAN and COL2 expression were significantly higher in the less mature F-D2 microtissues, which suggests the development of a more cartilage-like phenotype in this group with time in culture.

The less mature F-D2 microtissues were then employed to engineer larger grafts by increasing the density of microtissues per tissue construct. Previous studies [13,36] have explored microtissue fusion by varying the number of cells per microtissue while keeping the number of microtissues constant. These studies revealed that the number of cells per microtissue directly impacts the final graft cellular homogeneity by impacting the kinetics of fusion and the final tissue morphology. In our study, we investigated microtissue fusion by modifying the microtissue density per final construct, resulting in an increase in overall tissue size. A recent study by Kopinski-Grünwald et al. (2024) [37] attempted to engineer larger tissues using spheroids as building blocks with a scaffold. While the microtissues fused, the individual microtissues were still discernible after 28 days in culture. In our study, the microtissues in all groups compacted cohesively and the tissue exhibited an increase in diameter, that might be related to an increase in GAGs production which was prominent across all groups. Interestingly, no obvious signs of hypertrophy were observed after 42 days of culture in the presence of TGF β 3, suggesting the development of stable hyaline cartilage. A number of factors may explain the absence of hypertrophy within such

microtissue based grafts. The high cell density within these engineered tissues likely leads to the development of a low oxygen microenvironment, which is known to suppress of hypertrophy of chondrogenically primed MSCs [38–40]. It has also previously been shown that extended chondrogenic induction *in vitro* allows MSCs to form stable cartilage tissue *in vivo* [41]. Therefore the high levels of cell-cell interactions and low oxygen conditions within cell-rich microtissues, coupled with extended chondrogenic induction *in vitro*, may allow for the development of stable hyaline cartilage. Further *in vivo* studies would obviously be required to evaluate whether our proposed approach can be used to generate phenotypically stable cartilage grafts resistant to calcification and endochondral ossification.

Our final goal was to fabricate a functional cartilage graft with a size comparable to a human clinical defect. After 42 days of *in vitro* culture, the tissues macroscopically appeared similar to cartilage, characterized by a white and translucent colour. Histological analysis of the tissue revealed positive staining for all chondrogenic markers. Nutrient transfer limitations within large, engineered tissues are widely recognized as a key challenge that can hinder matrix deposition [42–44], particularly in cell-dense constructs such as microtissue-based grafts with high metabolic demands. In our study, when the microtissue density was increased up to 300 microtissues per construct, we observed changes in the histomorphology of the graft. While there was no significant change in GAG deposition, collagen deposition was notably impacted, with higher deposition observed in the annulus of the construct compared to the core. When culturing larger tissues, we sought to address this challenge by culturing the grafts under higher oxygen levels (20% O_2), which may have improved oxygen transport within the tissue. However, in TE, the size of the construct is not the sole consideration. Cell density within the graft is another crucial factor. Increasing the cellularity of MSC-based cartilage grafts has been shown to reduce oxygen levels within the core of the construct, which correlated with increased sGAG but reduced collagen accumulation in the centre of the engineered tissue [45]. Given that native AC exhibits a zonal architecture with spatial gradients in biochemical content, introducing appropriate gradients in oxygen, glucose, and other regulatory factors [46] may actually support the engineering of more biomimetic, scaled-up cartilage tissues. However, additional research is needed to identify conditions that could further enhance nutrient and oxygen transport in a quantifiable and controllable manner when engineering using cell-rich microtissues and organoids.

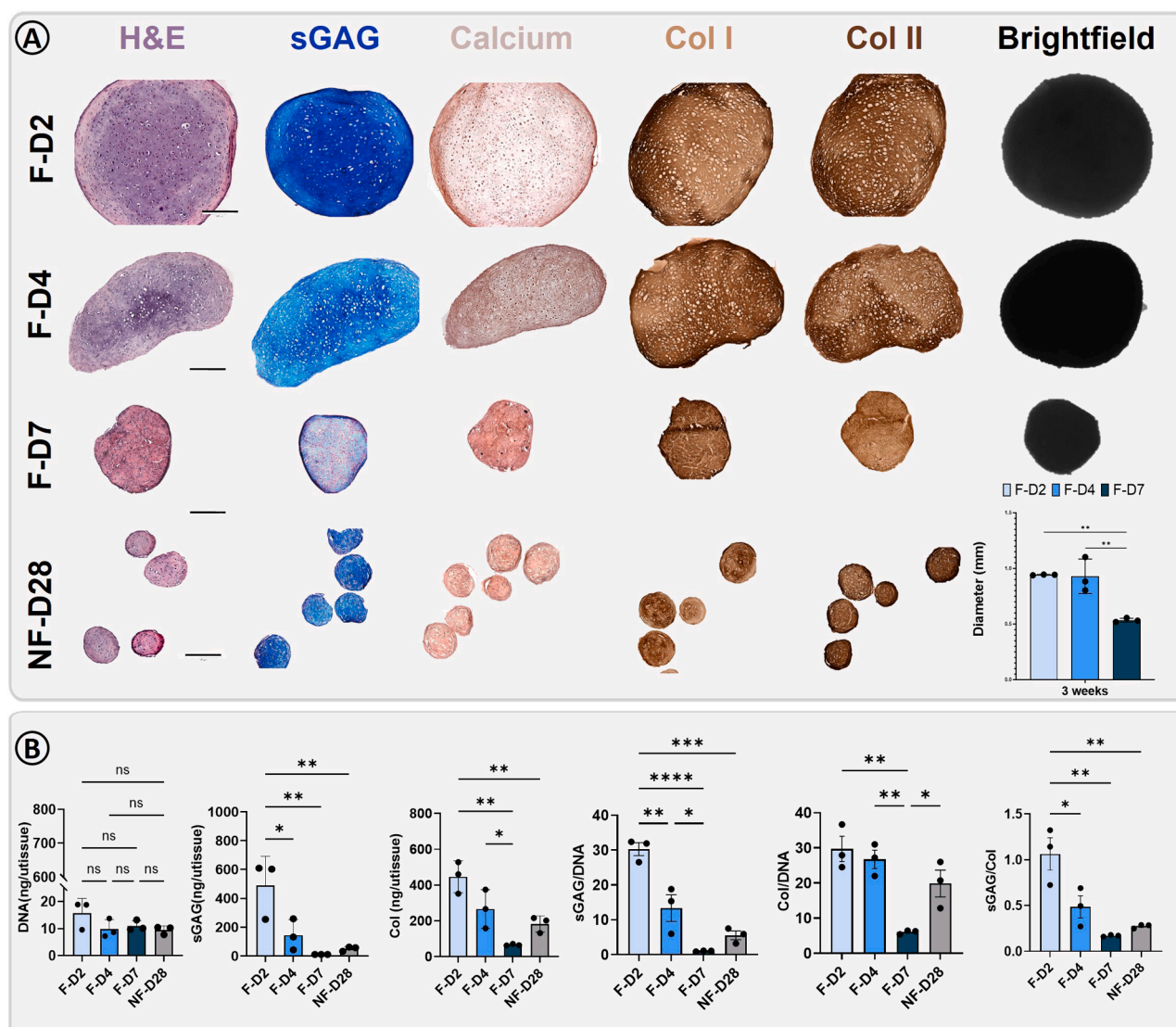


Fig. 5. Fusion of microtissues at day 2 supports superior cartilage tissue formation. A) Histological panel for standard chondrogenic markers: haematoxylin and eosin (H&E) staining, alcian blue staining, alizarin red staining, immunohistochemistry for collagen type I and collagen type II) after 28 days in chondrogenic media (scale bar: 250 μ m). Last column is a brightfield microscope image of fused microtissues at 28 days in chondrogenic media. B) Biochemical quantification of the microtissues, levels of sGAG and collagen normalised to DNA content. Significant differences are denoted as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ when tested using a one-way ANOVA test ($n = 3$) for biochemical analysis and a two-way ANOVA for diameter measurements. F-D2: microtissues fused at day 2 maturation level. F-D4: microtissues fused at day 4 maturation level. F-D7: microtissues fused at day 7 maturation level.

One potential approach to overcome nutrient transport challenges is the utilization of perfusion bioreactors [47–49], or the addition of rapidly degradable supporting gels [50,51], which could aid in the transport of oxygen and nutrients within the self-organized tissue construct. In our study, cartilage constructs were cultured under static conditions (in petri dishes) and dynamic conditions (in a stirring flask) using media that was refreshed three times per week. No dramatic improvements in tissue development were observed with the introduction of dynamic culture, which may possibly be linked to the limited perfusion of nutrients through the graft in this particular culture set-up. Perfusion bioreactors have previously been used to support the development of scaffold-free engineered cartilage [52], with the delayed application of perfusion found to be optimal as it gave time for cells to self-aggregate and consolidate prior to its application. A similar ‘delayed’ approach to perfusion is worthy of consideration for microtissue based grafts. Despite concerns associated with nutrient transport in our system, the resulting grafts were mechanically robust, with a tensile modulus approaching 1.5 MPa, albeit falling short of the reported

values for AC, which can exceed 100 MPa for human cartilage [53]. These limitations are attributed to insufficient collagen deposition, crosslinking, and organization. Therefore, it is imperative to explore additional strategies to improve the final ECM organization to achieve a more mechanically robust cartilage construct when using a high density of microtissues.

5. Conclusion

In this work, we successfully showed the potential of utilizing microtissues derived from human MSCs as biological building blocks to engineer large cartilage grafts. Our study demonstrated that promoting an earlier fusion led to a superior chondrogenic phenotype. Overall, these findings suggest that by carefully controlling microtissue maturation levels and density that it is possible to engineering scaled-up and mechanically competent cartilage grafts. However, a challenge that still needs to be addressed is the fabrication of hierarchical organized and functional cartilaginous constructs. For this purpose, additive

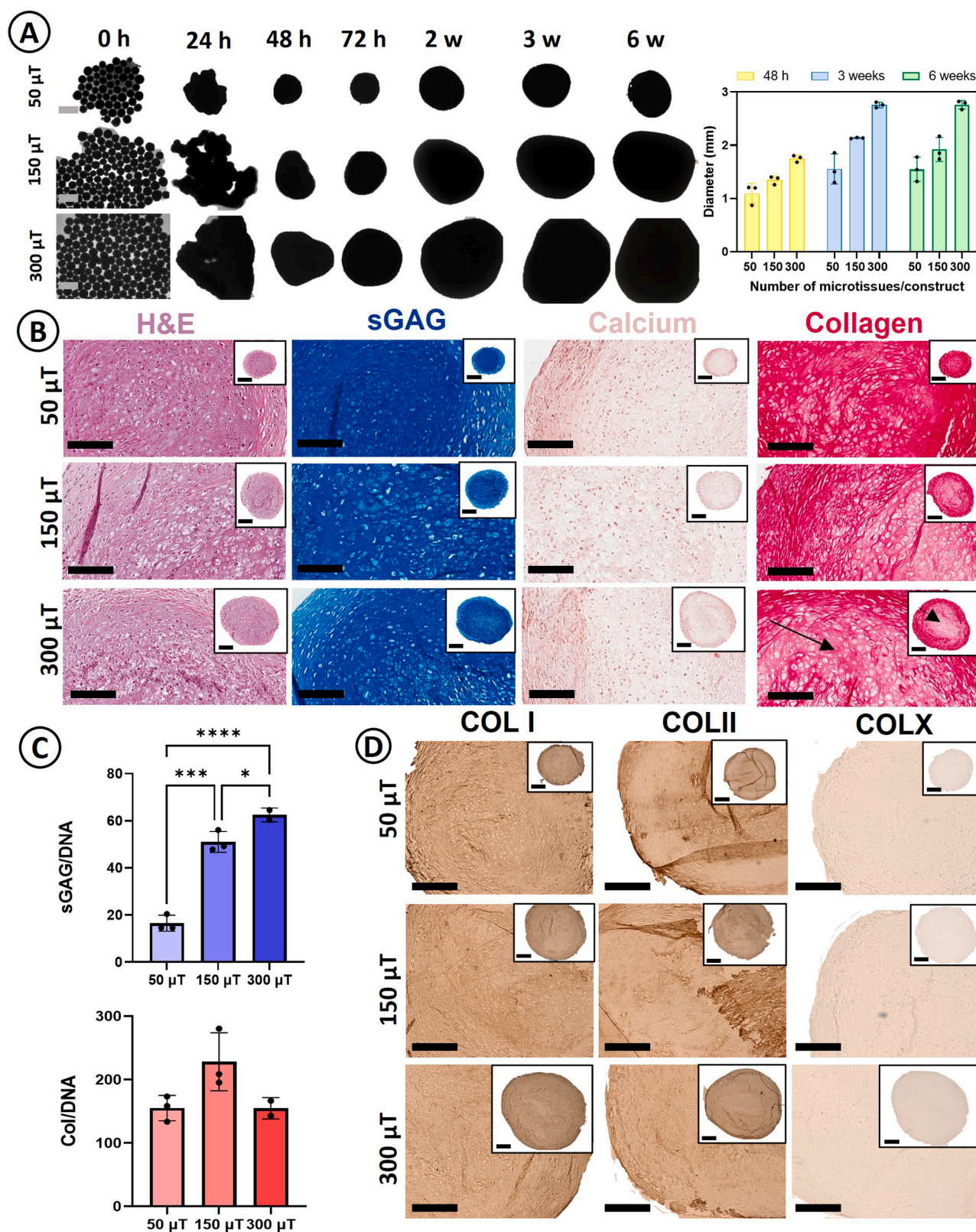


Fig. 6. Increasing the density of microtissues per fused construct improves chondrogenesis. A) brightfield imaging of fused microtissues over a period of 42 days in culture in chondrogenic media followed by diameter measurement. Scale bar: 500 μ m. B) histological panel of fused microtissues after 42 days in culture for standard chondrogenic markers: haematoxylin and eosin (H&E) staining, Alcian blue staining (AB), alizarin red staining (AR) and picosirius red staining (PSR). Scale bars: 200 and 400 μ m. C) Biochemical quantification for chondrogenic markers (sGAG, Collagen). D) Immunohistochemistry for collagen types I, II and X. Significant differences are denoted as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ when tested using a one-way ANOVA test ($n = 3$). μ T: microtissues. Scale bars: 300 and 400 μ m.

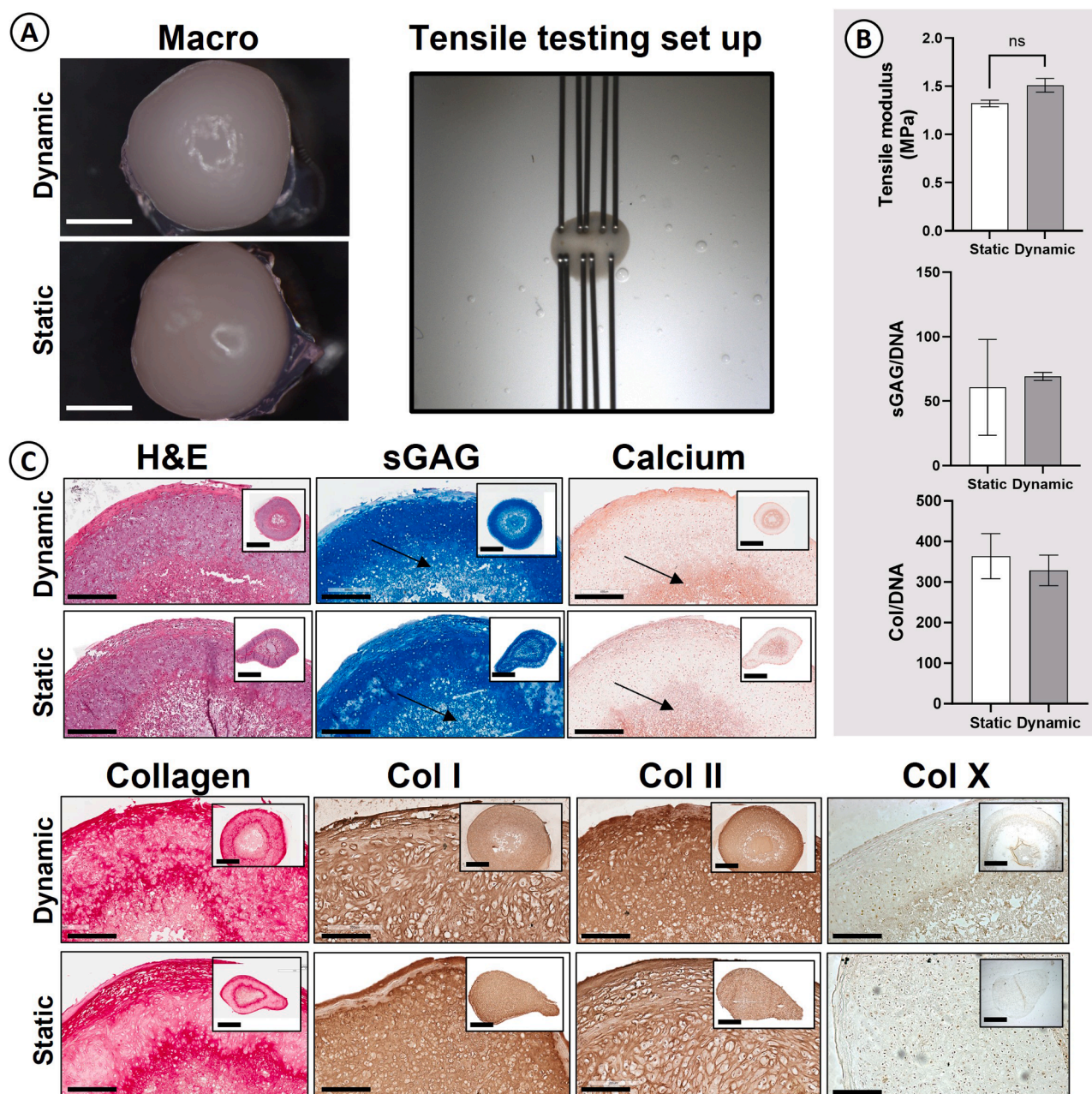


Fig. 7. Engineering of scaled-up grafts using cartilage microtissues. A) Stereomicroscopy (macro) images of a scaled-up engineered construct after 42 days of chondrogenic induction (scale bar: 2 mm); set up for tensile testing of fused microtissues after 42 days in culture using a tensile biaxial tester (CellScale). B) Tensile modulus quantification of fused microtissues after 42 days in culture in CDM. Biochemical quantification of constructs after 42 days of culture. C) Histological and immunohistochemical panel of chondrogenesis markers: H&E, Alcian Blue, Alizarin Red, Picrosirius Red, collagen 1 (COL I), collagen 2 (COL II), collagen 10 (COL X). Scale bar: 400 μ m and 2 mm.

manufacturing (AM) techniques such as 3D bioprinting can offer a solution by allowing precise spatial patterning of microtissues layer by layer [54,55]. Furthermore, 3D bioprinting strategies can potentially support the development of tissues within specific ECM organization, which is essential to cartilage biomechanical function [56]. Therefore, our future studies will focus on 3D bioprinting strategies using early maturation stage microtissues as building blocks for musculoskeletal TE.

CRediT authorship contribution statement

Francesca D. Spagnuolo: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Gabriela S.**

Kronemberger: Writing – review & editing, Validation, Supervision, Methodology, Investigation, Conceptualization. **Kyle J. Storey:** Writing – review & editing. **Daniel J. Kelly:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.2025.01.024.

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