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RESEARCH ARTICLE

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Neo-cartilage formation using human nondegenerate versus osteoarthritic chondrocyte-derived cartilage organoids in a viscoelastic hydrogel

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Abstract

Current regenerative cartilage therapies are associated with several drawbacks such as dedifferentiation of chondrocytes during expansion and the formation of fibrocartilage. Optimized chondrocyte expansion and tissue formation could lead to better clinical results of these therapies. In this study, a novel chondrocyte suspension expansion protocol that includes the addition of porcine notochordal cell-derived matrix was used to self-assemble human chondrocytes from osteoarthritic (OA) and nondegenerate (ND) origin into cartilage organoids containing collagen type II and proteoglycans. Proliferation rate and viability were similar for OA and ND chondrocytes and organoids formed had a similar histologic appearance and gene expression profile. Organoids were then encapsulated in viscoelastic alginate hydrogels to form larger tissues. Chondrocytes on the outer bounds of the organoids produced a proteoglycan-rich matrix to bridge the space between organoids. In hydrogels containing ND organoids some collagen type I was observed between the organoids. Surrounding the bulk of organoids in the center of the gels, in both OA and ND gels a continuous tissue containing cells, proteoglycans and collagen type II had been produced. No difference was observed in sulphated glycosaminoglycan and hydroxyproline content between gels containing organoids from OA or ND origin after 28 days. It was concluded that OA chondrocytes, which can be harvested from leftover surgery tissue, perform similar to ND chondrocytes in terms of human cartilage organoid formation and matrix production in alginate gels. This opens possibilities for their potential to serve as a platform for cartilage regeneration but also as an in vitro model to study pathways, pathology, or drug development.

KEYWORDS

cartilage, chondrocytes, human organoids, osteoarthritis, tissue engineering

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1 | INTRODUCTION

Regenerative cartilage therapies for focal defects are aimed at restoring cartilage structure and mechanical properties to limit the progression of the focal defect into osteoarthritis (OA) and thereby prevent or postpone the need for total knee replacement (TKR) surgery in young or middle-aged patients. Current golden standard therapy is autologous chondrocyte implantation (ACI). Success rates are variable, common problems are the lack of integration between newly formed tissue and native cartilage, as well as the formation of fibrous cartilage tissue that is not as mechanically strong as native cartilage and therefore deteriorates quickly.[1–4] One of the reasons for fibrocartilage formation in treated defects, is dedifferentiation of the chondrocytes caused by the two dimensional (2D) monolayer expansion culture.[5,6] This dedifferentiation can be avoided by culturing chondrocytes in a three dimensional (3D) environment.

Recently, a technique was developed that can boost chondrocyte expansion while preserving their chondrogenic phenotype: cartilage organoids were generated from bovine chondrocytes and porcine notochordal cell-derived matrix (NCM) in a 3D fashion in spinner flasks. NCM is a biologic matrix product, that is obtained by lyophilizing porcine nucleus pulposus tissue. Composition of this mixture resembles that of cartilage as it is rich in collagen type II and glycosaminoglycans, and the NCM has been shown to have a positive impact on chondrocytes in terms of proliferation and matrix production.[7–9] When a chondrocyte spinner flask culture is supplemented with NCM, this results in quick proliferation and self-assembly into organoids containing matrix (NCM components) and cells, while chondrocytes maintain their chondrogenic phenotype.[10] Embedding these organoids in nondegradable alginate gels with different molecular weights (Mw) revealed a viscoelasticity-dependent behavior of the cells.[11] In more viscoelastic gels, cell proliferation, migration, and matrix deposition were enhanced, resulting in fusion of encapsulated organoids into a homogeneous piece of neocartilage that had an interconnected matrix similar in composition and organization to native cartilage.[10]

By implanting cartilage spheroids or organoids in a chondral defect, chondrocytes already embedded in a cartilaginous matrix are introduced, which can speed up the regenerative process and improve matrix integration and early load-bearing properties of the newly implanted cells compared with ACI.[10,12,13] As a result, the long postoperative procedure related to ACI can be shortened, allowing for earlier rehabilitation of the patient, leading to lower costs, earlier mobility, and a higher quality of life.

Previous work showed initial integration between native human cartilage and organoids derived from human chondrocytes and NCM.[14] These chondrocytes were isolated from the smooth cartilage of leftover tissue from TKR surgeries, being the most readily available human articular chondrocyte source. These OA-chondrocytes are likely a mixture of normal and diseased chondrocytes and some progenitor cells, and have a different phenotype than healthy chondrocytes.[15–17] Nevertheless, several groups have studied the use of OA-chondrocytes for cartilage repair purposes and found that when cultured in the right 3D circumstances, OA-chondrocytes might exhibit similar properties as healthy chondrocytes and might provide a source for repair with autologous chondrocytes.[18,19] Previous research has shown that notochordal cell-conditioned medium helps to get a regenerative effect on human articular chondrocytes derived from OA tissue, as the treatment restored matrix production of the OA chondrocytes to healthy chondrocyte baseline levels.[9] Besides, NCM has shown to exhibit strong matrix anabolic effects and is suggested to have anti-inflammatory and anticatabolic potential.[8]

To gain knowledge about the implications of studies using these OA chondrocytes to create organoids and to better understand whether these organoids can be used to treat focal defects, there is a need to compare the performance of these OA-chondrocyte-derived organoids to organoids derived from a nondegenerate (ND) chondrocyte source. It was hypothesized that OA and ND organoids derived from spinner flask cultures supplemented with NCM generate similar tissues and have similar gene expression profiles.

NCM-containing organoids were produced from chondrocytes derived from both OA- and ND-tissue and cell proliferation, number of organoids, and organoid size were compared between the two cell sources. Subsequently, organoids were cultured in a viscoelastic alginate gel for 28 days in chondrogenic medium to evaluate matrix deposition. Gene expression profiles were evaluated before organoid encapsulation and after 28 days of culture, while biochemical and histological analyses were done after 1 and 28 days of alginate culture.

2 | MATERIALS AND METHODS

2.1 | NCM retrieval

Nucleus pulposus tissue from porcine intervertebral discs (~3 months old, $n = 5$, obtained from the local abattoir) was pooled, lyophilized (Labconco), pulverized, and stored at −80°C.

2.2 | Chondrocyte isolation

OA chondrocytes were isolated from cartilage tissue derived from patients undergoing TKR surgeries (4 subjects, 3 female and 1 male, aged 61.3 ± 4.0 years, range 56–66 years). The usage of this residual tissue for research purposes was approved by the Máxima Medical Center Local Research Committee (METC, number N16.148) and not subject to the Dutch Medical Research Involving Human Subjects Act. Full-depth cartilage slices were removed from the smooth-looking parts of the tibial plateau and femoral condyles, minced and digested overnight (high glucose Dulbecco's Modified Eagle Medium [hgDMEM, 41966; Gibco], 10% fetal bovine serum [FBS; Gibco], 1% penicillin/streptomycin [P/S; Lonza], 0.15% collagenase 2 (Worthington Biochemical Corporation), 0.01% hyaluronidase (Merck KGaA), 37°C, 5% CO_2). The cell suspension was strained using a 70 µm cell strainer and chondrocytes were collected, resuspended in a mixture

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of 90% FBS and 10% dimethylsulphoxide (DMSO; Sigma-Aldrich), and stored in liquid N_2.

ND chondrocytes were previously collected from healthy-graded cartilage of deceased (nonjoint related) donors (ETB-BISLIFE, three subjects, all male, aged 47.3 ± 0.5 years, range 47–48 years) in a similar fashion.

2.3 | Organoid culture

Upon use, OA and ND chondrocytes were thawed. Cell count and viability were determined using a nucleocounter (NC-100; Chemometec) and for every donor, 3.4×10^6 viable chondrocytes were resuspended in 68 mL culture medium in a coated spinner flask (coating: Sigmacote, Sigma-Aldrich; Spinner flask: Wheaton™ Magna-Flex™, DWK Life Sciences). Spinner flask culture medium consisted of hgDMEM, 5% FBS, 1% P/S, 10 mM 4-(2-hydroxyethyl)−1-piperazineethanesulfonic acid (HEPES; Gibco), 1% Non-Essential Amino Acids (NEAA; Gibco), 1% Insulin-Transferrin-Selenium-Plus (ITS + premix; Corning, Fisher Scientific), 0.2 mM L-ascorbic acid-2-phosphate (Sigma-Aldrich) and pulverized NCM was dissolved in the culture medium using an ULTRA-TURRAX® disperser (T10 basic; IKA) at a concentration of 0.25 mg/ml NCM (Day 0) or 1 mg/ml (Days 4, 8, 13 (50%, 100% and 100% of culture medium refreshed, respectively). Chondrocytes were cultured at an agitation of 60 rpm using a magnetic stirrer (Variomag Biosystem 4 and Biomodul 40B; Thermo Scientific) at 37°C, 5% CO_2 and 2.5% O_2. On Days 4, 8, 12, 15, and 18, a 4.0 mL sample was removed from the spinner flasks, digested overnight in organoid digestion mix (hgDMEM supplemented with 0.01% hyaluronidase and 0.2% collagenase A (Roche, Basel, Switzerland)), and subsequently cell count and viability were determined.

On Day 18, the content of each spinner flask was strained through a 100 µm cell strainer and organoids were collected in the strainer. Organoids samples were collected for analysis of amount, size, gene expression and histology.

2.3.1 | Count and size

Organoids were counted and size was determined using brightfield microscopy (Observer Z1, Carl Zeiss). The maximum (F_{max}) and minimum (F_{min}) Feret diameter were measured in ImageJ software, the root mean square (F_{mean}) of F_{max} and F_{min} was determined, and subsequently organoid volume was determined using F_{mean} by assuming organoids were perfect spheres ($V = 4/3\ \pi(F_{mean}/2)^3$).

2.3.2 | Gene expression

Organoids and Day 28 gels were snap frozen in liquid N_2 and stored at −80°C. Frozen samples were crushed using a pestle to homogenize the samples, and subsequently RNA was extracted and purified using the Qiagen mini kit (Qiagen) and DNAse I (Qiagen). cDNA was synthesized using M-MLV Reverse transcriptase (Invitrogen). Gene expression was evaluated by real-time qPCR (CFX384; Bio-Rad) using a Fast SYBR green master mix (Applied Biosystems) and reported according to the $2^{-\Delta Ct}$ method (primer sequences in Table 1). A panel of primers for several human reference genes was tested for variation in the specific groups and samples, after which *TOP1* and *ATP5F1B* were selected as the most stable reference genes.

TABLE 1 Primer sequences of genes analyzed.

Gene	5′–3′
ADAMTS5	F -GCAGCACCAACACAACCAG
	R -CCAGGGTGTCACATGAATGATG
MMP3	F -CAGCACTCTGAGGGGAGAAAT
	R -GTGTGGATGCCTCTTGGGTAT
MMP13	F -GGAGCATGGCGACTTCTAC
	R -GAGTGCTCCAGGGTCCTT
SOX9	F -GGCAAGCTCTGGAGACTTCTG
	R -CCCGTTCTTCACCGACTTCC
ACAN	F -TGCTTTGTAGACAGACTTGAGG
	R -CCAGCGTAGCATTGTGAGATT
COL1A1	F -AATCACCTGCGTACAGAACGG
	R -TCGTCACAGATCACGTCATCG
COL2A1	F -GGGAGAGCCTGGAGATGAC
	R -GACCGACGATGCCTCTCTG
COL6A1	F -CACTCAAAAGCAGCGTGGAC
	R -GTCGGTCACCACAATCAGGT
COL10A1	F -GTGGACCAGGAGTACCTTGC
	R -CATAAAAGGCCCACTACCCA
TOP1	F -GTCCAGTATTTGCCCCACCATA
	R -GCTACTTCCTCTGCTTTGGGG
ATP5F1B	F -CCAGCAGATTTTGGCAGGTGA
	R -AGACCCCTCACGATGAATGC

2.3.3 | Histology

Organoids were fixed in zinc formalin (Sigma, 1 h), embedded in 3% agarose (Sigma), fixed again (1 h), dehydrated (tissue processor, Microm) and embedded in paraffin (Paraplast; Sigma-Aldrich). For each donor, 10 µm sections were prepared using a microtome (Leica).

2.4 | Alginate gel formation

Alginate (Mw: 48 kDa) was prepared using a protocol described earlier.[10] In short, a 2% w/v alginate solution (Sigma, Mw: 298 kDa)

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was treated with 1.5% hydrogen peroxide (Sigma) for 2 h at 85°C, dialyzed, and lyophilized.

2.4.1 | Gel preparation

Molds were created by punching a Ø4 mm cylinder from a 4 mm layer of 1% agarose solution containing 1 mM $CaCl_2$. A 2.5% w/v alginate solution and a 275 mM $CaSO_4$ (Sigma) crosslinker solution were prepared in hgDMEM. Organoids were mixed in the alginate solution and the crosslinker solution was added, creating a volume ratio of 4:20:1 (alginate solution:organoids:crosslinker solution). Immediately after mixing, for each donor, 10 gels of 25 µL each were pipetted in the molds and were allowed to solidify for 45 min at room temperature. Gels were cultured at 37°C, 5% CO_2, and 2.5% O_2 in gel culture medium (hgDMEM, 1 mM $CaCl_2$, 10% FBS, 1% P/S, 10 mM HEPES, 1% NEAA, 0.2 mM L-ascorbic acid-2-phosphate). Medium was changed twice a week, and after 7 days the gels were removed from the molds and transferred to agarose-coated wells.

After 1 day, for each donor, five gels were harvested (three for biochemical analysis, two for histology). Remaining gels were cultured for 28 days, and thereafter prepared for biochemical analysis (3) and histology (2). For 2 donors in the OA group and 1 donor in the ND group, gels were also mechanically tested.

2.5 | Gel analysis

2.5.1 | Histology

Samples were fixed in formalin (24 h), processed for paraffin embedding, and 10 µm microtome-sections were prepared. Upon use, sections from organoids and gels were dried overnight, rehydrated, and stained with alcian blue (pH 1.0) or picrosirius red (to visualize GAGs and collagen, respectively). Stained sections were visualized with brightfield microscopy. Other sections were treated with 0.5% hyaluronidase (Sigma, 30 min at 37°C) and 0.05% pronase (Sigma, 30 min at 37°C) for Coll-II antigen retrieval or citrate buffer (Dako, 20 min at 96°C for Coll-I antigen retrieval. Then, sections were blocked and incubated with primary antibodies against Coll-II (DSHB II-II6B3, 1:50) or Coll-I (ab34710, 1:200) overnight at 4°C, and subsequently with 4′,6-diamidino-2-phenylindole (DAPI) (1:500) and secondary antibodies (A21127 (Coll-II) or A21428 (Coll-I)) for 1 h at room temperature. Sections were visualized using fluorescent microscopy (Observer 7, Zeiss). All images were processed using ImageJ software.

2.5.2 | Biochemical analysis

Samples were lyophilized, weighed to determine their dry weight and digested overnight at 60°C in papain digestion buffer (100 mM phosphate buffer, 5 mM L-cysteine, 5 mM EDTA, 140 µg/mL papain, all from Sigma). GAG content was determined using a dimethyl methylene blue (DMMB, pH 1.5) assay with shark cartilage chondroitin sulfate (Merck) as a reference. Hydroxyproline (HYP) content, as measure for collagen content, was determined with a chloramine-T assay with a trans-4 hydroxyproline (Sigma-Aldrich) reference. DNA content was determined with the Qubit dsDNA high-sensitivity kit (Invitrogen) according to the manufacturer's instructions.

2.5.3 | Mechanical testing

Gels harvested at Day 1 (2 per donor, $n = 2$ for OA group and $n = 1$ for ND group) and 28 (3 per donor, $n = 2$ for OA group and $n = 1$ for ND group) were evaluated for their relative stiffness with a microtester (MTG2; CellScale) using a Ø 1 mm spheroid indenter. Samples were submerged in a PBS bath and were compressed at a speed of 500 µm per minute. Force was recorded at 20, 50, 100, and 150 µm compression.

2.6 | Statistical analyses

All statistical analyses were performed using Prism 9.1 (GraphPad Software; www.graphpad.com). Data are presented as mean ± standard deviation (SD). A Shapiro–Wilk test was conducted to check for normal distribution within the groups, and an F test (two groups) or Brown–Forsythe test (three or more groups) to check for equal variances. Organoid size and count were compared between groups with a Mann–Whitney test and an unpaired t test, respectively. Two-way analysis of variance was performed to analyze the effect of culture time and chondrocyte donor on biochemical content (blocking for cell donor) and on gene expression levels. If appropriate, a nested t test was used to test for significance between groups within the same timepoint or within the same donor group, or a Sidak's post hoc test was used. Statistical significance was assumed when $p < 0.05$.

3 | RESULTS

3.1 | Organoid analysis

3.1.1 | Count and size

OA and ND chondrocyte proliferation and viability patterns were similar (Figure 1A,B). Eighteen days of culture in the 3D spinner flasks resulted in a 6.4 ± 0.4 and 6.7 ± 1.8-fold increase for OA and ND cells, respectively. Cell viability stabilized over time to >95% for both groups. The number of organoids formed in the spinner flasks was significantly larger for the OA group compared with ND group (Figure 1C, 656 ± 30 and 501 ± 12 organoids/mL for OA and ND, respectively, $p = 0.0004$). However, diameter of OA organoids was significantly smaller than of

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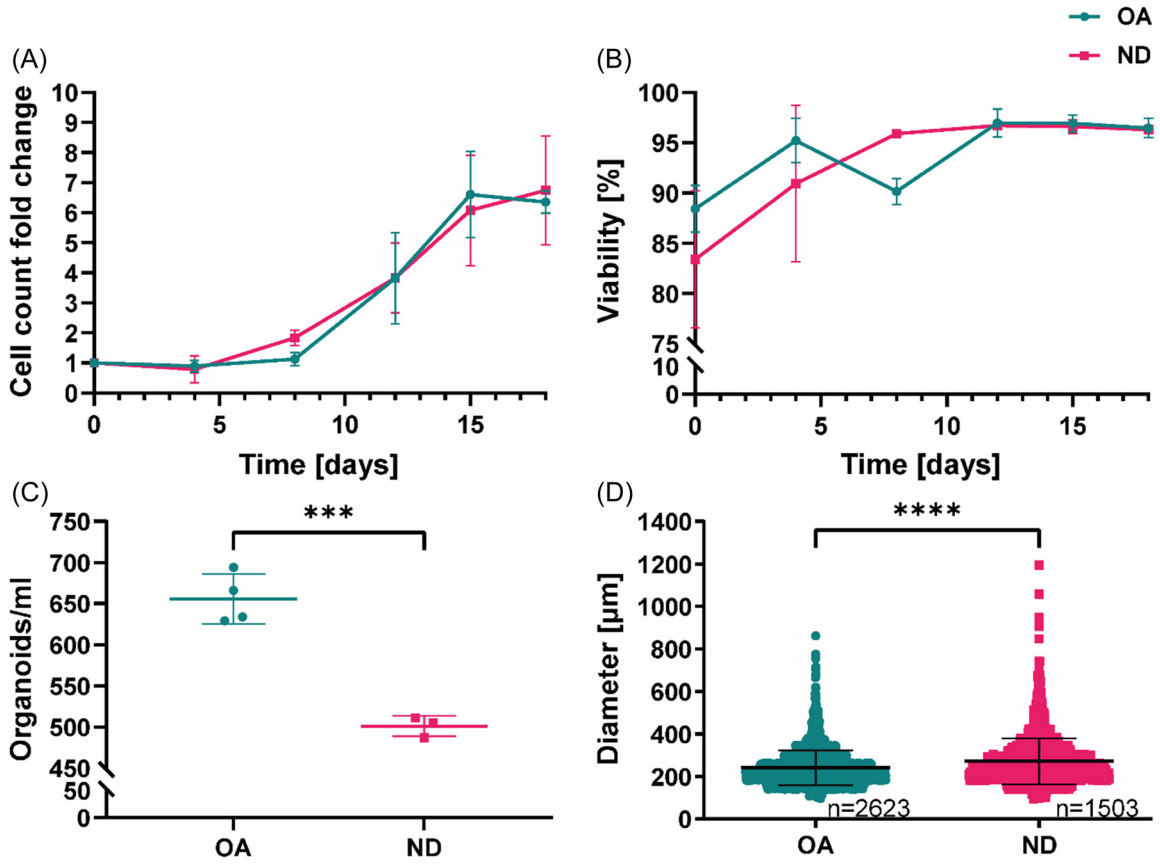

FIGURE 1 Top: Cell count (A) and cell viability (B) of the chondrocytes during the spinner flask culture of OA and ND chondrocytes. Bottom: Number of organoids/ml (C) and organoid diameter (D) of OA and ND organoids. ***$p < 0.001$; ****$p < 0.0001$. ND, nondegenerate; OA, osteoarthritic.

ND organoids (Figure 1D, 241 ± 83 µm and 271 ± 108 µm for OA and ND groups, respectively, $p = 0.0001$).

3.1.2 | Histology

Organoids from both groups were rich in proteoglycans and collagen (Figure 2). The center of the organoids mainly consisted of Coll-II, while the outside was covered in a small ring of Coll-I for both OA and ND organoids (Figure 2). For ND organoids, some Coll-I was also deposited in the middle of the organoids.

3.2 | Gel analysis

3.2.1 | Gel appearance

Organoids were encapsulated in an alginate gel immediately after spinner flask culture. In 28 days, the gels visually matured from a gel in which individual organoids could be identified (Figure 3A,B) to a more compact gel in which individual organoids were not macroscopically visible anymore (Figure 3C,D). There was no difference in macroscopic appearance between OA and ND samples.

3.2.2 | Histology

Proteoglycan and collagen distribution were similar in alginate gels from OA and ND organoids (Figures 4 and 5). In the center region of the gels, organoids merged and space between the separate organoids was filled up with proteoglycan-rich tissue. Some collagen (mainly Coll-II, with a slight staining of Coll-I) was also deposited in this region; however, the space between organoids was not completely filled with collagen in the OA gels. In the ND gels, space between the organoids filled up mainly with Coll-I. In the peripheral region of the gels, a continuous tissue formed containing both proteoglycans and collagen. Immunofluorescent staining revealed that this tissue was rich in Coll-II, but also some Coll-I was present. In regions with less Coll-II, more Coll-I was observed. The peripheral layer of the gels contained flattened cells surrounded with Coll-I.

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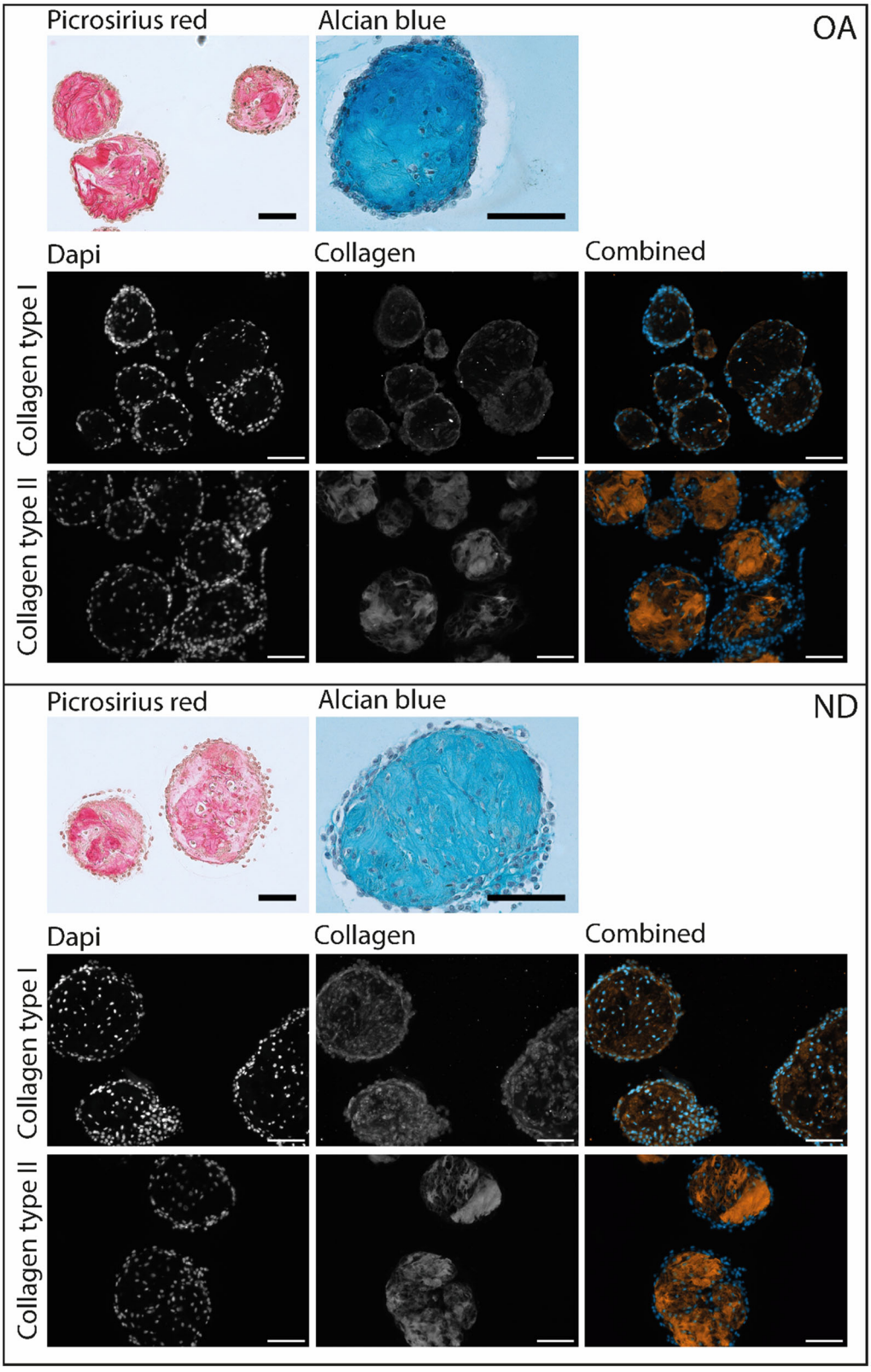

FIGURE 2 Representative images of histological analysis of paraffin sections of OA (top) and ND (bottom) organoids after the spinner flask culture (18 days) stained with picrosirius red and alcian blue (top row), immunofluorescence of DAPI + collagen type I (middle row) and DAPI + collagen type II (bottom row of each panel). Scalebar = 100 µm. DAPI, 4′,6-diamidino-2-phenylindole; ND, nondegenerate; OA, osteoarthritic.

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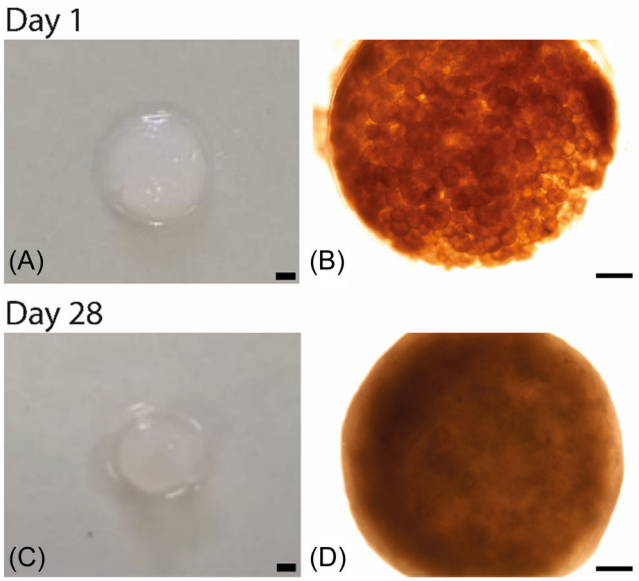

FIGURE 3 Macroscopic view (A, C) and light microscopy image (B, D) of OA alginate gels after 1 (A, B) and 28 (C, D) days of culture. Scalebar = 1 mm (A, C) or 500 µm (B, D). ND, nondegenerate; OA, osteoarthritic.

3.2.3 | Biochemical analysis

Biochemical content of OA and ND gels was similar after 1 day of culture (Figure 6), and DNA content of the samples was stable during the culture duration. Main effect analysis showed a significant effect of culture time ($p = 0.014$), but not of cell type ($p = 0.087$) on the sGAG content and a near-significant effect of culture time ($p = 0.079$) and no significant effect of cell type ($p = 0.916$) on HYP content. Subsequent nested *t* tests (blocking for donor) within cell type showed a significant increase of sGAG content from Day 1 to Day 28 in the OA group ($p = 0.001$) but not for the ND group ($p = 0.197$). Note the high donor-dependent variability that is observed after 28 days, especially for the ND samples.

3.2.4 | Gene expression

Gene expression profiles for *ADAMTS5*, *MMP3*, *MMP13*, *SOX9*, *and COL6A1* were similar for OA and ND organoids before encapsulation (Figure 7, D0), although *ACAN*, *COL1A1*, and *COL2A1* seem to be expressed stronger (though nonsignificant) in ND compared with OA organoids. After 28 days of culture encapsulated in alginate (D28), RNA could be extracted for only two donors from the OA group and two donors from the ND group (Day 28). Main effect models showed a significant decrease in expression of *MMP3* ($p = 0.049$) and *COL6A1* ($p = 0.040$) over time while expression of *ADAMTS5* ($p = 0.019$) and *COL2A1* ($p = 0.042$) increased. Also, the *COL2A1/COL1A1* ratio increased in the encapsulated and cultured samples compared with the organoids before encapsulation ($p = 0.0001$). Within timepoints, no significant differences were observed between OA and ND donors, except for *COL2A1/COL1A1*, which was significantly higher for OA compared with ND after 28 days ($p = 0.003$). No *COL10A1* expression was detected in the samples.

3.2.5 | Mechanical testing

Immediately after culture, gels from three different donors were subjected to a linear compression test. Day 28 gels were stiffer than Day 1 gels (Figure 8). For two OA donors and one ND donor, relative stiffness increased 4.9 ± 0.6x, 32.6 ± 11.5x, and 12.0 ± 4.0x, respectively.

4 | DISCUSSION

This study shows the ability of human chondrocyte-derived organoids to form a cartilaginous piece of tissue when embedded in low Mw alginate. Histologically, constructs of OA and ND chondrocyte origin were similar. After encapsulating the organoids in alginate gel, within 28 days a proteoglycan matrix was deposited bridging the space between organoids. A cell- and Coll-II-rich layer of matrix surrounded the bulk of organoids in the center of the constructs.

The organoid production process of OA and ND chondrocytes showed similar trends in terms of proliferation and viability, and were in alignment with previous results.[14] Although the number of cells in the spinner flasks was similar for OA and ND donors, in the OA group there was a higher number of smaller organoids, and in the ND group a lower number of bigger organoids. The size distribution (Figure 1D) shows that most of the organoids are between 100 and 400 µm in both groups, and it was observed that some very large organoids formed by merging of smaller organoids in the ND group. Therefore, the difference was not considered biologically relevant as later in the alginate gels the organoids merge anyway. Analysis of the organoids before encapsulation showed similar cell density and matrix arrangement for both groups, indicating similarity of the organoids. However, more Coll-I seemed to be deposited within the ND organoids. The Coll-II likely originates from the NCM, as the NCM, which is rich in Coll-II and sGAGs, is embedded within the organoids, serving as a cartilage-like matrix that the chondrocytes attach to during organoid formation.[8,10,14]

It was shown before that notochordal cell-conditioned medium has a regenerative effect on human articular chondrocytes, providing a chondroprotective environment and restoring the matrix production capacity of OA chondrocytes to healthy chondrocyte levels while reducing the production of pro-inflammatory cytokines.[9] While matrix content in the constructs is variable from donor to donor, the data from this in vitro study demonstrate that chondrocytes from both OA and ND donors, even though ND donors were younger than OA donors, have similar ability to secrete matrix, without the addition of growth factors or steroids other than the native proteins from the NCM within the organoids.[20,21] GAG and HYP content were similar for OA and ND donors at both timepoints, but large standard

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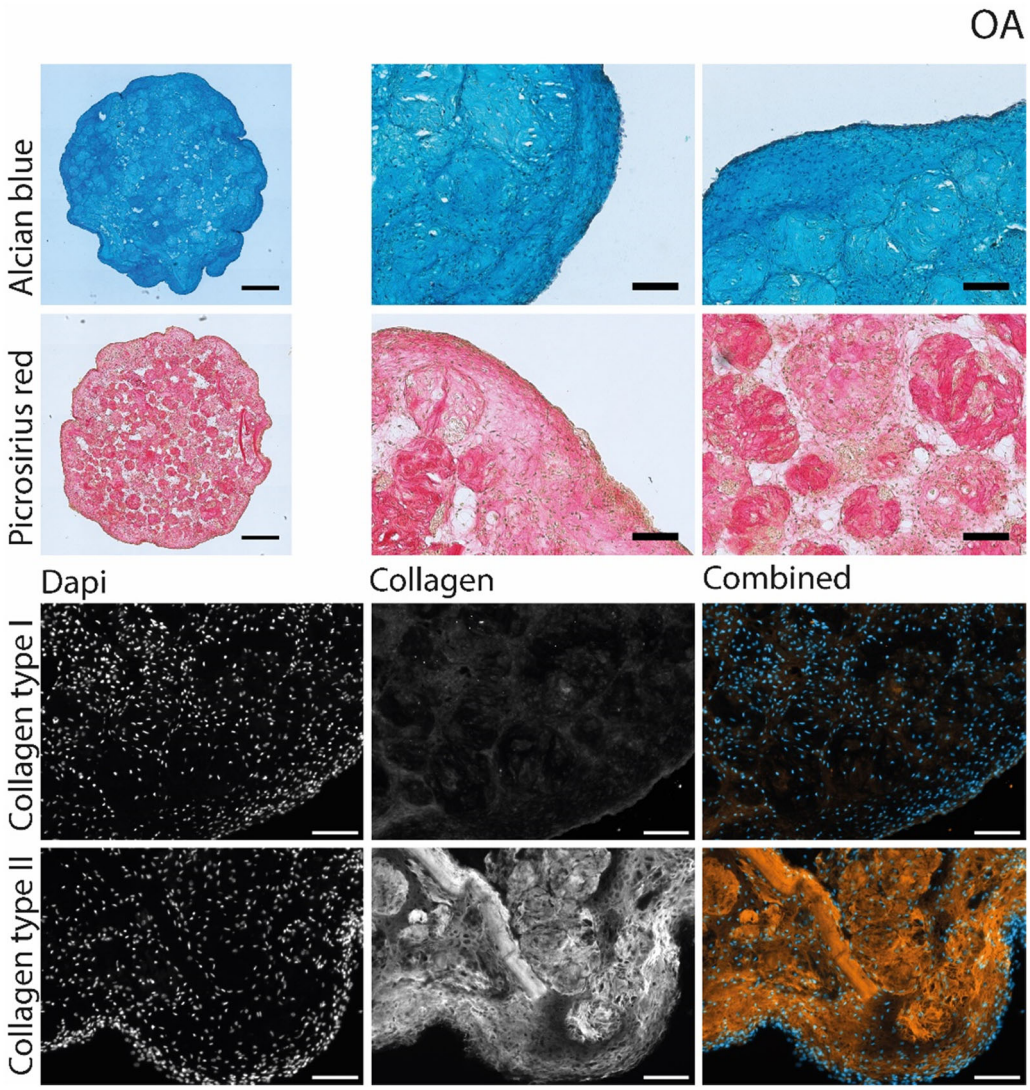

FIGURE 4 Representative examples of paraffin sections of OA gel samples after 28 days of culture stained with alcian blue (top row), picrosirius red (second row), immunofluorescence of DAPI+ collagen type I (third row) and DAPI + collagen type II (bottom row). Scalebar overview images = 500 µm, others: scalebar = 100 µm. DAPI, 4′,6-diamidino-2-phenylindole; OA, osteoarthritic.

deviations limit the statistical significance of the analysis. Collagen synthesis was rather slow and culture time might be too short to find a significant increase. However, *COL2A1* is highly expressed in the Day 28 samples, indicating a longer culture time might reveal a more significant increase in collagen content. An increase in sGAG content was seen when comparing samples from Day 28 with Day 1. Studies have shown that although some sGAGs are released from traditional elastic alginate constructs into the surrounding medium, the release rate of newly produced sGAGs from chondrocyte-alginate constructs is low.[22,23] However, in porous hydrogels or scaffolds, this can be relatively high.[22,23] Hence, with a viscous relaxing alginate hydrogel, this could be moderate. Future studies could include analysis of the medium to validate this for viscoelastic organoid-alginate constructs.

Results indicate that the matrix-producing performance of chondrocytes in the spinner flask and as organoids is mainly related to the individual donor rather than the group (OA or ND) the donor was in, which is biologically relevant information. Strikingly, variation in the results was larger for ND donors than for OA donors. This was not expected, as ND chondrocytes were derived from healthy cartilage donors of a similar, younger age (n = 3, range 47–48 years), while OA donors (n = 4, range 56–66 years) were older, greatly varied in age and had diseased cartilage.[24] Further, more detailed analysis of the proteoglycan and collagen network could be useful to understand this finding. Previous research has shown that OA repair tissue contains less proteoglycans with shorter sGAG chains, altered sulfation patterns, and atypical proteoglycan synthesis.[25–27] Also, the Coll-II structure, orientation, and crosslinking within the collagen network are important features that provide insight in the quality of the obtained tissue.[28,29] This could be done by either more extensive biochemical assays, or by advanced microscopic techniques. Another option to be considered is to evaluate the mechanical properties of the single organoids.

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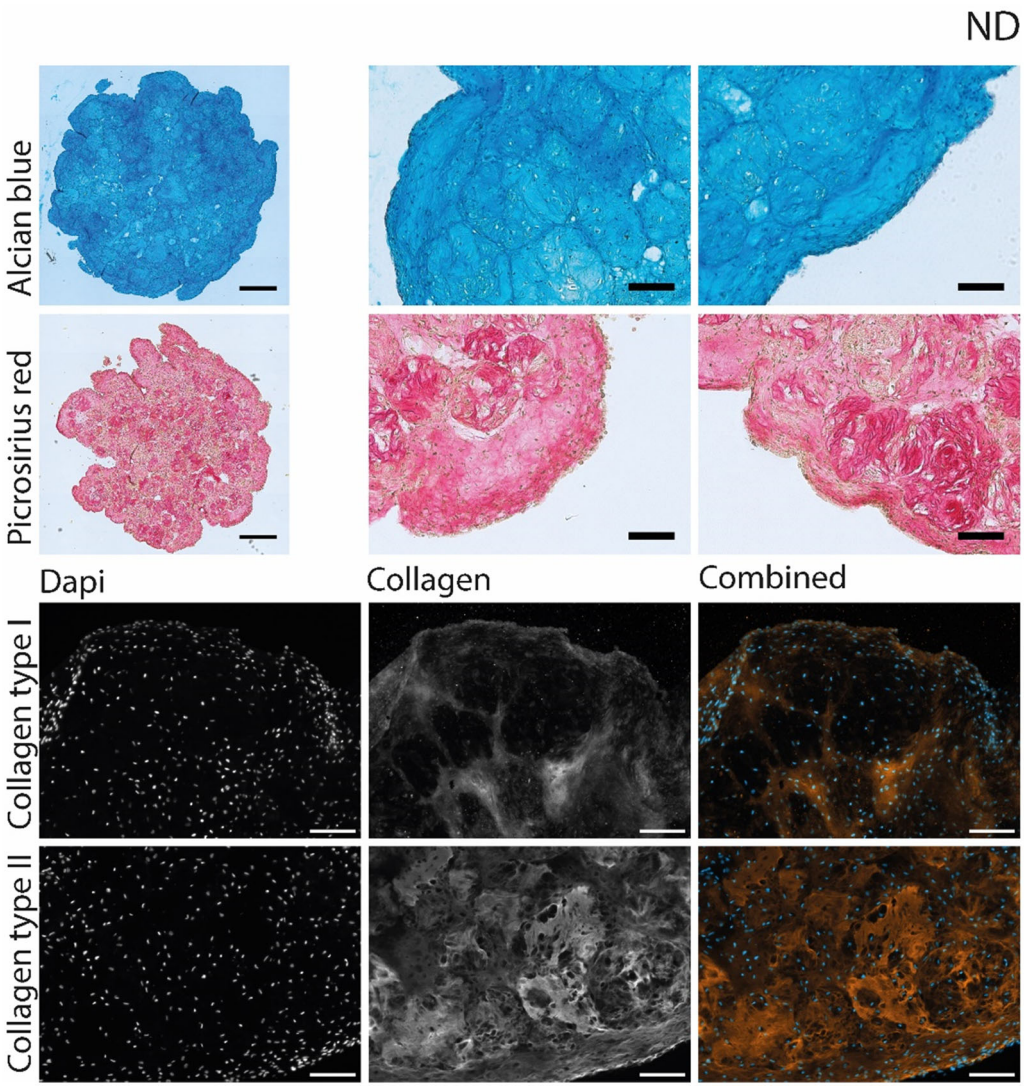

FIGURE 5 Representative examples of paraffin sections of ND gel samples after 28 days of culture stained with alcian blue (top row), picrosirius red (second row), immunofluorescence of DAPI + collagen type I (third row) and DAPI + collagen type II (bottom row). Scalebar overview images = 500 µm, others: scalebar = 100 µm. DAPI, 4′,6-diamidino-2-phenylindole; ND, nondegenerate.

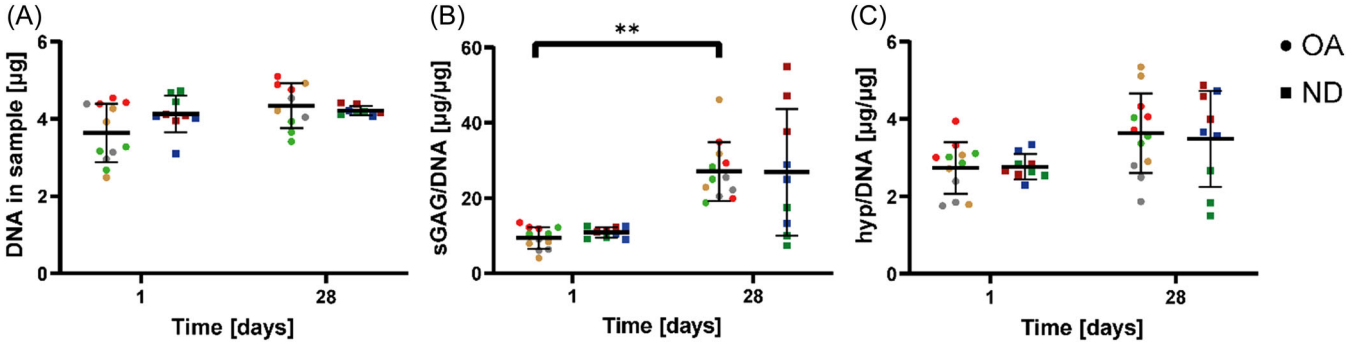

FIGURE 6 DNA (A), sGAG (B), and HYP (C) content (mean ± SD) of the OA and ND gels after 1 and 28 days of culture. Significant *p* values for analysis of variance main factors are not shown in the graphs (culture time [sGAG*]). Significant *p* values were indicated for nested *t* tests. $^{*}p < 0.05$; $^{**}p < 0.01$. ND, nondegenerate; OA, osteoarthritic.

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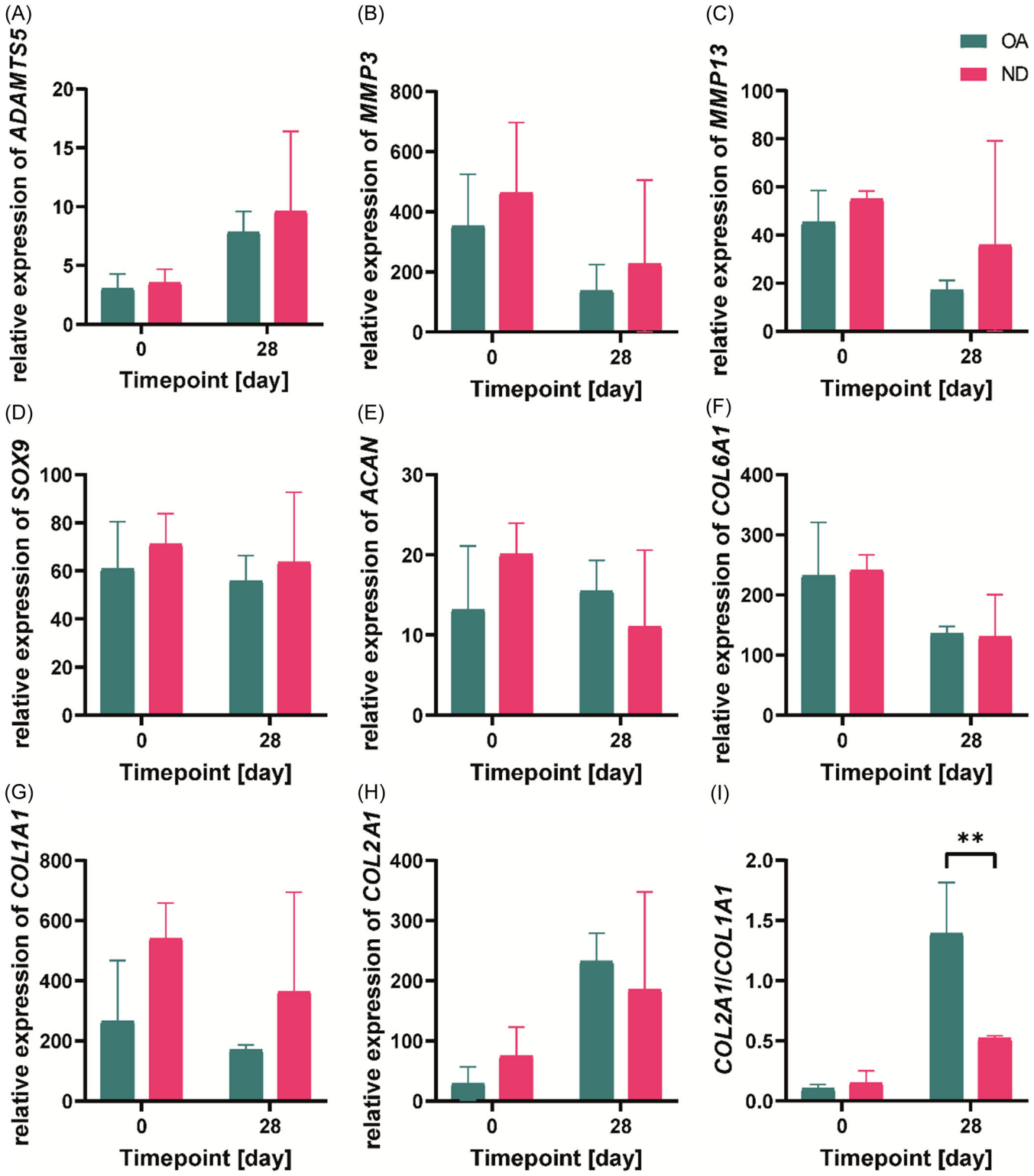

FIGURE 7 Gene expression measured in the organoids before encapsulation (Day 0) and after 28 days of culture encapsulated in an alginate gel (Day 28) for OA and ND groups. Values displayed as 2-dCt to ATP5F1B/TOP1. Significant p values for ANOVA main factors are not shown in the graphs (culture time [(a) ADAMTS5*, $p = 0.019$], [(b) MMP3*, $p = 0.049$], [(f) COL6A1*, $p = 0.040$], [(h) COL2A1*, $p = 0.042$], [(i) COL2A1/COL1A1****, $p = 0.0001$]). *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$.

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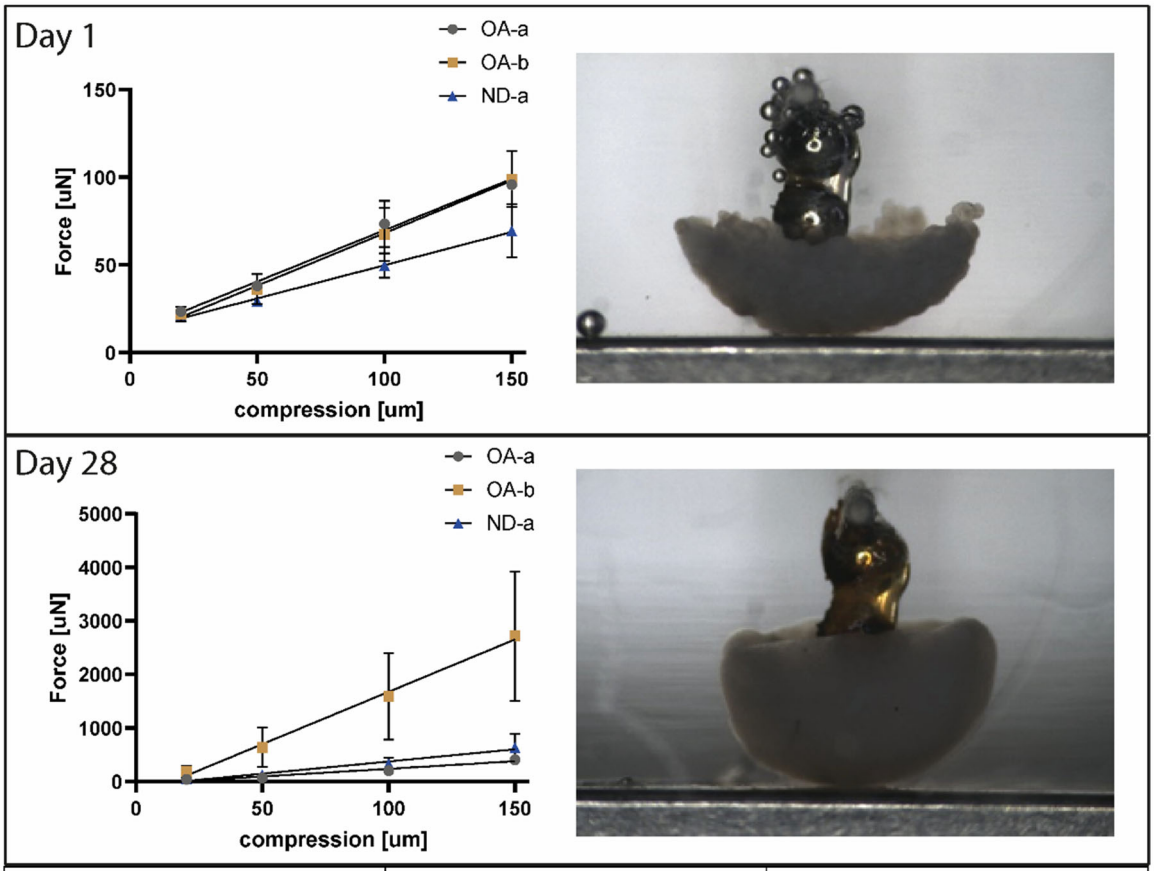

Donor	Stiffness [N/m] - day 1	Stiffness [N/m] -day 28
OA-a	0.59 ± 0.10	2.86 ± 0.41
OA-b	0.60 ± 0.07	19.55 ± 6.95
ND-a	0.38 ± 0.08	4.56 ± 1.51

FIGURE 8 Force-compression data of some of the OA and ND gels after 1 (top) and 28 (bottom) days of culture, accompanied by an example of what the samples look like during the mechanical test. Note the different range of the *y*-axis for Days 1 and 28. ND, nondegenerate; OA, osteoarthritic.

Spinner flask cultures supplemented with NCM resulted in similar gene expression profiles for OA and ND chondrocytes. Matrix genes *ACAN*, *COL1A1*, and *COL2A1* seemed to be expressed somewhat stronger in ND compared to OA organoids, though not significantly. It has been observed before that the addition of NCM does not specifically induce *COL2A1* expression in chondrocytes, so these differences probably originate from native tissue.[8,10] Besides the known reductive effect of NCM on catabolic factors, also the hypoxic culture conditions might have contributed to similar gene expression profiles of the chondrocytes, as hypoxia is reported to decrease *MMP13* expression, and ADAMTS5 and aggrecanase activity in human cartilage chondrocytes and explants.[8,30–32]

After the 28-day alginate culture, catabolic marker *ADAMTS5* expression increased over time, while *MMP3* (significant) and *MMP13* (nonsignificant) expression decreased. Moreover, histological analysis of the samples showed that cells migrated into, and matrix had appeared bridging the space between organoids. Although the exact enzyme content and mechanism is unknown, it is hypothesized that cells remodel ECM components in the organoids, deposit matrix to bridge the space between organoids, as well as rearrange the surrounding hydrogel network to create space for their cellular activity.[10] Increased expression of *ADAMTS5* combined with increased sGAG content could indicate proteoglycan remodeling in the gels.[33–36] *MMP3* expression is upregulated early in the OA process and decreases throughout the progress.[37,38] MMP3 digests ECM components and mediates cartilage breakdown by activating other MMPs and ADAMTS5.[38] MMP13 plays a role in healthy cartilage matrix turnover as well as in cartilage disease.[39] It is upregulated mostly in late-stage OA as the main enzyme digesting the triple-helical structure of Coll-II.[37,40] The decreased expression of *MMP3* and *MMP13*, combined with the increased sGAG and stable HYP content could therefore be a positive signal.

SOX9 and *ACAN* expression were stable for both groups while *COL10A1* could not be detected, indicating no dedifferentiation or hypertrophy of the chondrocytes. *COL1A1* expression tended to decrease for both groups while *COL2A1* expression was increased on

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Day 28 compared with Day 0. Collagen immunohistochemistry revealed that Coll-I was mainly deposited in between the organoids, probably early in the culture, while Coll-II was deposited later in a thick outer layer of the gel, in line with the gene expression results. This confirms that an alginate culture system supports expression, production, and deposition of Coll-II by chondrocytes and is consistent with data from other studies.[41,42] Further studies could analyze gene expression profiles and matrix deposition for cells in the core and on the outside of the gels separately, to be able to differentiate between them.

Of particular interest is the increase in *COL2A1/COL1A1* gene expression ratio, indicating that the environment stimulates *COL2A1* expression in both groups. This *COL2A1/COL1A1* ratio was significantly higher for OA compared with ND donors. This is in line with the immunofluorescent analysis of Coll-I and Coll-II deposition, where more Coll-I was seen in the gels of the ND group. The flattened cells and Coll-I localized on the outer rim of the organoids and gels resemble the typical superficial layer in human adult cartilage and have been observed in other spheroid cultures before.[12,13,43–47]

Organoid production in spinner flasks combines proliferation and self-aggregation of chondrocytes into single organoids while maintaining their chondrogenic phenotype, limiting the amount of work, and shortening production time. Research has shown ability of the organoids to coalesce and integrate with native cartilage and the elevated sGAG and HYP content of the organoid-filled gels (incorporated and produced during self-assembly and expansion) may be beneficial for early load bearing and mechanical stimulation upon implantation when compared to low-passage expanded chondrocytes.[14] Scaling up the production process of spheroid approaches is often difficult because of laborious aggregation processes, as chondrocyte expansion is still in 2D and subsequent aggregation into single-coated wells is required.[12,48,49] In contrast, the method presented here holds great scale up-potential by simply increasing the working volumes of the spinner flasks, that is, culture medium, cells, and NCM, which would not significantly increase labor or time.[10,14]

For some alginate gels, micro indentation tests were performed to evaluate mechanical properties. Results (Figure 8) indicate that a 5- to 33-fold higher force was needed to compress the gels at Day 28 compared with Day 1. This increase can be attributed to merging of organoids and the tissue layer in the periphery of the gels, as seen in histology. Longer cultures might result in more homogeneous tissues and therefore reduced variability in stiffness. Compared with native healthy bovine cartilage (bovine and human cartilage stiffness are in the same order of magnitude[50–52]), the force needed to indent 20 µm was still about 20-fold lower.

Alginate is a biomaterial that can aid in the 3D culture of chondrocytes, as it supports maintenance of the chondrogenic phenotype and chondrocytes embedded in alginate synthesize matrix components that closely resemble those in native articular cartilage.[22,53] Alginate was successfully used in the clinic for the transplantation of human articular chondrocytes to treat cartilage defects in knee joints and for acellular cartilage repair in elbow joints.[54–56] Implanting chondrocytes already embedded in a cartilaginous matrix can speed up the regenerative process and improve early load-bearing properties of the newly implanted cells.[10,12,13] Crispim et al. hypothesized that chondrocytes in the organoids can only rearrange the alginate network if it behaves like a viscoelastic material. They observed that when organoids were embedded in alginate with a higher Mw and therefore increased elasticity, the organoids were not able to merge.[10] In the current study their best-performing alginate formulation was used, and some merging was observed, however not in similar quantities as for bovine chondrocytes. Human organoids might require a longer culture time to achieve this merging. Alternatively, human organoids may require other viscoelastic carrier properties, indicating further optimization of the alginate carrier is needed for human organoids.

Overall, it was observed that chondrocytes from an OA and ND source have similar proliferation rates in a 3D spinner flask culture supplemented with NCM, and they both self-assemble into cartilage organoids. Histological appearance and gene expression profiles are very similar, and they show comparable behavior when encapsulated in low Mw alginate, where OA chondrocytes even show a higher *COL2A1/COL1A1* ratio in gene expression and matrix deposition. This indicates that OA chondrocytes can be used for organoid formation and subsequent embedding in alginate gels. These constructs have the potential to serve as a platform for cartilage regeneration as well as in vitro models to study pathways, pathologies, or drug development.

AUTHOR CONTRIBUTIONS

Meike W. A. Kleuskens designed and conducted the experiments and wrote the manuscript. Meike W. A. Kleuskens and Marina van Doeselaar interpreted and analyzed the data. Corrinus C. van Donkelaar and Keita Ito secured the funding, contributed to experiment design, data interpretation, and manuscript review. Rob P. A. Janssen contributed to tissue procurement and harvesting as well as data interpretation and manuscript review. João F. Crispim provided expertise with the organoid and alginate production and contributed to manuscript review. All authors have read and approved the final submitted manuscript.

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CONFLICT OF INTEREST STATEMENT

Keita Ito has patents pending on the production of decellularized NCM additive and is CSO of NC Biomatrix BV.

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