BMP9 induces cell and collagen reorganisation in immature articular cartilage to generate adult-like tissue

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INTRODUCTION: For proper function of synovial joint weightbearing cartilage, repair or regenerated tissue should adopt the collagen orientation of adult mature articular cartilage, where fibres are orientated parallel at the tissue surface with deeper fibres becoming more perpendicularly aligned (1). This contrasts with immature cartilage where the deeper fibres remain parallel to the surface or have no definitive orientation. The switch in the organisation of the collagen framework occurs during the process of postnatal maturation.

Studying postnatal maturation is difficult as collagen reorientation in large mammals takes many months as it is induced by progressively increased weightbearing. However, Morgan *et al* (2) showed that exposure of isolated immature bovine chondrocytes to bone morphogenetic protein-9 (BMP9) *in vitro* generates a collagen structural framework similar to adult-like mature cartilage.

In this study we show BMP9 also induces postnatal cell and collagen reorganisation in native immature articular cartilage.

METHODS: 4mm discs of articular cartilage were explanted from metacarpophalangeal joints of immature bovine steers and cultured *ex vivo* in serum-free chondrogenic medium for 21 days at 37°C. Treated explants were supplemented with 100ng ml^{-1} BMP9 (Peprotech Ltd, UK). Sectioned explants were stained with 1% picrosirius red (PSR) to visual collagen orientation using polarised light microscopy. Collagen orientation was determined using ImageJ and Fibril tool plugin.

RESULTS: Picrosirius red staining of control and BMP9 treated immature cartilage, **Fig.1A** show chondrocytes in treated explants are found at lower density, have larger chondrons, and, deeper in the tissue have begun to organise in columns with adjacent chondrons aligning vertically (*black arrowheads* in **Fig.1A**). Polarising light microscopy shows the profound change in collagen organisation in treated explants with fluorescent signal orientated in a perpendicular direction from the surface. Image analysis using Fibril tool, **Fig.1B** where each line represents collagen fibril direction and magnitude in defined quadrants, illustrates the effect of BMP9 on immature cartilage.

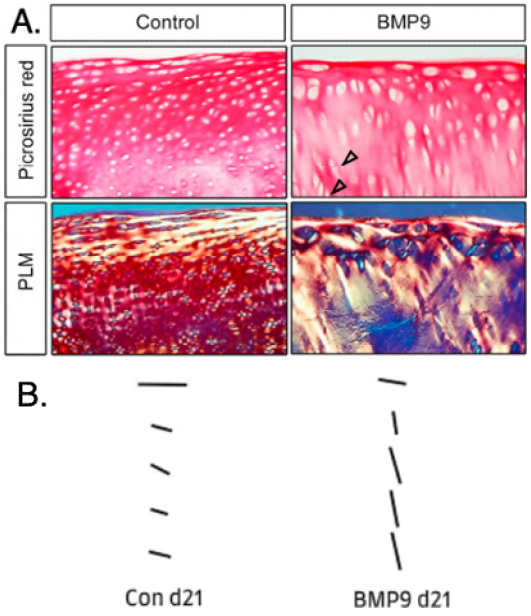

***Fig. 1**: **A**. PSR sections of control and BMP9 treated explants after 21 days (d21) in culture. **B**. Fibril tool analysis of collagen fibril orientation. Fibril direction and fluorescence intensity is signified by each line.*

DISCUSSION & CONCLUSIONS: These data show BMP9 rapidly generates adult-like cartilage from an immature precursor. First there is a seismic shift in collagen fibre organisation. Then follows shape, size and position of chondrocytes, matching the observations of Decker *et al* (3) when analysing early postnatal growth and maturational development of cartilage in mice. Form follows function and these findings will help enable effective tissue engineering of functional implants for cartilage repair and regeneration.

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Development of electrochemical nitric oxide microsensors for the real time monitoring of inflammation in chondrocytes

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INTRODUCTION: During inflammation cells release nitric oxide (NO) a metabolite that can be used as biomarker[1]. Common methods imply the use of Griess method or paramagnetic electron resonance (EPR) for indirect NO measurement in a solution. However, all these methods cannot provide real time monitoring, limiting the investigation of drug response and the clinical translation. The use of electrochemical sensors can overcome this issue, however the selectivity of this sensing tool could be critical if not specifically designed for the biological use[2].

METHODS: Platinum wires were used bare or modified with polymers and/or Carbon Black (CB) nanomaterial[3]. Two different modifications were performed: with poly-o-phenylenediamine (p-OPD/PPD) applying +700 mV vs an Ag/AgCl reference electrode and CB dispersion in Nafion™ by drop casting and oven drying (200°C for 2 min). The sensors were calibrated with known (S-nitroso-N-acetylpenicillamine, SNAP) concentrations (0 to 100 µM) using a potential at +865 mV. Ascorbic (AA) acid, L-Glutamine (Glu), and H_2O_2 were tested as common biological interference. Nitric oxide was measured in real time in 2D chondrocyte culture for 48 hours in the presence or in the absence of 10 ng/ml IL1b by applying the same potential vs an Ag/AgCl reference electrode directly in cell culture plate.

RESULTS: p-OPD and CB were homogenously distributed on sensor surface. The coating with p-OPD showed lower background noise for AA and none for Glu but considerable for H_2O_2. Contrarily, the coating with CB strongly decreased all the interferences. The real time monitoring of NO in chondrocytes showed significant difference in signal between the groups. The use of CB coating increased the analytical performance in terms of selectivity and reproducibility during real time monitoring in inflamed biological system (Fig. 1).

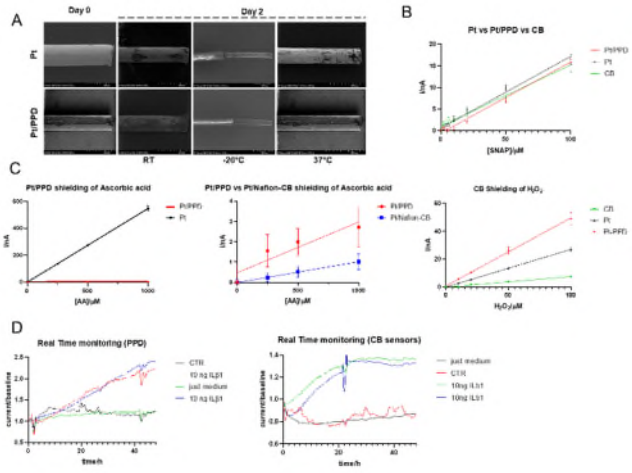

Fig. 1: (A) Sensor stability and modification, (B) Calibration curve with SNAP, (C) interference analysis with AA and H202, (D) real time monitoring with PPD and CB sensors in 2D chondrocytes culture during inflammation.

DISCUSSION & CONCLUSIONS:

The monitoring of NO release by electrochemical sensor during the inflammation *in vitro* and eventually *in vivo* could help to determine the progression/status of widespread pathologies or infections from pathogens in biological fluids and cell culture medium. However, we provide evidence that in a complex system such as cell culture medium, it is of paramount importance to properly modify the active surface of the sensor to avoid the interference with analytes not of interested. Particularly, during inflammation the cells produce peroxide. Herein, we prove how the modification with CB-Nafion does not limit the sensor performance while increasing the selectivity for the NO.

ACKNOWLEDGEMENTS: AO Foundation, Innosuisse funding scheme.

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In-Situ-Forming Zwitterionic Hydrogel for Cartilage Protection in Early-Stage Osteoarthritis

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INTRODUCTION: Persistent inflammation in joint cavity plays a central role in osteoarthritis (OA) onset and progression [1-2]. In this study, we have designed a novel in-situ-forming hydrogel based on highly hydrophilic zwitterionic (ZI) polymers that can halt inflammation-induced cartilage degeneration and provide cartilage lubrication and reinforcement. ZI polymers can effectively penetrate cartilage and form an interpenetrating network with the tissue. This hydrogel system can be used as a minimally invasive treatment to prevent OA progression or post-traumatic OA induction.

METHODS: Novel ZI copolymer, poly(carboxybetaine-co-tyramine acrylamide) is synthesized which crosslinks in presence of horseradish peroxide (HRP) as activating enzyme and hydrogen peroxide (H_2O_2) as crosslinker. Bovine cartilage explants are used as *ex vivo* model to test polymer penetration and in-situ-crosslinking, as well as the hydrogel effect on tissue protection, lubrication, and reinforcement. Cartilage explants digested with collagenase are used to represent "degraded" tissue for penetration and reinforcement study. IL-1β stimulation of cartilage explants is used to mimic inflammation-induced tissue loss in OA for tissue protection study.

RESULTS: Fluorescently labelled ZI copolymers are shown to penetrate whole thickness of cartilage and form an interpenetrating network with the tissue, making them retain in both healthy and degraded cartilage even after 7 days of extensive washing (Fig.1). ZI hydrogel is shown to effectively inhibit IL-1β induced tissue loss up to 3 weeks of culture, compared to non-ZI control, emphasizing the importance of zwitterionic nature of the hydrogel (Fig.2). Moreover, ZI hydrogel is shown to effectively protect collagenase-degraded explants from progressive degradation and decrease coefficient of friction in degraded tissue, thus having regenerative effects on cartilage and helping in restoring tissue function.

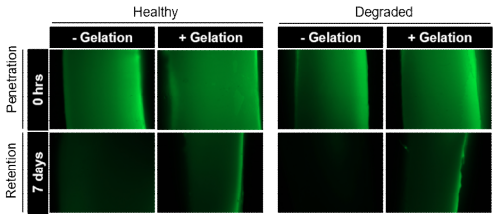

***Figure 1. Penetration and in-situ-gelation**. Microscopic images of fluorescent ZI polymers penetrated bovine cartilage explants*

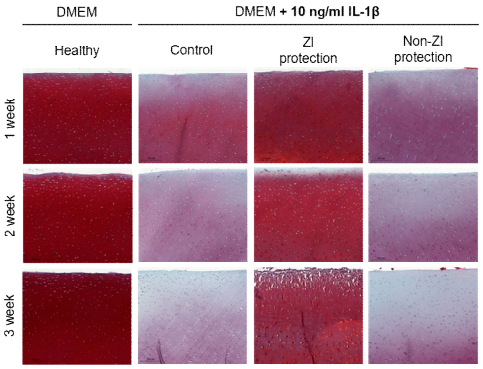

***Figure 2. Cartilage protection in inflammatory environment**. Safranin O staining of bovine cartilage explants cultured in DMEM + 10% FBS + 10 ng/ml IL-1β for up to 3 weeks (except for healthy condition which was cultured in media without IL-1β)*

DISCUSSION & CONCLUSIONS: In this study we have introduced a novel in-situ-forming ZI hydrogel as a non-invasive treatment for early-stage OA. The crosslinking method is fast and biocompatible and enables tissue adhesiveness to cartilage extracellular matrix. This hydrogel can effectively inhibit inflammation-induced tissue loss and protect tissue from progressive degradation. Moreover, the hydrogel system can reinforce and lubricate the tissue, helping in retaining healthy cartilage structure and function.

ACKNOWLEDGEMENTS: The authors would like to thank the Swiss National Science Foundation (project number 192656 to MZW) for providing financial support

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Effect of therapeutic IL1Ra and IL10 mRNA vehiculated by polymeric nanoparticles in osteochondral ex-vivo model: a new approach for osteoarthritis treatment.

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INTRODUCTION: Currently the most widely used drugs for the treatment of Osteoarthritis (OA) are non-steroidal and steroidal drugs that aim to attenuate the inflammation[1]. Local administration is limited due to the low penetrability of the drugs through the various tissues in the joint with extracellular matrices. Therefore, new types of treatments, which can target all tissues within the joint, are of extreme necessity and urgency. A recent innovation is based on the use of biological therapies[2], in which nucleic acids are administered using nanocarriers. This study investigated the effect of mRNA therapeutics in an inflammatory osteochondral explant model.

METHODS: Cylindrical osteochondral plugs were obtained from bovine stifle joints using a custom-made coated trephine drill. After 24h the explants were treated with 1 ng/ml IL1b (Kingfisher) and respectively incubated with nanogel (NG) loaded with IL1Ra or IL10 mRNA, NG, only Inflamed CTR or culture medium. NG based on multifunctional poly-(amidoamines) (20Med) at concentration of 1.5 mg/ml were loaded with mRNA before the transfection[3]; therapeutic mRNAs were diluted to 120 ug/ml in 20mM Hepes to obtain a final mRNA:NG ratio (w/w) of 1:12.5. mRNAs containing chemically modified nucleotides were produced by using in vitro transcription with T7 RNA polymerase (Ethris). For capping, anti-reverse cap analogue (ARCA) was also included in the in vitro transcription reaction. mRNAs were carrying a 120 polyA-tail. Content of nitric oxide (NO) and glycosamino-glycan (GAG) in conditioned medium was measured using Griess reagent and Dimethyl-methylene Blue Assay (DMMB). Real-Time qPCR was performed using gene expression assays for IL8, IL6, IL10, ADAMTS5 and RPLP0 as endogenous control. Histological evaluation was carried out by Saf-O / Fast Green staining at day 0 and day 14.

RESULTS: An upregulation of IL6, IL8, and ADAMTS5 gene expression was observed after 7 days which persisted over 14 days, confirming that the treatment with inflammatory cytokines was constant over time, also in terms of NO and GAG released into the conditioned medium compared to control. The use of GFP-labeled mRNA showed the capacity of penetration of mRNA encapsulated within NG. We further tested the effect of therapeutic IL1Ra and IL10 mRNAs on inflamed explants. Interestingly, mRNA treatment (IL1Ra, IL10) reduced the GAG and NO ($p<0.001$) release into the medium compared to the inflamed control. The concentration of IL6 was as well attenuated by the administration of IL1Ra mRNA, though no significant effect was observed for IL10 mRNA. Histology showed larger Safranin-O positive stained area after treatment with IL1Ra compared to all other groups over the 14 days in constant presence of inflammation.

DISCUSSION & CONCLUSIONS: We provided a suitable and stable model able to mimic the human chronic inflammation of OA, furthermore we showed the feasibility of using alternative drugs based on nanoparticles and therapeutic mRNA able to penetrate the cartilage. The IL1Ra and IL10 mRNA could be a possible alternative as local treatment for the modulation of inflammation that can possibly counteract the progression of inflammation and cartilage degeneration.

ACKNOWLEDGEMENTS: EuroStars grant JOINT-APPROACH E!12792.

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X-ray scattering imaging of intervertebral discs under load

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INTRODUCTION: An improved understanding of intervertebral disc (IVD) structure and function is required for treatment development. Loading induces micro-fractures at the interface between the nucleus pulposus (NP) and the annulus fibrosus (AF), which is hypothesized to induce a cascade of cellular changes leading to degeneration. However, there is limited understanding of the structural relationship between the NP and AF at this interface and particularly response to load. This study utilised X-ray scattering to provide hierarchical morphometric information of collagen structure across the IVD, especially the interface region under load.

METHODS: Bovine IVDs were imaged using the I22 SAXS/WAXS beamline at Diamond Light Source. Peaks associated with the D-banded structure of collagen fibrils were fitted to quantify their azimuthal distribution, as well the magnitude and direction of internal strains. WAXS imaging with the new high-throughput VMXi beamline was performed of non-degenerate and degenerate bovine IVDs under 0-20% applied strains to investigate the hierarchical response to load. Total collagen distribution and tropocollagen molecule orientation determined together with Azimuthal distribution of intermolecular peaks during load.

RESULTS: SAXS imaging provided structural "AF-like" and "NP-like" fingerprints, with clear bidirectionality within the AF, whilst the NP showed multidirectional strains. D-band peak intensities between the AF and NP also alluded to variations in charge distribution along tropocollagen molecules. With highest D period seen within the NP demonstrating high internal strains on collagen fibers, likely a result of internal tissue swelling due to high proteoglycan content. The interface region exhibited a linear rearrangement from AF-like to NP-like structure over a region approximately 500µm wide with no other distinguishing features. WAXS imaging of non-degenerate and degenerate IVDs demonstrated clear changes in collagen content and orientation within degenerate discs (Figure 1). Increased strain in healthy discs appeared to increase collagen distribution within the interface zone although no clear changes in orientation were observed (Figure 1). Degenerate discs showed higher levels of collagen within a fibrotic region which disrupted the collagen orientation particularly in the NP and decreased orientation in the AF. With hot spots seen during loading and potential fissure propagation.

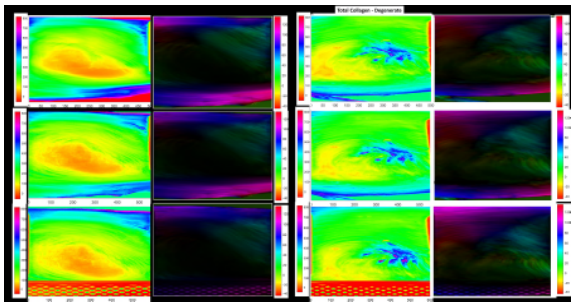

Figure 1: Total collagen and preferential tropocollagen orientation in normal and degenerate bovine IVDs under 0 &10 % strain. Scales show collagen content (Left) and Tropocollagen Orientation (Right), where an angle of 0 corresponds to vertical alignment of collagen molecules and thus the fibers they form

DISCUSSION & CONCLUSIONS: SAXS and WAXS have been utilised to develop an improved understanding of collagen structure across the intervertebral disc. Demonstrating high internal strains on collagen fibers particularly within the NP region of the disc. AF and NP regions showed distinct collagen orientation and internal strains with an apparent lack of bracing structure seen at the interface between the differential mechanical tissues (NP and AF). This could lead to the generation of shear forces under load, leading to micro-fractures and failure seen during degeneration at the interface region. WAXS imaging of normal and degenerate discs under strain demonstrated clear differences in collagen content and orientation between normal and degenerate discs, with potential increased collagen organization within the interface zone under load in normal but not degenerate discs. Current work is investigating the D period internal strains on collagen fibers on these data to determine changes in collagen strains during external loading.

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Anti-degradative and pro-chondrogenic properties of liraglutide, a Glucagon-Like-Peptide 1 Receptor agonist: evidence from preclinical studies and implication for osteoarthritis

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INTRODUCTION: Osteoarthritis (OA) is a degenerative joint disease affecting millions of individuals worldwide. Its development has been reported to be associated with cartilage degradation and inflammatory responses leading to pain, swelling and reduced function. Although OA is a disorder of the whole joint, the progressive destruction of cartilage extracellular matrix is considered as its hallmark. To date, approved OA treatments are only symptomatic. Therefore, there is an urgent need to explore disease-modifying OA drugs (DMOADs) that can mitigate, stop, or even reverse the development of OA. In this context, the objective of this study was to assess the effect of liraglutide, a Glucagon-Like-Peptide 1 Receptor (GLP-1R) agonist approved for type 2 diabetes, on chondrogenesis, catabolism/inflammation and cartilage protection in *in vitro* and *in vivo* preclinical models of OA.

METHODS: The capacity of liraglutide to induce chondrogenesis was evaluated using primary human mesenchymal stem cells (hMSCs). Alcian blue staining was used to assess differentiation of hMSC into chondrocyte spheroids. IL-1β-stimulated mouse articular chondrocytes were treated with different concentrations of liraglutide for 24h. Production of matrix metalloproteinase MMP-13, prostaglandin E2 (PGE2) and nitrite was measured by ELISA and Griess reaction, respectively. Exendin 9-39, a GLP-1R antagonist, was used to confirm target engagement in the *in vitro* experiments. Intra-articular (IA) injections of liraglutide or vehicle were performed in the type II collagenase rat model. Histopathological analyses (OARSI scores[a]) were conducted blindly by one investigator.

RESULTS: After 21 days of treatment, liraglutide but not vehicle induced their differentiation into chondrogenic 3D spheroids (Liraglutide 10nM= 5 alcian-blue positive spheroids out of 6 counted wells, $p<0.05$; Liraglutide 100nM= 4/6, $p=0.06$, vs vehicle= 0/6). Liraglutide significantly reduced dose-dependently the IL-1β-induced production of PGE2 (5808±178 for vehicle vs 4560±140, 2933±171 and 2365±85 pg/mL for liraglutide 10, 100 and 500nM, respectively, $p\leq0.001$), nitrite (24.9±0.4 for vehicle vs 20.9±1.5, 19.1±0.9 and 16.5±0.5 µM for liraglutide 10, 100 and 500nM, respectively, $p\leq0.001$) and MMP-13 (686±9 for vehicle vs 553±3, 402±5 and 297±8 pg/mL for liraglutide 10, 100 and 500nM, respectively, $p\leq0.001$) in murine chondrocytes. Effects of liraglutide were GLP-1R dependent since exendin 9-39 significantly counteracted both chondrogenesis and inflammation/catabolism markers expression. Histological assessment of rat collagenase-injected knee joint revealed a significant ($p\leq0.05$) decrease of the total joint score in the IA Liraglutide treated group (8±4) compared to vehicle (11±4).

DISCUSSION & CONCLUSIONS: Liraglutide induced chondrogenesis, decreased metalloproteinase and inflammatory mediators production by chondrocytes and protected cartilage in *in vitro* and *in vivo* preclinical OA models, opening the way for repositioning this drug as a potential DMOAD.

ACKNOWLEDGEMENTS: All the people who contributed to the InOsteo project: the members of 4P-Pharma, INSERM UMR S938 research team, SATT Lutech, 4Moving Biotech and Sorbonne University.

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Catabolic Phenotype Induction of NP and AF Cells in 3D Culture

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INTRODUCTION: Intervertebral disc (IVD) degeneration is considered a key contributor for low back pain, which is the leading cause of disability worldwide[1]. In the last decades, a wide range of cytokines have been applied to IVD cells *in-vitro* to investigate the degenerative cascade[2,3]. However, a generic stimulation model defining dose and duration is lacking. Hence, we aimed to explore the catabolic response of bovine IVD cells after TNF-α or IL-1β stimulation at different concentrations and timepoints *in-vitro.*

METHODS: Primary nucleus pulposus (NP) and annulus fibrosus (AF) cells were isolated from bovine tails and expanded for two weeks. Afterwards, IVD cells were encapsulated in 1.2% alginate beads (4 x 10^6 cells/ml) and cultured for two weeks for phenotype recovery. Then, alginate beads were stimulated with 0.1, 1 and 10 ng/ml TNF-α or with 0.01, 0.1 and 10 ng/ml IL-1β for one week. Beads were collected on the stimulation day (Day 0) and on Day 1 and 7 after stimulation. A nonparametric distribution was assumed and Kruskal–Wallis test followed by Dunn's multiple comparisons test was performed.

RESULTS: A dose-dependent upregulation of catabolic markers was observed in NP and AF cells after one day of TNF-α or IL-1β stimulation. Particularly, 10 ng/ml TNF-α stimulation revealed a significant upregulation ($p<0.05$) of *ADAMTS4*, *MMP3* and *MMP13* in AF cells compared to the control group after one day of stimulation. Although the significance was only observed in AF cells, *MMP3* upregulation in NP cells showed a strong trend ($p=0.0643$) (Fig. 1). Nevertheless, the dose-dependent upregulation tendency was less pronounced or even lost after seven days of treatment. In addition, no significant difference between treatments was observed in *COL2*, *COL1* and *ACAN* expression. Noteworthy, the cell viability was not reduced after seven days in both NP and AF cells regardless of the treatment.

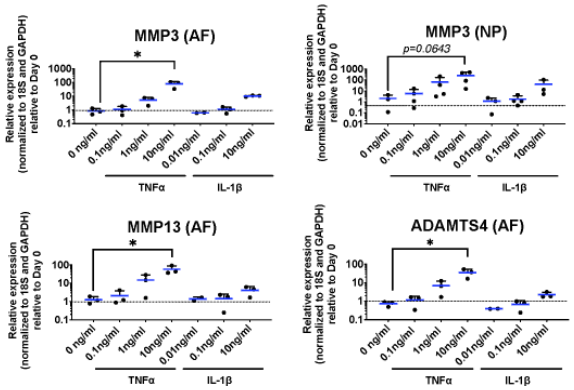

*Fig. 1: Relative gene expression of MMP13 ADAMTS4 and MMP3 in AF and NP cells after one day of stimulation. Shown are means + SD, N = 2–4, p-value: * <0.05.*

DISCUSSION & CONCLUSIONS: We demonstrate a dose dependent upregulation of catabolic markers in NP and AF cells under TNF-α or IL-1β stimulation. Moreover, the significant upregulation of *ADAMTS4*, *MMP3* and *MMP13* genes was found in AF cells after one day of treatment. Notably, after seven days of treatment, the dose-dependent effect could be counter-regulated due to a possible adaptation of the cells to the catabolic stimuli, suggesting a possible mechanism to face the metabolic shift.

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Notochordal cells encapsulated within hydrogel in an enzymatic bovine IVD degeneration model

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INTRODUCTION: Low back pain is closely associated to the degeneration of intervertebral discs (IVD). In search of treatment options, the regenerative potential of different cell-based therapies and biomaterials have been investigated [1]. *In vitro* studies showed possible beneficial effects of notochordal cells (NCs) on resident IVD cells [2]. Laponite crosslinked pNIPAM-co-DMAc (NPgel) has been shown to promote tissue integration into native nucleus pulposus (NP) while promoting the differentiation of incorporated hMSCs into NP phenotype; furthermore, biomechanical properties could be restored [3]. We hypothesized that combining NPgel with NCs would enhance the regenerative effects of both therapeutic approaches.

METHODS: Enzymatic degeneration was induced by injecting 100 µL of 0.5 U/mL collagenase II into the center of bovine caudal IVDs. The negative control group received 100 µL of PBS (PBS, n=4). At week 2 of the study, the IVDs were separated into three groups, either receiving 500 µL of $4x10^6$ porcine NCs/mL (NC+NPgel, n=4) encapsulated within NPgel, 500 µL of NPgel only (NPgel, n=3) or 500 µL of PBS (enzyme only, n=4). The IVDs were cultured with daily physiological loading for a total of 18 days. Histology was performed with Safranin-O/Fast Green Staining. Disc height and diameter were measured daily. IVD height versus time was plotted and fit to a linear trendline, with the slope taken as the rate of disc height change over time.

RESULTS: Macroscopic voids could be observed in all untreated collagenase-digested specimens at day 18 of culture. Contrarily, voids were not persistently present in the other groups. Histology demonstrated the presence of NPgel in both NC+NPgel and NPgel groups (black arrows figure 1A). Possible tissue integration into the hydrogel could be seen in the NPgel group (yellow arrow figure 1A). During the treatment period of IVD culture, all groups trended toward faster height loss than the PBS group, but the difference was significant only for the enzyme and NPgel groups ($p<0.05$, unpaired t-test) (figure 1B).

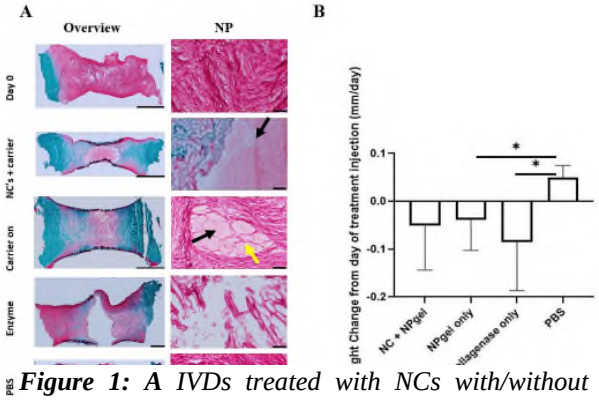

Figure 1: ***A*** *IVDs treated with NCs with/without NPgel. Representative Safranin O/ Fast Green results. Black arrows highlight NPgel, yellow arrow possible tissue integration.* ***B*** *Disc height change over time: groups trended toward faster height loss compared to PBS, but the difference was significant only for enzyme and NPgel groups ($p<0.05$)*

DISCUSSION & CONCLUSIONS: We successfully injected NPgel with/without NCs into a bovine enzymatic IVD degeneration model. Both treatments tended to slow down height loss. Tissue integration into the hydrogel could be observed in the NPgel group. We are currently evaluating further regenerative effects on gene and protein expression.

ACKNOWLEDGEMENTS: The work described is part of the iPSpine project, which has received funding from the European Union's Horizon 2020 Research and Innovation Programme (Grant No 825925).

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Using methacrylated gellan gum in combination with novel silk to repair the annulus fibrosus in a dynamically loaded bovine organ culture model

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INTRODUCTION: Annulus fibrosus (AF) rupture within the intervertebral disc (IVD) can lead to herniation and is a key contributor to low back pain [1]. A potential solution could be the implementation of a methacrylated gellan gum (MA-GG) hydrogel. MA-GG is a polysaccharide produced by the bacterium *Sphingomonas paucimobilis* and is considered as a promising biomaterial for IVD tissue engineering applications [2]. Therefore, we aimed to create an AF repair approach using 2% MA-GG as a filler material and sealing it with an embroidered silk yarn fabric, which mimics the AF's morphology.

METHODS: For this *ex vivo* organ culture model, coccygeal IVDs were isolated from fresh bovine tails. Next, the AF was injured using a biopsy puncher (⌀ 2 mm) [3]. The damaged site was either left untreated or filled with 2% MA-GG and then sealed with an embroidered silk yarn fabric sutured directly onto the IVD. Then, the IVDs were cultured for 14 days under different loading profiles, i.e. i) no load, ii) static load at 0.2 MPa, and iii) dynamic load at 0.2 ± 0.1 MPa and a superimposed torsion of 2° (Figure 1a). After the culture period, changes in the IVDs' height were recorded, the AF was dissected, and the tissue and the culture medium were harvested. The AF tissue was further analysed for its metabolic activity, glycosaminoglycan (GAG) content, nitric oxide (NO) content, and relative gene expression.

RESULTS: After 14 days of culture, no IVD herniation occurred, and the silk ribbon successfully managed to seal the repaired site regardless of the loading profile (Figure 1b). Moreover, repaired IVDs showed a better disc height recovery than damaged samples under the dynamic loading profile. However, the metabolic activity and the amount of GAG and NO remained mostly unchanged. Furthermore, repaired samples showed generally a higher expression of anabolic genes like *Aggrecan* and *Collagen type II* compared to damaged samples under dynamic load, and also inflammatory genes were often more highly expressed in the repaired samples than in the damaged ones.

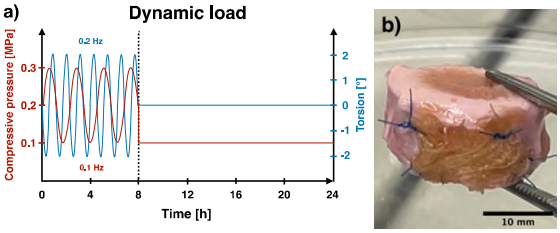

Fig. 1: a) Daily dynamic loading profile, repeated for 14 days. b) Injured bovine IVD, repaired with MA-GG, sealed with silk and cultured for14 days under dynamic load.

DISCUSSION & CONCLUSIONS: The presented approach using MA-GG and silk fibroin to repair an AF injury was a success as no displacement of the biomaterials and no IVD herniation was observed. Furthermore, the cytocompatible properties of MA-GG and silk were confirmed as the tissue's metabolic activity remained stable. Nevertheless, results should be viewed with caution as the data are still preliminary due to the small sample size of N = 1-2.

ACKNOWLEDGEMENTS: We would like to thank the Swiss National Science Foundation # 310030E_192674/1 for funding.

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Effects of Dynamic Compression on Cartilage Endplate Cells in Agarose

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INTRODUCTION: Intervertebral disc (IVD) degeneration is one of the main causes of low back pain. However, the initiating and risk factors of IVD degeneration are poorly understood[1]. The cartilage endplate (CEP) covers the top and bottom of the IVD and acts as a semipermeable barrier between the discs and the vertebrae[2]. However, with aging and degeneration the CEP experiences structural changes with altered permeability[3]. Although most research focuses on the nucleus pulposus and annulus fibrosis, early CEP degeneration is believed to be a cause of nucleus dehydration and play a key role in IVD degeneration[4].

METHODS: Bovine tail CEP cells were expanded until passage three. Afterwards, a 1:1 mixture of CEP cells (2 x 10^7 cells/ml) and 4% agarose was pipetted into silicon molds to create constructs of 6 mm in diameter and 3 mm in thickness and then cultured for 2 days for phenotype recovery. Cell-agarose constructs were placed in custom-made chambers, stimulated with 10 ng/ml TGF-β1 throughout the entirety of the experiment and dynamically compressed at 7% strain for 1 hour every day for up to 14 days. Those that were not dynamically loaded were considered to be statically loaded as they had the constant weight of a lid (~18.8 g) imposed. Constructs were collected on Days 0, 7, and 14 for downstream analysis of cell viability and gene expression.

RESULTS: After seven and 14 days of culture, the cell-agarose constructs in all conditions demonstrated increased expression of the anabolic genes aggrecan (ACAN) and collagen II (COL II), alongside decreased expression of catabolic gene matrix metalloproteinase 3 (MMP3) (Fig. 1a-c). Cell viability was unchanged after seven days but decreased after 14 days for all conditions, except for the dynamically loaded chondrogenic constructs which kept increasing after 14 days (Fig. 1d). Cell viability was generally higher at the edge of the construct in comparison to the center.

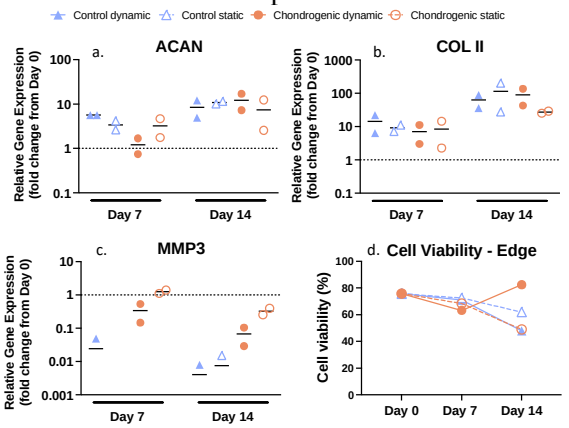

Fig. 1: Relative gene expression of (a) ACAN, (b) COL II, (c) MMP13 in CEP cells, and (d) cell viability at the edge of the cell-agarose constructs. Shown are the means, N=2.

DISCUSSION & CONCLUSIONS: Although the data presented is preliminary and additional biological replicates are needed to make any significant conclusions, increased expression of anabolic genes ACAN and COL II convey that the agarose constructs provided an anabolic environment for CEP cells under static and dynamic compression. However, it appeared that the chondrogenic media and type of compression did not affect gene expression. Future work will quantify glycosaminoglycan content and then assess its distribution using alcian blue and histology. Likewise, possible boundary effects shall be quantified.

ACKNOWLEDGEMENTS: This project received funds from the European Commission (H2020-MSCA-ITN-ETN-2020-955735).

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Self-assembling peptide hydrogels for nucleus augmentation of the intervertebral disc

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INTRODUCTION: Low back pain affects 80% of adults and has estimated costs 1 – 2% of GDP[1,2]. Nucleus augmentation aims to restore healthy disc anatomy and biomechanical function through the delivery of a biomaterial into the nucleus of a degenerated intervertebral disc. Self-assembling peptides (PEP) can be used with glycosaminoglycans (GAG) to form a PEP-GAG hydrogel for use as a nucleus augmentation material. This work focuses on assessing three different peptides and their viability for nucleus augmentation.

METHODS: All samples contained peptide (20 mg/ml) with or without chondroitin sulfate (136 mg/ml). Different cytotoxicity assays were used to assess the PEP-GAG hydrogel and counterion cytotoxicity with L929 and BHK cells. Rheometry was used to assess mechanical properties after different modes of delivery. PEP samples were made at a range of concentrations to determine the critical concentration (c*) for self-assembly using ^{1}H NMR. CryoSEM and FIB-SEM images were obtained using FEI – Helios G4 CX Dual beam FIBSEM.

RESULTS: Cytotoxicity testing of different PEP-GAG hydrogels showed some slight cytotoxicity in the indirect and extract assays but not in the direct contact assay with the three different peptides behaving similarly across the assays (Fig 1). A greater difference in sample cytotoxicity was seen when comparing trifluoroacetate (TFA), acetate and HCl counterions with HCl showing the least cytotoxicity compared to TFA and acetate.

As the end polar amino acids change from serine to glutamine, the c* decreased as a result of the increased number and strength of intermolecular hydrogen bonding. When injected down different needle lengths, gauges and designs, there was a small reduction in gel stiffness (G') when the needle design was changed, however this small change was not clinically relevant. CryoSEM showed that all three PEP-GAG hydrogels were able to form fibrous networks (Fig 2).

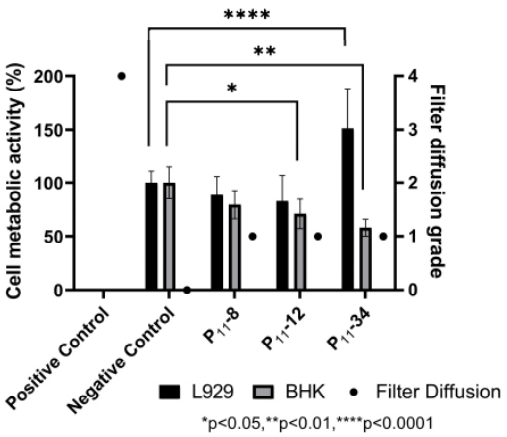

Figure 1 – Cytotoxicity comparison of PEP-GAG hydrogels

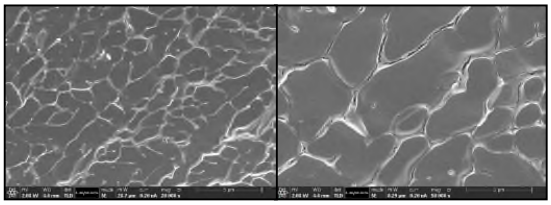

Figure 2 – CryoSEM of P_{11}-34-GAG hydrogel.

DISCUSSION & CONCLUSIONS: All three peptides investigated were able to form PEP-GAG hydrogels with low levels of cytotoxicity. Changing the end polar amino acid between glutamine and serine allows for tuning of the material properties. The mechanical properties as determined by rheology suggest that the PEP-GAG hydrogels are suitable for nucleus augmentation. The use of a minimally invasive clinical delivery device slightly reduced the gel stiffness but provides the advantage of reduced damage to the annulus and better mixing *in situ*. The PEP-GAG hydrogels offer a promising new approach to repair the degenerated intervertebral disc to treat lower back pain.

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Bioreactor Culture Model Of Human Nucleus Pulposus

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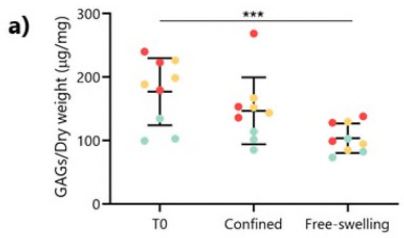
INTRODUCTION: Intervertebral disc (IVD) degeneration (IVDD) is one of the leading causes of chronic LBP. However, the pathogenesis of IVD-related LBP is still controversial. To study IVD degeneration and develop novel treatments, models for studying of IVD are important; these models range from in vitro cell culture to animal models. Organ or tissue culture systems that employ living, degenerate disc tissues from human donors may be the ultimate platform to effectively mimic the biological complexity of the in vivo cellular microenvironment, particularly in bioreactors that restrict swelling or even apply physiological loading.
The aim of our study was to validate the use of a bioreactor culture system for human NP tissue. The first step was to show the added value of culturing in confined condition, so inside the bioreactor, compared to free-swelling conditions.

METHODS: Human NP explants (n=4/donor, 8 mm diameter) were cultured in free-swelling condition or in the bioreactor for 28 days (3 donors, Thompson grade=III). T0 explants were used as baseline control. Tissue was harvested and processed for wet weight and dry weight measurement, paraffin histology (Alcian blue and picrosirius red) and IHC (collagen type I&II, aggrecan), biochemical assays (DMMB (GAG tissue and medium), hydroxyproline (collagen), PicoGreen (DNA)).

RESULTS: Free swelling culture of NP tissue resulted in a reduced GAG content of the tissue (Fig 1a). GAG release over time was higher in confined culture (Fig1b). Collagen content was higher in explants cultured in free swelling conditions (Fig 1c).



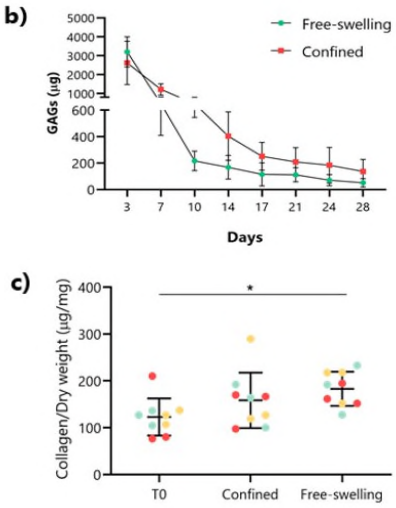


*Fig. 1: ECM content and release in NP explants after culture in free swelling or confined condition a) glycosaminoglycan (sGAG) content normalized to dry weight, b) GAG release in bioreactor and free swelling culture for 28 days, c) collagen content normalised to dry weight. *P<0.05*

DISCUSSION & CONCLUSIONS: NP tissue cultured in confined conditions appears to retain the original matrix composition better than if cultured under free swelling conditions. Whether the increased release of GAGs in bioreactor culture results from degradation of synthesis is unclear. In vitro IVD study models will make an important contribution to studying IVD degeneration and developing new treatments.

ACKNOWLEDGEMENTS: This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No:825925 and from the Turkish government under Republic of Turkey Ministry of National Education scholarship programme.

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A better way to repair large osteoarthritic cartilage lesions. 3D Bioprinting with spheroids of human chondrocytes in biocompatible hydrogels.

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INTRODUCTION: Cartilage lesions that progress to severe osteoarthritis affect millions of people worldwide. In contrast to focal cartilage defects, treatments such as autologous chondrocyte implantation (ACI) are ineffective in osteoarthritis. Crosslinked hydrogels have shown great potential when injected into joints as a stabilizing cushion. A novel medical-grade and biocompatible hydrogel isolated from marine aquaculture of tunicates, containing nanofibrillated cellulose (TUNICELL) were successfully evaluated in *in vivo* studies for cartilage and soft tissue reconstruction (1, 2). This project investigates an alternative treatment for the repair of cartilage lesions by combining spheroids of human chondrocytes with TUNICELL using 3D Bioprinting.

METHODS: Various compositions of TUNICELL (Ocean TuniCell, Norway) with hyaluronic acid (Corgel, USA); TUNICELL-HA or alginate (NovaMatrix, Norway); TUNICELL-A, were used to formulate bioinks to mimic biomechanical properties of cartilage. Spheroids of human chondrocytes were prepared as described elsewhere (3). They were precisely placed into hydrogels in the XYZ plane with predetermined 3D architecture (3D Bioprinter BioX, CELLINK, Sweden). An *in vitro* study was carried out to evaluate cartilage regeneration when spheroids were partly or fully encapsulated in hydrogels.

RESULTS: Figure 1 shows the design of 3D architecture of the constructs and 3D Bioprinted constructs with model particles and chondrospheres. Measurements after three weeks of cultivation showed increases in spheroid diameter of 10-30% in TUNICELL-A, and 10-15% in TUNICELL-HA and in control samples.

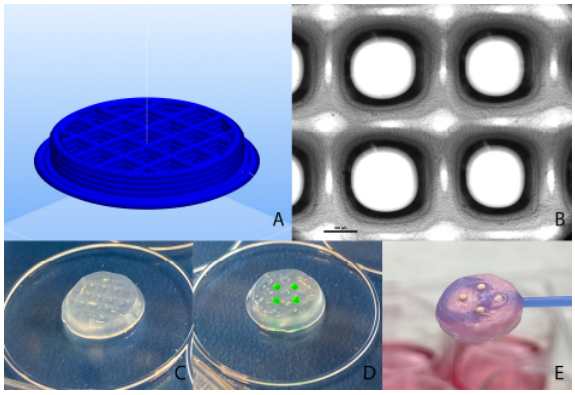

Fig. 1 (A) CAD design, (B) Printability test, (C-E) 3D Bioprinted constructs, (D) With fluorescent particles, (E) With spheroids.

DISCUSSION & CONCLUSIONS: This indicates a potential increase in extracellular matrix production when spheroids are placed in TUNICELL-A. Cartilage formation within the spheroids encapsulated in different hydrogels and with different 3D placements is determined by histology and immune-histochemistry. Therefore, 3D Bioprinting with spheroids of human chondrocytes in biocompatible hydrogels such as TUNICELL can be developed as a procedure for future repair of large osteoarthritic cartilage lesions.

ACKNOWLEDGEMENTS: Research Council of Norway is acknowledged for financial support.

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Influence of mechanical loading regimes on spontaneous response of dorsal root ganglion neurons

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INTRODUCTION: Mechanical loading of the intervertebral disc (IVD) is often discussed as a low back pain (LBP) risk factor. Aberrant loading was found to induce IVD degeneration [2], but its influence on pain-associated neurology remains unknown. Dorsal root ganglion (DRG) neurons are the first-order neurons that transduce nociceptive/pain signals. Particularly, the spontaneous activation of these DRG neurons is correlated with spontaneous pain [3]. In this study, intensive loading of IVD was hypothesized to induce an increased spontaneous activation of DRG neurons.

METHODS: Bovine tail IVDs were compressively loaded in a bioreactor:

1). high static loading (force control loading, FC) of 0.2 MPa 24h/day was applied for 6d to represent the 'long-term sitting and standing' and was compared with a low loading keeping the disc height of day 0 (displacement control, DC).

2). cyclic loading of high frequency and intensity (degenerative loading, DL) was applied for 5d to represent the 'wear and tear' and was compared with a low frequency and intensity loading (physiological loading, PL) (0.32~0.5 MPa at 5 Hz versus 0.02 ~0.2 MPa at 0.2 Hz, 3 h/day).

IVD degeneration was evaluated using real-time RT-PCR. Viability of the disc cells was analysed using lactate dehydrogenase (LDH) and ethidium-homodimer-1 staining of IVD cryosections. Conditioned media (CM) were collected to stimulate primary bovine DRG cell cultures. Calcium imaging (Fluo-4) was used to evaluate the spontaneous calcium oscillation in the outer region (calcium influx) of DRG neuronal soma. The nociceptive neurons were labelled using immunofluorescence of calcitonin gene related peptide (CGRP).

RESULTS: High static loading elevated the expression of interleukin 6 (IL-6) and matrix metalloproteinase 13 (MMP-13) in nucleus pulposus (NP) cells by 43.7 (p=0.07) and 13.2 (p=0.07) fold, respectively. High static loading also induced higher number of dead cells compared with low static loading in inner annulus fibrosus (AF) (15.7% versus 3.9%, p=0.03) and NP regions (18.5% versus 14.3%).

CM of IVDs subjected to both forms of intensive loading enhanced the frequency of spontaneous calcium oscillation in CGRP(+) neurons compared with their control IVD CM, respectively (Fig. 1A and B).

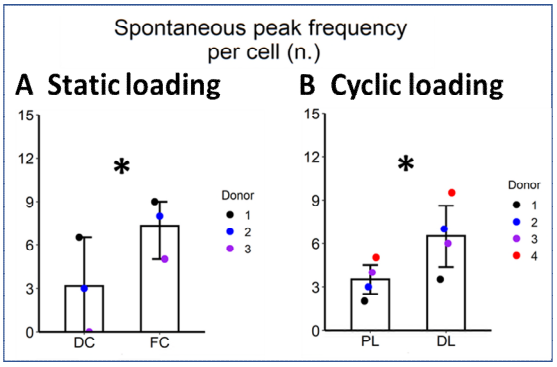

*Fig. 1: Calcium imaging of DRG nociceptors (CGRP(+) neurons); *p<0.05.*

DISCUSSION & CONCLUSIONS: The high static loading induced a degenerative-like change in the inner AF and NP region of the IVD. Both static and cyclic intensive loadings caused an altered disc-nerve communication and a sensitization in DRG nociceptors. To better understand the LBP biology, the molecular mechanism of the disc-nerve communication will be investigated in the future. This model takes advantage of using large animal tissue and is promising to replace/ reduce animal pain models in line with '3R'.

ACKNOWLEDGEMENTS: This work is supported by AO Foundation and AO Spine.

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Cortical microarchitecture impacts distal clavicle fracture morphology

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INTRODUCTION: Common distal clavicle fracture classification systems are based on fracture morphology relative to the coraco-clavicular entheses. Fracture stability is thus determined by ligamentous integrity. Like in other skeletal locations, osteoporotic transformation is discussed as a causative factor regarding incidence peaks in the distal clavicle. In this study, we aimed to characterize the cortical microarchitecture of the distal clavicle according to common fracture classification systems and to investigate the potential effects of age, gender, and osteoporosis.

METHODS: XtremeCT scans (Scanco Medical) at an isometric voxel size of 82 µm were prepared from n = 20 left human cadaveric distal clavicles (n = 10 male, n = 10 female, 25-87 years). Cortical porosity and bone mineral density (BMD) were evaluated in eleven sections of approx. 7 mm thickness for each dataset. According to common fracture regions, three distinct zones were defined as lateral (lateral to the trapezoid attachment; L), intertubercular (between trapezoid and conoid ligament attachment; I), and medial (medial to the conoid attachment; M). Parameters were further evaluated regarding age and gender.

RESULTS: Cortical porosity increased >20fold in the mediolateral axis ($p \leq 0.001$). Significant differences were present between L and I ($p \leq 0.001$) but not between I and M ($p=0.09$). >60-year-old specimen featured significantly higher cortical porosity in I compared to younger donors ($p=0.01$). BMD decreased >2fold towards the distal apex ($p \leq 0.001$) and was significantly greater in I compared to L ($p \leq 0.001$) and in M compared to I ($p=0.02$). BMD was significantly lower in I and M of >60-year-old compared to younger donors ($p<0.01$). Occurrence of low bone mass did not significantly differ between <60year-olds and >60year-olds in any of the three fracture regions.

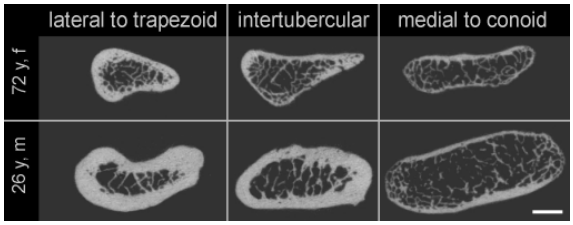

Fig. 1: XtremeCT scans of clavicle sections reveal high inter-individual variance with a remarkable flattening and cortical thinning towards the lateral apex in a 72-year-old female (upper row) and a 26-year-old male (lower row) specimen. Scale bar = 5mm.

DISCUSSION & CONCLUSIONS: This work detected distinct characteristics of the cortical microarchitecture in important fracture zones of the distal clavicle regardless of age, gender, and osteoporosis. The antidromic progression of cortical texture showing an increase in cortical porosity as opposed to a decrease in bone mineral density towards the distal apex should be carefully considered when planning surgical intervention in the treatment of distal clavicle fractures.

Free calcium and the Extracellular Calcium-Sensing Receptor Regulate Intervertebral Disc Degeneration and Calcification

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INTRODUCTION: Degenerative disc disease (DDD) is a common cause of lower back pain. Calcification of the intervertebral disc (IVD) has been correlated with DDD, and is especially prevalent in scoliotic discs. The appearance of calcium deposits has been shown to increase with age, and its occurrence has been associated with several other disorders such as hyperparathyroidism, chondrocalcinosis, and arthritis. Trauma, vertebral fusion and infection have also been shown to increase the incidence of IVD calcification. Our data indicate that Ca^{2+} and expression of the extracellular calcium-sensing receptor (CaSR) are significantly increased in mild to severely degenerative human IVDs. In this study, we evaluated the effects of Ca^{2+} and CaSR on the degeneration and calcification of IVDs.

METHODS: IVD tissue and cells were isolated from donor tissue. Immunohistochemistry (IHC) was performed to determine expression of CaSR. Free calcium levels were measured and compared between grades. Western blotting and RT-qPCR were performed on cultured NP and AF cells to demonstrate expression of CaSR, matrix proteins aggrecan and collagen, catabolic enzymes and calcification markers. IVD cells were cultured in increasing concentrations of Ca^{2+} [1.0-5.0 mM], CaSR allosteric agonist (cincalcet, 1 uM), and IL-1b [5 ng/mL] for 7 days. Ex vivo bovine IVD organ cultures were prepared using PrimeGrowth Disc Isolation System (Wisent Bioproducts). IVDs were cultured in 1.0, 2.5 mM Ca^{2+} or with cinacalcet for 21 days. Complex modulus and structural stiffness of disc tissues was determined using the MACH-1 mechanical testing system (Biomomentum, Laval, Quebec).

RESULTS: Ca^{2+} dose-dependently decreased matrix protein synthesis (proteoglycan and Col II) in disc cells (n = 4). Ca^{2+} and cincalcet did not significantly increase the expression of catabolic enzymes save ADAMTS5. Similar effects were observed in whole organ cultures, as Ca^{2+} and cinacalcet decreased proteoglycan and collagen content. Although both Ca^{2+} and cinacalcet increased the expression of alkaline phosphatase (ALP), only in Ca^{2+}-treated IVDs was there evidence of calcium deposits in NP and AF tissues. Biomechanical studies on Ca^{2+} and cinacalcet-treated IVDs demonstrated decreases in complex modulus ($p<0.01$ and $p<0.001$, respectively; n=5), however, only in Ca^{2+}-treated IVDs were there significant increases in stiffness of NP and AF tissues ($p<0.001$ and $p<0.05$, respectively; n=3).

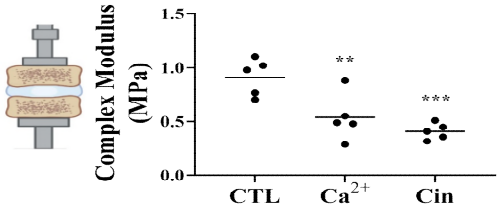

Fig 1. Effect of Ca^{2+} and CaSR agonist on IVD biomechanics. *IVDs were cultured in 1.0 (CTL), 2.5 mM Ca^{2+} or cinacalcet for 21 days. Complex modulus of IVD was performed using the MACH-1 mechanical testing system (Biomomentum, Laval, Quebec). ANOVA posthoc Dunnett's, **,p<0.01; ***,p<0.0001; n=5.*

DISCUSSION & CONCLUSIONS: Our results suggest that changes in the local concentrations of calcium and activation of CaSR affects matrix protein synthesis, calcification and IVD biomechanics. Ca^{2+} may be a contributing factor in IVD degeneration.

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IN VITRO EVALUATION OF OVINE IVD CELLS INTERACTIONS WITH A COLLAGEN/HYALURONIC ACID BIOMATERIAL INK: ON THE WAY TO A BIO-PRINTED IVD MODEL

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INTRODUCTION: Intervertebral disc (IVD) degeneration is a major cause of low back pain (LBP), affecting over 600 million individuals worldwide. Mechanisms associated with IVD degeneration remain poorly characterized, in part due to the lack of adequate IVD models. Mimicking the complex structure of an IVD, with its gelatinous core Nucleus Pulposus (NP) surrounded by the anisotropic lamellae of the Annulus Fibrosus (AF), represents a challenge for tissue engineers. To develop a bioprinted IVD model, we selected collagen type I (Col) and Tyramine-conjugated Hyaluronic Acid (THA) with different ratios, inspired by the native composition of the IVD, and we evaluated the interactions of ovine NP and AF cells with these bioinks.

METHODS: Bioinks with Col 4%-THA 6% (ratio 1:1.5) and Col 4% [w/v]-THA 1% [w/v] (ratio 1:0.25) were selected to mimic the NP and AF tissues, respectively. To determine the effect of THA concentration on the stiffness of the hydrogels, Young's modulus values were measured with a MicroTester equipment (CellScale). NP and AF cells were harvested from healthy discs of lambs (6 month-old), in collaboration with the Nantes ONIRIS veterinary school. We cultured NP and AF cells (up to 6 million per mL) in Col-THA 1:1.5 and Col-THA 1:0.25, respectively for up to 28 days. To characterize the interaction of the cells with the bioinks, cell viability was assessed with a Live/Dead assay and cell morphology was visualized by cytoskeletal actin labeling with fluorescent phalloidin-Alexa Fluor 647. Cell proliferation was assessed by a DNA assay (PicoGreen assay) and by a nucleoside incorporation assay during the cell cycle (EdU assay).

RESULTS: The two bioinks exhibited different mechanical properties with a Young's modulus of 2.0 kPa and 0.6 kPa for the 1:1.5 and 1:0.25 ratios, respectively. In addition, an increase in THA concentration increased the stiffness of the bioinks. NP and AF cells cultured in both formulations were alive for 28 days, confirming that the bioinks are cytocompatible. While actin labeling revealed a rounded morphology of AF and NP cells in Col-THA after 1 day of culture, the cells showed an elongated morphology afterwards, suggesting cell adhesion to the polymer network. While AF and NP cells proliferated both in 2D and when seeded on top of a Col-THA gel, no proliferation was observed in the bioinks, as shown by PicoGreen and EdU assays. Interestingly, we also evidenced a significant contraction of the bioink volume over time, as a function of the initial cell density, suggesting a cell-mediated remodeling of the bioink.

DISCUSSION & CONCLUSIONS: Col-THA bioinks are not cytotoxic, allow the encapsulation of ovine NP and AF cells, and promote their adhesion. Interestingly, cell seeding induces a bioink contraction upon culture, allowing to control the final dimensions of the bioprinted IVD construct.

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Activated neutrophils degrade cartilage endplates

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INTRODUCTION: Modic changes (MC) are painful vertebral endplate bone marrow (BM) lesions that occur around a degenerated intervertebral disc and colocalize with endplate damage. Neutrophils are activated in MC BM. Activated neutrophils mediate articular cartilage damage in rheumatoid arthritis. However, little is known about the role of neutrophils in disc tissue damage. The aim of this study was to discover the effects of activated neutrophils on cartilage endplate (CEP) composition.

METHODS: Circular 4 mm CEP biopsies from L4/5 and L5/S1 (n=6) were collected from patients undergoing anterior lumbar interbody fusion surgery at the University of California San Francisco. From each donor, 3 CEP biopsies were halved. One half-biopsy from each pair was exposed to either: 1) 0.75 U/ml collagenase P (positive control); 2) 25 mio/ml activated (100nM PMA, 3h, 37°C) neutrophil supernatant; or 3) 12.5 mio/ml activated neutrophil supernatant. The other half-biopsy from each pair was used as control and exposed to: 1) HBSS buffer; 2) 25 mio/ml non-activated neutrophil supernatant; or 3) 12.5 mio/ml non-activated neutrophil supernatant. Exposure supernatant and CEP tissues were collected after 18h and assayed for sulphated glycosaminoglycans (sGAG) and hydroxy-proline using a dimethylmethylene blue and chloramine-T assay. Relative sGAG / hydroxy-proline release from the biopsies were determined by normalizing sGAG / hydroxy-proline release from the half-biopsy exposed to activated neutrophil supernatants to that released from the half-biopsy exposed to the biopsy-specific control (100%). Relative release was tested against null hypothesis (μ0=100%) using a one sample t-test. P-values<0.05 were considered statistically significant.

RESULTS: Exposure of CEP tissues to neutrophil supernatants caused significant release of sGAG from the CEP tissues in a dose-dependent manner (25 mio/ml: 380.1% ± 177, p=0.012; 12.5 mio/ml: 123.7 % ± 22.3, p=0.048, positive control: 545.0 % ± 302.8, p=0.016) (Figure 1). In contrast, there was no significant effect of neutrophil supernatant on hydroxyproline release (25 mio/ml: 162 % ± 90.74, p=0.155; 12.5 mio/ml: 110.1 % ± 117.9, p=0.842; positive control: 2536 % ± 1321, p=0.006) (not shown).

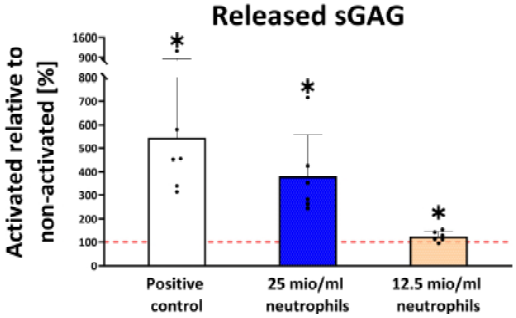

***Fig. 1**: Relative sGAG released from CEP tissues exposed to collagenase P (white bar) or neutrophil supernatants (blue/yellow bar). Relative sGAG release was compared with one sample t-test. *P<0.05, CEP donors: n=6. Relative sGAG release was 3.1-fold higher in CEPs exposed to supernatant from 25mio/ml neutrophils compared to 12.5mio/ml (P=0.022).*

DISCUSSION & CONCLUSIONS: We established a neutrophil-mediated CEP damage model using activated and non-activated blood neutrophils, and we showed that CEP exposure to activated neutrophil supernatant leads to a significant loss of proteoglycans. The relative sGAG release from CEPs following exposure to activated neutrophil supernatant for only 18 hours was similar to relative sGAG lost *in-vivo* over 20 years of natural ageing. This supports the hypothesis that activated neutrophils might exacerbate CEP damage present in MC. Importantly, CEP damage may not only be crucial in the etiology of MC as a facilitator of enhanced inflammatory disc/marrow crosstalk, but CEP damage coincides with increased nerve fibre density. Therefore, the present findings could have implications for treatment strategies to mitigate CEP damage in MC.

Fibre-reinforced bone dECM hydrogel scaffolds for differentiation of human mesenchymal stem cells towards hypertrophic chondrocytes

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INTRODUCTION: Articular cartilage (AC) consists of a layer of hyaline cartilage attached to subchondral bone through a layer of calcified cartilage; the latter improves adhesion of the former to bone, enhances transfer of mechanical properties and prevents unwanted bony ingrowth in the cartilage region.[1] However, this tissue is often omitted when designing scaffolds for regeneration of AC.
The goal of this study was to develop fibre-reinforced bone decellularized extracellular matrix (bdECM)-based hydrogels for enhanced hypertrophic differentiation of chondrogenicly-induced human bone marrow MSC (hMSC).

METHODS: The hydrogels were composed of either 10 mg/ml type I collagen (Col I) or bdECM mixed with alginate (30 mg/ml). 3D fibrous scaffolds were fabricated using PCL and PCL-based composites containing 25 wt% of β-TCP and FDM technology as the reinforcement of the hydrogels. The lay down patter of the scaffolds was designed as squares (architecture S), circles (architecture O) and as smaller squares deposited within bigger squares (architecture L). HMSC were encapsulated in the pre-gel at density of $20x10^6$/ml, infused into the 3D scaffolds and cultured in chondrogenic medium for 28 days. To measure cell viability, live/dead staining and MTS assay were performed. Secretion of cartilaginous matrix was evaluated by staining for safranin O, collagen type I, II and X. Moreover, quantification of GAGs was performed using 1,9-dimethylmethylene blue assay and collagen using total collagen assay. Additionally, alkaline phosphatase (ALP) activity was measured using colorimetric assay with para-nitrophenyl phosphate as a substrate.

RESULTS: Viability of hMSC measured as metabolic activity did not decrease upon culture; on the other hand, number of dead cells visualized by live/dead staining increased, suggesting cell proliferation. ALP activity was up to 54% and 79% higher in the PCL/βTCP-bdECM/Alg constructs than in the, respectively, PCL-ECM and S_COL. Moreover, more intense staining against type X collagen (Col X) was observed in the PCL/βTCP-bdECM/Alg constructs.

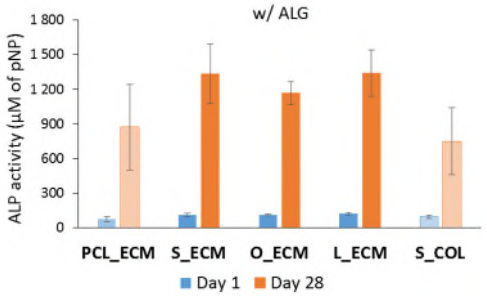

Fig. 1: Activity of ALP measured in MSC-laden dECM hydrogels.

Table 1. Composition of the hybrid constructs.

Hybrid construct	FDM-reinforcement	Hydrogel
PCL_ECM	PCL	bdECM/Alg
S_ECM	PCL-β-TCP	bdECM/Alg
O_ECM	PCL-β-TCP	bdECM/Alg
L_ECM	PCL-β-TCP	bdECM/Alg
S_COL	PCL-β-TCP	Col I/Alg

DISCUSSION & CONCLUSIONS: The increased ALP activity and enhanced expression of Col X, both being markers of hypertrophy of chondrocytes[2], suggests a synergistic effect of the bdECM and β-TCP on hypertrophic differentiation of chondrogenicly-primed hMSC. Therefore, hybrid constructs containing those materials could be used in TE of calcified cartilage. However, this layer has to be integrated with a layer of hyaline cartilage in order to reconstruct full thickness articular cartilage lesion.

ACKNOWLEDGEMENTS: Research was funded by POB Technologie Materiałowe of Warsaw University of Technology within the Excellence Initiative: Research University (IDUB) programme.

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TARGETED PROTEOMIC ANALYSIS TO EXPLORE THE ANTI-INFLAMMATORY EFFECTS OF NOTOCHORDAL-CELL DERIVED MATRIX

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INTRODUCTION: During maturation and degeneration of the intervertebral disc (IVD), the large and vacuolated notochordal cells (NCs) are replaced by chondrocyte-like nucleus pulposus cells (NPCs). In previous studies, porcine NC-derived matrix (NCM), containing matrix and biologic factors secreted by NCs, induced regenerative and anti-inflammatory effects on human, canine, and bovine NPCs *in vitro* and degenerated canine IVDs *in vivo*[2,3]. This indicates that NC-based approaches may be a promising therapeutic strategy to address lower back pain related to IVD degeneration[4]. The precise mechanism behind NCM remained elusive. Therefore, we aimed to determine the mode of action of NCM in the degenerative IVD environment.

METHODS: Canine and human NPCs were cultured in monolayers with and without NCM for 6, 24, and 72 hours, whereafter RT-qPCR was performed on inflammatory markers. At 72 hours, targeted proteomics was performed with DigiWest, a proprietary immunoassay technology which transfers Western Blot to a high-throughput bead-based microarray platform[5]. Lastly, immunohistochemistry was performed on *in vivo* canine IVDs injected with NCM at 6 months follow up[2].

RESULTS: RT-qPCR analysis indicated that NCM induced an initial inflammatory response after 6 hours, since *IL-6, IL-8,* and *COX2* mRNA expression was increased in human and canine NPCs. DigiWest analysis showed that NCM mainly induced changes in the Mitogen-activated protein kinase (MAPK) pathway after 72 hours of treatment, *i.e.* after the initial pro-inflammatory response. MAPK signalling (pERK1/2, pJNK, and pPKC expression), was mostly inhibited by NCM, whereas expression of proteins that are known to dephosphorylate MAPK key signaling molecules, DUSP1, 5, and 6, was increased in NCM-treated NPCs. Protein expression of KRT19, a healthy NP marker, was induced by 72 hours of NCM treatment. Confirming the DigiWest results, *in vivo* canine IVDs treated with intradiscal NCM injection demonstrated increased DUSP5 immunopositivity compared with degenerate controls at 6 months follow up. Furthermore, these IVDs showed increased immunopositivity for KRT19, PAX1, and FOXF1 after NCM injection indicating an improved NPC phenotype. Ongoing studies investigate expression of inflammation markers.

DISCUSSION & CONCLUSIONS: NCM induces an initial and short inflammatory response, but thereafter exerts prolonged anti-inflammatory effects inhibiting MAPK signalling. The latter leads to a reduced expression of inflammatory cytokines and improved nucleus pulposus cell phenotype.

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Modic change 1 bone marrow stromal cells increase neurite outgrowth

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INTRODUCTION:
Vertebral bone marrow lesions known as Modic type 1 changes (MC1), are a major cause of unspecific lower back pain. Pain may relate to higher innervation of MC1 endplates[1]. Blocking neo-innervation in MC1 could be a promising treatment approach for MC1. However, the neurotrophic mechanisms in MC1 are unknown. Bone marrow stromal cells (BMSC) can produce neurotrophic factors and are dysregulated in MC1[2]. The aim of the study was to identify if BMSC in MC1 support neo-innervation through release of neurotrophic factors.

METHODS: BMSC were isolated from vertebral bone marrow biopsies from a MC1 and an intra-patient control region of patients undergoing spinal fusion surgery (n=4+4). BMSC were co-cultured with the neuroblastoma cell line SH-SY5Y for 8 days. Neurite outgrowth from SH-SY5Y was quantified as a measure for neurotrophic activity.
Briefly, SH-SY5Y cells were pre-differentiated for 48h in B27 supplemented serum free media using retinoic acid (10µM). BMSC were seeded on cell culture inserts and SH-SY5Y cells in 6-well plates before being co-cultured for 8 days. Neurite outgrowths of SH-SY5Y were analyzed on a widefield microscope and with the Image J Ridge Detection Plugin. Average neurite length from three images was calculated. Fold-change to day 0 was calculated and compared between MC1 and intra-patient control using paired t-tests of log2 fold changes.
Thirty neurotrophic cytokines in the conditioned media were analyzed with C-Series Human Neuro Discovery Array C2 (RayBiotech Life, Inc.). Images were analyzed using Protein Array Analyzer Plugin for ImageJ. Relative signal intensities between MC1 and control were compared with paired t-tests.

RESULTS: After four days of co-culturing MC1 BMSC with SH-SY5Y cells, neurite outgrowth was significantly increased compared to intra-patient control (Figure 1). Cytokine array analysis revealed significantly more brain-derived neurotrophic factor (BDNF) (21.49 ± 4.81 vs. 29.53 ± 3.48, p = 0.021) and ciliary neurotrophic factor (CNTF) (9.28 ± 2.42 vs. 11.52 ± 2.68, p = 0.030) (Figure 2).

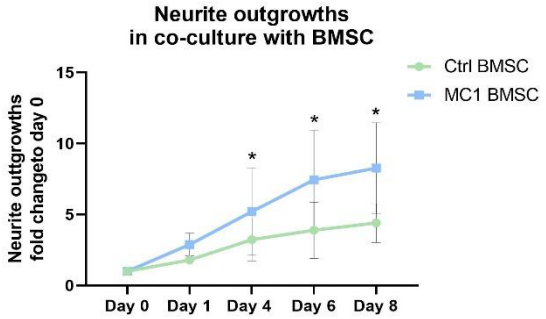

Figure 1. *Effect of BMSC on neurite outgrowth of SH-SY5Y during 8 days in co-culture normalized to day 0 (n=4). * p < 0.05.*

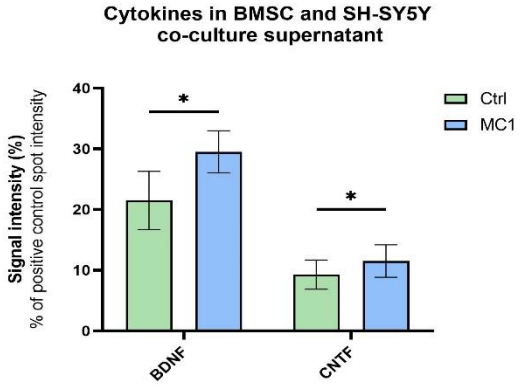

Figure 2. *Cytokine array spot intensities of BMSC and SH-SY5Y co-culture conditioned media (n=4). Spot intensities calculated as percentage of positive control spot. * p < 0.05.*

DISCUSSION & CONCLUSIONS: BMSC from MC1 have an increased neurotrophic activity, potentially facilitated by BDNF and CNTF. Therefore, BDNF and CNTF may represent interesting novel treatment approaches for MC1 that directly target pain mechanisms.

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Hyaluronan-Collagen Composite Bioink for the Printing of Nucleus Pulposus-Like Structures

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INTRODUCTION: Intervertebral disc (IVD) degeneration is still insufficiently understood and treated, despite its high prevalence and impact on patients and the healthcare system. This is in part due to models which are either oversimplistic or poorly representative of human tissue. Bioprinting offers precise and reproducible cell microenvironment control, suitable for recreating the disc's heterogeneous composition, structure, and mechanics in vitro. We developed a bioink for the fabrication of structures resembling the gel-like nucleus pulposus (NP) of the IVD, the first step towards a better in vitro disc model.

METHODS: Type I collagen (20 mg/ml) was combined with a tyramine derivative of hyaluronic acid (THA, 30 mg/ml) and gelled via pH increase, enzymatic oxidation, and visible light. First, a homogeneous soft gel was prepared via shear-induced fragmentation of collagen as it underwent fibrillogenesis, followed by enzymatic gelation of THA with hydrogen peroxide and horseradish peroxidase. This was extruded and further strengthened, after deposition, via exposure to green light in the presence of photoinitiator eosin Y. Gelation, elastic recovery, as well as the shear-, strain-, and shear rate-dependent responses were studied rheologically. Response to compression was evaluated with an incremental stress-relaxation test. Cell compatibility was assessed by embedding bovine NP cells in cast gels. Simple lattice-based structures were printed.

RESULTS: We observed good extrudability prior to light crosslinking, and a peak storage modulus of 4.6 kPa after exposure to green light (Fig. 1A & 2A). Flow was observed under high strains, followed by recovery of elasticity when it was decreased (Fig. 1B). Shear thinning of the material was also confirmed (Fig. 1C). Under compression, the light crosslinked gels exhibited a 5.3 kPa equilibrium linear-region modulus (Fig. 1D). Embedded NP cells demonstrated good viability and proliferation after 5 days of culture (Fig. 2B).

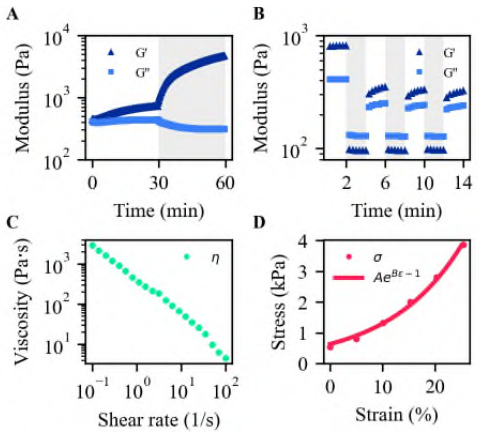

Fig. 1: Shear and compressive properties of the composite. A) Enzymatic and light (shaded) crosslinking. B) Elastic recovery of the initial soft gel during intervals of high/low strain. C) Shear thinning of the soft gel. D) Exponential model of the equilibrium compressive stress under progressively increasing strain, after full crosslinking.

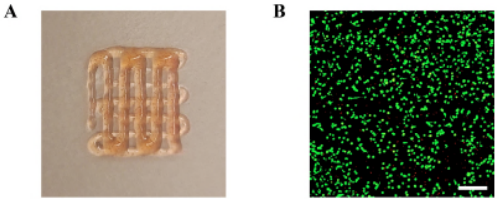

Fig. 2: A) 2-layer lattice extruded with a 25G needle. B) Bovine NP cells in cast gels, proliferating and demonstrating good viability at day 5. Stained with calcein AM (green, live) and ethidium homodimer-1 (red, dead).

DISCUSSION & CONCLUSIONS: We present a bioink with rheological and compressive properties within the range of healthy human NP. To our knowledge, this is also the first bioink simultaneously composed of biochemically suitable components representative of native NP, and approaching the high concentrations observed in tissue. This work brings us a step closer to better, reproducible, and representative 3D printed IVD models, and the promise of new insights into the treatment of disc degeneration.

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Intervertebral disc cells *in vivo* internalize bacteria: What is their potential influence?

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INTRODUCTION: Low back pain is the leading cause of morbidity worldwide. Its aetiology is complex and multifactorial with degeneration of the intervertebral disc (IVD) being a key cause. Causality between infection of the IVD and its degenerative process gained great interest. In a systematic review, Granville Smith *et al.*[1] (2021) identified 36 articles from 34 research studies investigating bacteria in human IVDs. In 27 studies bacteria were identified. To date, all the studies detecting bacteria found Gram-positive bacteria with *Cutibacterium acnes* being the most abundant. However, there are too few quantitative studies investigating bacterial infection to show whether bacteria are present *in vivo* or represent perioperative contamination. This study aims to investigate bacterial presence within the disc and their potential influence.

METHODS: Immunohistochemical staining for Gram-positive bacteria was performed on 100 human disc specimens to identify whether bacteria are present. Only samples with an intact annulus fibrosus (AF) were included to minimize the risk of contamination from adjacent tissue. Staining with a second anti Gram-positive bacteria antibody is ongoing. Nucleus pulposus (NP) cells in monolayer were stimulated with lipopolysaccharides (LPS) and peptidoglycans (PPG) (0.1-50 µg/ml) for 24, 48 and 72 hours. Factors associated with disc degeneration were analyzed by qPCR, ELISA and Luminex. Experiments with NP cells resuspended in 1.2 w/v alginate treated with 5 µg/ml and 50 µg/ml LPS respectively PPG are ongoing.

RESULTS: Gram-positive bacteria were internalized by human disc cells and influenced the cell and nuclei morphology (Fig.1, a). Luminex data supported by qPCR and ELISA results shows an increase in cytokines such as IL-1β, IL-6, IL-8, TNF-α, IFN-γ, IL-4, IL-17A, matrix degrading enzymes MMP1, MMP9 and neurotrophic and angiogenic factors NGF and VEGF in treated samples compared to the untreated control, with greatest effects seen following 50 µg/ml PPG stimulation (Fig.1, b).

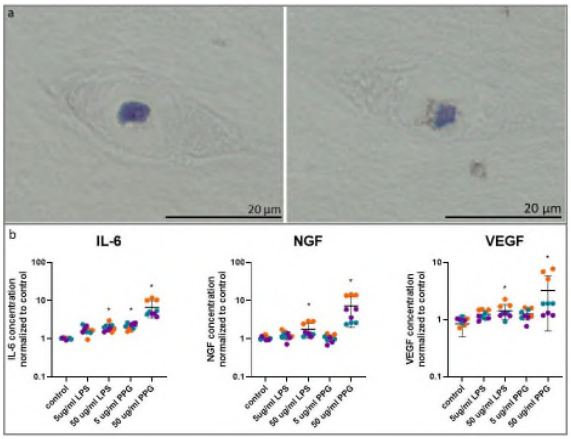

Figure 1 a) *Immunohistochemical staining for Gram-positive bacteria within human IVD tissue. Bacteria, stained brown, are internalized by the disc cells in vivo and with altered nuclei morphology observed in cells in presence of bacteria. b) Human NP cells in monolayer were stimulated for 48 hours with 5µg/ml or 50 µg/ml LPS or PPG respectively. Secretome was analyzed with Luminex, normalized to the untreated control of each donor (n=3). The biological replicates are indicated by different colors. Statistical analysis was performed using Kruskal-Wallis test (p≤0.05). *=P<0.05.*

DISCUSSION & CONCLUSIONS: This study demonstrated the presence of bacteria within human IVD tissue samples of patients undergoing spinal surgery. Whether the detected bacteria are representative for the disc microbiome or for an infection is unclear and will be further investigated. Exposure of human NP cells to the bacterial components LPS and PPG showed enhancement of several cytokines, chemokines, matrix degrading enzymes and other factors associated with disc degeneration. Demonstrating the capacity of both LPS and PPG to induce catabolism within disc cells.

ACKNOWLEDGEMENTS: This project received funds from the European Commission (H2020-MSCA-ITN-ETN-2020-955735).

REFRENCES: 1. Granville Smith, I. *et al.* Evidence for infection in intervertebral disc degeneration: a systematic review. *European spine journal* 1 (2021).

Intracellular trafficking of bioreducible poly(amidoamine) nanoparticles for mRNA delivery

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INTRODUCTION: Disulfide bond-poly(amidoamine) (SS-PAA) is a bioreducible polymer that can be used as a carrier for therapeutic mRNA delivery in the treatment of osteoarthritis (OA). SS-PAA nanoparticles (NPs) have a cationic core which allows for condensation of nucleic acids[1], and payload release is mediated by intracellular glutathione (GSH) through degradation of the redox-sensitive disulfide bridges from the polymer[2]. Successful transfection of resident cells from the joint was shown previously[3] but cell uptake, endosomal escape and nanoparticle degradation were not studied in detail. PEG-coated NPs can additionally enhance colloidal stability and reduce immunogenicity, but may also impact electrostatic interactions with cell membrane during uptake. Therefore, we aim to obtain further insight in the intracellular trafficking of SS-PAA NPs in C28/I2 human chondrocytes to optimize delivery efficiency of nucleic acids.

METHODS: Cy5-labelled SS-PAA NPs (PEG-coated or uncoated) were evaluated regarding (1) NP internalization efficiency, (2) endosomal escape kinetics and (3) payload release over time. To find the optimal formulation of SS-PAA NPs for delivery in C28/I2 cells, we investigated transfection efficiency and cytotoxicity using a GFP reporter, with different mRNA-nanoparticle ratios (1:12.5, 1:25 and 1:50 w/w) and mRNA dosages (0.8 to 3.2 µg/mL). Finally, we explored the effects of endosomal escape enhancers and modulators of the intracellular reducing environment on the behaviour of SS-PAAs NPs.

RESULTS: Cell uptake was higher for uncoated SS-PAA NPs, although virtually all cells showed internalization (Fig. 1) of both coated and uncoated NPs. Endosomal entrapment of NPs decreased over time during 48h after transfection. The optimal mRNA-NP formulation ratios were 1:25 and 1:50 w/w, at a dosage of 3.2 µg/mL mRNA, with transfection levels up to 80% and without toxic effects. Furthermore, cell treatment with calcein at a self-quenching concentration of 3 mM, after incubation with chloroquine alone or coated SS-PPA NPs, made possible to visualize endosomal escape 4h after transfection.

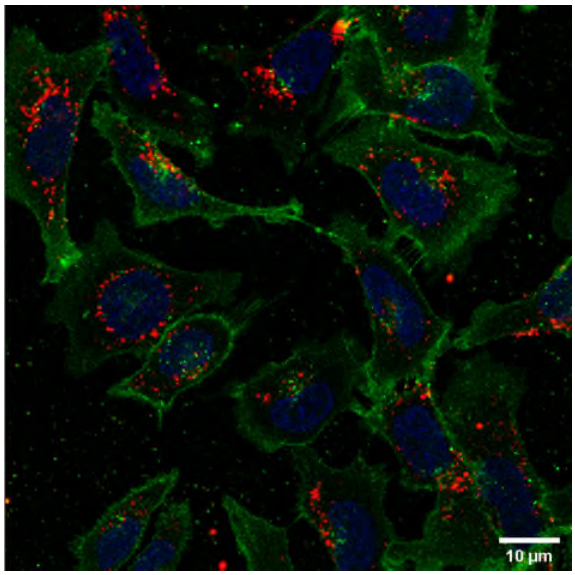

Fig. 1: Uptake efficiency in C28/I2 cells after 3h incubation with Cy5-labeled coated SS-PAA NPs (in red); CellMask-stained plasma membrane is colored in green.

DISCUSSION & CONCLUSIONS: This study provides insight into the cell uptake and endosomal escape in C28/I2 human chondrocytes of SS-PAA NPs. The high transfection efficiency of this system, without toxicity, paves the way for future 3D cultures and *in vivo* studies in OA treatment.

ACKNOWLEDGEMENTS: This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 955335.

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Detergent-free decellularization yields a regenerative biomaterial for intervertebral disc regeneration

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INTRODUCTION: Decellularized scaffolds have been gaining increasing attention in intervertebral disc (IVD) regenerative approaches. Published protocols use detergents to lyse cells for DNA removal. However, detergents are reported to affect tissue composition and later cell infiltration into decellularized tissues.[1] We therefore aimed to create a detergent-free protocol for porcine notochordal cell-derived matrix (NCM). NCM has been shown to exert proliferative, anabolic and anti-inflammatory effects onto nucleus pulposus cells (NPCs).[2] A decellularized NCM is there-fore promising for use in degenerated IVDs.

METHODS: NCM was lyophilized for >72 h for cell lysis, followed by a nuclease treatment, brief washout period, second lyophilization and pulverization to yield decellularized NCM (dNCM). Removal of DNA was tested with the Qubit assay. Compositional changes were evaluated using DMMB, hydroxyproline and BCA assays for GAG, collagen and total protein, respectively. The remaining bioactivity of dNCM was tested by adding it to medium of bovine NPCs in alginate bead culture for 4 weeks. Il-1β was used to simulate inflammatory conditions. Beads were analysed for GAG and collagen deposition by NPCs, and anti-inflammatory action was investigated on RNA level. Lastly, we investigate dNCM's suitability for 1) disc swelling pressure restoration and 2) for cell delivery by LIVE/DEAD staining.

RESULTS: We found a DNA-content reduction of 93.9±3.1% to ≈85 ng/mg tissue in dNCM. No significant loss in GAG- and HYP-content was seen, while 43% of protein-content remained within the dNCM.
NCM-treatment significantly increased DNA content/alginate bead to circa threefold the amount of non-treated cells, and double of dNCM-treated NPCs. dNCM stimulated the same amount of sGAG production as NCM, both groups being significantly higher than non-treated cells. dNCM-treated cells exhibited less collagen II deposition than NCM-treated groups, but slightly more than the base medium group. Collagen I was mostly present in the base medium group and was less visible in NCM- and dNCM-treated groups.
NCM-treated groups exhibited significantly lower *IL-8* gene expression than dNCM treated cells. NCM significantly reduced *IL-1β* and *TNFα* gene expression compared to untreated groups. A significant inflammatory effect of dNCM was seen only in combination with IL-1β-stimulation for *IL-6* gene expression after 28 days compared to unstimulated non-treated cells.
Reconstituted dNCM swelled up to 300% in free swelling conditions. 10% w/v dNCM-suspension had a storage modulus of ≈100-200 Pa, was shear-thinning, and retained viability of injected MSCs >70% 24 h post-injection.

DISCUSSION & CONCLUSIONS: We successfully established a detergent-free decellularization protocol for porcine NCM, which preserves its anabolic effects, and partly retains its mitogenic potential, but may lose its anti-inflammatory properties onto NPCs. We hypothesize that altered protein composition after decellularization explain the differential biological effects onto NPCs. We demonstrate that reconstituted dNCM is a soft & swelling material, usable as a cell-vehicle for injection into the IVD.

ACKNOWLEDGEMENTS: This project is funded by the European Union's Horizon 2020 research and innovation programme (grant agreement No 825925) as a part of the iPSpine project. MAT is financially supported by the Dutch Arthritis Society (LLP22).

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Serum Mass Spectrum Analysis of subjects with and without Modic Changes

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INTRODUCTION: Modic changes (MC) are vertebral bone marrow lesions that are associated with low back pain (LBP), one of the leading causes of disability. In the MRI, the inter observer-reliability is low. The aim of our study is to find a serum biomarker indicating the presence of MCs in patients, to add to the diagnostic.

METHODS: 103 serum samples of participants of the Northern Finland Birth cohort 1966 (1) with either "MC" (n = 50) or "no MC" (n=53) were subjected to a sequential window acquisition of all theoretical mass spectra (SWATH-MS) analysis with a stringent 1% false-discovery-rate (FDR) peptide filter. Normality of the resulting data was tested with the Shapiro-Wilk test. Differential expression of proteins between "MC" and "no MC" was analysed with the Mann-Whitney-U-test and FDR. We adjusted for the possible confounders BMI and sex with linear regression. Receiver operating characteristic curves (ROCs) of individual proteins were calculated to assess prediction power as biomarker. For all statistics, we used the software R.

Table 1. Basic characteristics of the serum donors. MC n=50, no MC n=53. BMI in kg/m^{2}with standard deviation. LBP: Percentage of subjects with LBP

	Sex (% female)	*BMI (Mean, STD)*	*LBP (%)*
MC	66	26.04, 3.72	50
no MC	62.26	25.51, 4.18	49.05

RESULTS: Sex, BMI, and percentage of subjects with LBP is shown in Table 1. All subjects were 47 years old. The SWATH-MS analysis identified 450 proteins. For 19 proteins, unadjusted Mann-Whitney-U-test showed a different expression between "MC" and "no MC" ($p < 0.05$). After FDR adjustment, no significances were found. Five peptides were parts of immunoglobulins, the others are shown in Table 2, together with the sex- and BMI-adjusted p-value of the linear regression and the area under the curve (AUC) of the ROCs.

Table 2. Result of Mann-Whitney-U-Test, proteins with p-Value < 0.05, BMI and sex-adjusted p-value, AUC of the ROC

Protein	p-value	adj. p-value	AUC
APOD	0.005	0.046	0.745
LRC8B	0.006	0.099	0.689
MBL2	0.008	0.049	0.662
HBB	0.009	0.178	0.678
APOC3	0.012	0.635	0.646
MMRN1	0.013	0.065	0.574
PLCH1	0.022	0.025	0.614
ENPL	0.024	0.207	0.622
FGD6	0.036	0.089	0.594
TENX	0.038	0.633	0.592
FETUB	0.038	0.117	0.654
FCGBP	0.042	0.307	0.626
AMPN	0.045	0.342	0.626
BTD	0.048	0.446	0.655

DISCUSSION & CONCLUSIONS: We identified proteins with altered abundance in serum of subjects with MC, although high FDR suggests, that the findings might be due to chance. Linear regression revealed, that adjusted for sex and BMI, APOD, MBL2, and PLCH1 had a significantly different expression. The AUC of APOD of 0.745 suggests, that is a potential biomarker for MC. A subgroup analysis of subjects with LBP shows similar results.

REFERENCES: (1) Rantakallio P. Paediatr Perinat Epidemiol. 1988 Jan;2(1):59-88.

A new generation of multiaxial spine bioreactor for advanced studies of intervertebral disc degeneration and repair

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INTRODUCTION: The course of intervertebral disc (IVD) degeneration remains the main riddle for the scientific community studying this commonly prevalent disease. Thereat high mechanical load on the spine is considered a major trigger of IVD degeneration. After more than a decade of research in our laboratory using uniaxial bioreactors for simulation of loads on *ex vivo* organ IVD models, we are now developing a new generation of bioreactors with integrated 6 degrees of freedom (6 DOF) and chamber for IVD culture in sterile biological conditions. The multiaxial bioreactor should mimic the natural spine load more accurately, thus reproducing near-reality *in vitro* degeneration models for studying IVD disease and repair strategies.

METHODS: The multiaxial bioreactor consists of a 6 DOF mechanical system, namely hexapod, customized force controller, force sensor and software for the load control. The chamber, specimen holder and mechanical interfaces were custom-made and user friendly-designed for easy assembly and culture in sterile conditions (Fig. 1b, c). A tubing system for medium exchange was introduced outside the chamber. An *ex vivo* bovine organ model was adapted by retaining 7 mm of bone on each side of the caudal IVD, on which a cross and a central hole for nutrient access through the cartilaginous endplate were machined (Fig. 1b). The model is assembled with a specimen holder via a counter cross of the same dimensions and side screws tightened onto the bone. The system was validated for stability and sterility after 9 days of dynamic compression loading under physiological conditions.

RESULTS: The concept of a multiaxial bioreactor was successfully transferred from the sketch to laboratory conditions. Validation tests in compression loading have demonstrated the ability of the holder to provide a tight grip on the IVD specimen, so as the chamber to maintain the sterility in a long term IVD culture.

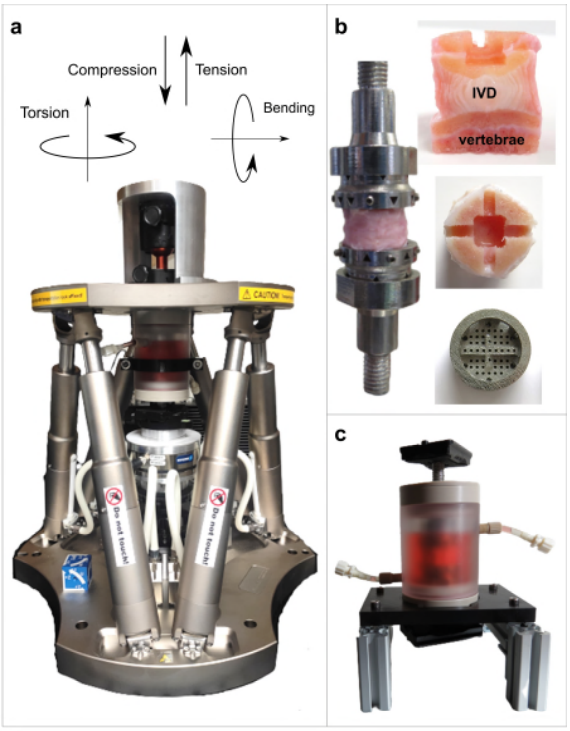

Fig. 1: A new generation of multiaxial bioreactor for in vitro mechanical loading of IVDs: a) bioreactor with 6 DOF mechanical system, b) individual and assembled ex vivo bovine IVD organ model and specimen holder, c) chamber for culture in sterile conditions with side tubing for medium exchange.

DISCUSSION & CONCLUSIONS: We have developed a new generation of bioreactors for advanced simulation of spine loads. Proof-of-concept experiments are currently ongoing to demonstrate the reproducibility of the system in a long-term IVD culture under physiological and degenerative compressive loading conditions. Protocols for 6 DOF loading have been computationally simulated and will be tested under multiaxial loading.

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Development of a bone remodelling in vitro model

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INTRODUCTION: Co-culture models of osteoblasts and osteoclasts offer an alternative to animal testing and are considered to have the potential to improve the drug development processes for bone related diseases in the future. Here, we report the development of a 2D in vitro model of the bone remodelling process containing osteoblast- and osteoclast-like cells.

METHODS: A collagen-supplemented ten times simulated body fluid like solution (10x SBF collagen) was used to deposit a calcium phosphate collagen composite coating on conventional tissue culture plastic (TCP). Human bone marrow derived osteoblast- (OB) and osteoclast-like cells (OC) were co-cultured on coated TCP and their remodelling activity evaluated using calcein green staining and via image analysis tools (ImageJ).

RESULTS: The SEM picture in Figure 1 A shows the morphology of the deposited calcium phosphate collagen composite on a coated polymer substrate.

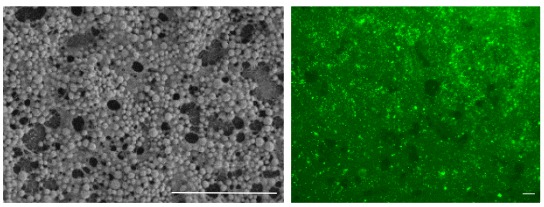

Figure 1: (A) SEM image of 10xSBF collagen coated polymer surface. Scale bar 10 µm. (B) Fluorescence image of a calcein green stained 10xSBF collagen coated TCP seeded with osteoblast- and osteoclast-like cells. Scale bar 100 µm.

The fluorescence image in Figure 1 B shows the co-culture of osteoblast-and osteoclast-like cells on 10xSBF collagen coated TCP. The remodelling activity of cells is visualised using the fluorescent dye calcein green.

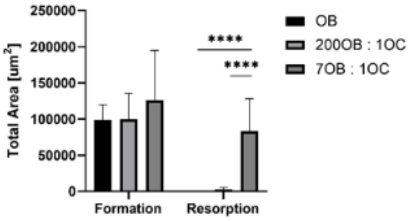

Figure 2: Quantification of cell remodelling activity in co-cultures after one week of co-culture.

Cellular mineral deposits are identified as bright green spots while the mineral coating appears in medium intensity. Osteoclastic resorption sites can be detected as non-fluorescent areas. The cellular remodelling activity of two OB to OCs ratios in co-culture was quantified using image analysis tools (Figure 2).

Calcein staining of the 10xSBF collagen coating also allows for live imaging of osteoclast activity (Figure 3).

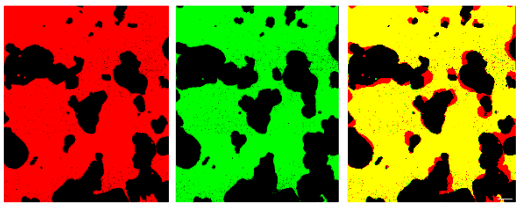

Figure 3 (A) Remaining coating at the start of imaging period. (B) Remaining coating at the end of imaging period. (C) Overlay image shows remaining coating in yellow and resorbed areas shown in red. Scale bar 100 µm.

Time-lapse recording of the osteoclast allowed to analyse the dynamics of osteoclast mediated activity and to collect cell specific data sets **DISCUSSION & CONCLUSIONS:** The developed model could be used in the future for drug screening purposes of osteoporosis drug candidates.

ACKNOWLEDGEMENTS: This research was funded by (i) the UKEPSRC Centre for 3D Printing (grant number EP/L01534X/1), (ii) the AO Research Institute Davos, Switzerland, and (iii) Newcastle University, UK

Bone Marrow Progenitor Cell delivery within a Thermally Triggered Hydrogel Regenerates Degenerate Nucleus Pulposus Tissue.

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INTRODUCTION: Intervertebral disc (IVD) degeneration is a major cause of low back pain (LBP). We have reported a hydrogel system (NPgel)[1,2], which following injection into bovine NP explants, integrates with nucleus pulposus (NP) tissue and promotes NP cell differentiation of delivered bone marrow progenitor cells (BMPCs) without the need for additional growth factors. Here, we investigated the injection of NPgel (+/- BMPCs) into human NP explants; the capacity of the delivered NPgel and BMPCs to halt catabolic processes and regenerate NP matrix was assessed to ascertain the future clinical success of this therapy.

METHODS: Human BMPCs were extracted from the femoral heads of patients undergoing hip replacement surgery for the treatment of osteoarthritis. Extracted BMPCs were incorporated into liquid NPgel and injected into naturally degenerate human NP explants, which were obtained from patients (n = 7) following microdiscectomy surgery. Explants were cultured for 6 weeks under hypoxic conditions (5% pO_2), semi-constrained within plastic rings, to limit tissue swelling. Histological and immunohistochemical analysis was performed to investigate altered matrix synthesis and catabolic protein expression.

RESULTS: Injected NPgel integrated with human degenerate NP tissue and native NP cells migrated into areas of the integrated NPgel. Histological staining indicated an increase in collagen and glycosaminoglycan (GAG) deposition within explants injected with NPgel (+/- BMPCs), compared to controls. Immunohistochemical staining confirmed the number of cells expressing collagen type II and aggrecan were significantly increased in NP explants injected with NPgel + BMPCs ($p \leq 0.05$) (Figure 1a & b). Conversely, the number of native NP cells expressing catabolic proteins such as MMP3, ADAMTS4 and IL-1β was reduced in explants injected with NPgel (+/- BMPCs), when compared to degenerate controls ($p \leq 0.05$) (Figure 1c, e, f). The expression of MMP13 also showed a downward trend in NPgel injected explants compared to controls, yet this was not significant (Figure 1d).

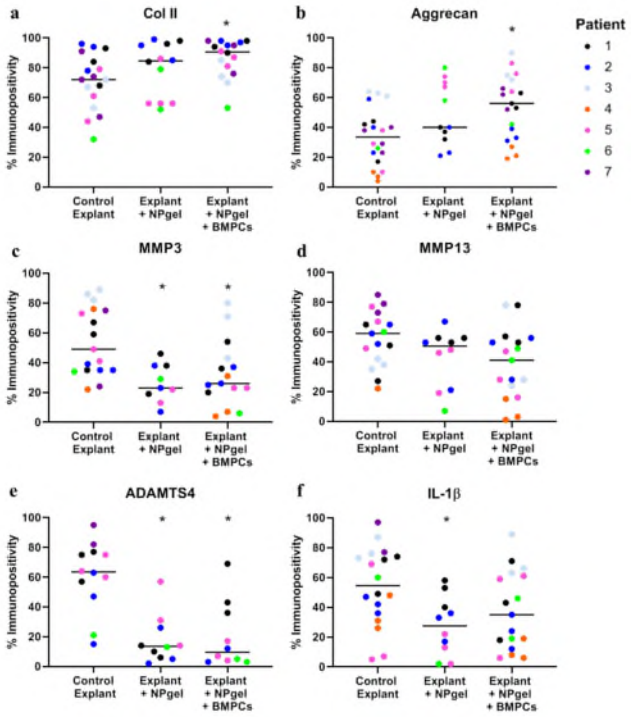

Figure 1. Immunohistochemical analysis of (a) collagen type II, (b) aggrecan, (c) MMP3, (d) MMP13, (e) ADAMTS4 and (f) IL-1β in non-injected control, NPgel-injected and NPgel + BMPC-injected human NP tissue explants following 6w culture at 5% pO_2. * ≤ 0.05.

DISCUSSION & CONCLUSIONS: In agreement with our previous findings[2] NPgel was sufficient alone to induce NP cell differentiation of BMPCs following injection into NP tissue explants under standard culture conditions without the need for additional growth factors. In addition we have shown that the injection of NPgel (+/- BMPCs) increases the expression of extracellular matrix proteins collagen type II and aggrecan, with both playing a vital role in maintaining NP tissue integrity. Whereas the expression of catabolic proteins such as MMP3, ADAMTS4 and IL-1β expression in NP explants injected with NPgel (+/- BMPCs) were all reduced. This potentially highlights that NPgel, with incorporated BMPCs, has the potential to regenerate the NP, provide mechanical support, whilst also reducing the catabolic phenotype of degenerate NP cells, which indicates its promising future use as a treatment strategy for IVD degeneration.

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Influence of traction on intervertebral disc mechanobiology: preliminary results in a bovine organ culture

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INTRODUCTION: Low back pain (LBP) is a major cause of long-term disability in adults worldwide and it is frequently attributed to intervertebral disc (IVD) degeneration. So far, no consensus has been reached regarding appropriate treatment and LBP management outcomes remain disappointing.
Spine unloading or traction protocols are common non-surgical approaches to treat LBP. These treatments are widely used and result in pain relief, decreased disability or reduced need for surgery. However, the underlying molecular mechanisms -i.e. the IVD unloading mechanobiology- have not yet been studied. The aim of this pilot study was to test a large animal organ culture IVD unloading set-up and to evaluate the impact of unloading on the mechanobiology of healthy discs.

METHODS: Bovine tail discs (diameter 16,0mm ± 1,1mm), including the endplates, were isolated from three different tails (cow age: 8months ± 4months and cow mass: 148,3kg ± 41,9kg) and prepared for culture. The three discs of each tail were distributed over the day0, physiological and traction groups. The day0 samples were directly processed while the six other discs were embedded in biocompatible resin -leaving the cartilage endplate free to permit nutrient diffusion- and fitted in the loading holder. The embedded discs were then loaded for 3 days. The physiologically loaded discs were compressed according to a previously established physiological loading protocol (2h/day, 0.2Hz) [1], whereas the discs in the traction group were subjected to cyclic traction loading (2h/day, 0.2Hz) corresponding to 30% of the animal body weight corrected for organ culture. These discs were processed on the day following the last loading day. In all groups, disc height, water content and gene expression in nucleus pulposus (NP), inner (AFi) and outer annulus fibrosus (AFo) were analysed.

RESULTS: The height of physiologically loaded samples decreased after each compression session whereas the height of the discs undergoing traction loading increased with each session.
Correspondingly, a higher water content in all zones of the discs of the traction group compared to the physiological group was found at the end of loading.
Compared to the physiologically loaded discs, the gene expression of the traction loaded discs presented with decreased COL1, MMP3 and ADAMTS5 but increased COL2 expression. These responses were zone-specific, with the AFo cells being most responsive to loading. ACAN was higher expressed upon traction than upon physiological loading in the AFo, on the contrary of the NP and AFi where ACAN was lower expressed for traction loading.

DISCUSSION & CONCLUSIONS: We can conclude that we could effectively unload discs in a large animal culture model as well as modulate the water content and the gene expression responses: **traction loading facilitated water uptake by the IVD** as reflected in the higher water content in the disc after traction compared to compression.
Furthermore, our preliminary gene expression data indicate that compared to physiological loading, traction tended to decrease catabolic markers and promote anabolic markers of the NP phenotype.
This work opens exciting new avenues for defining regenerative protocols for degenerated discs and opens the door to an evidence-based improvement of clinical spine traction protocols and LBP management overall.

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Synergistic Extracellular Vesicle delivery of Transcription Factors *FOXF1* & *Brachyury* to Human Nucleus Pulposus Cells

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INTRODUCTION: Intervertebral disc (IVD) degeneration is a major contributor of low back pain (LBP), yet current interventions do not address the underlying pathology[1,2]. Biological therapies such viral gene delivery and cell-based therapies are hampered by limitations such as immunogenic responses and cell survivability. Thus, minimally invasive non-viral gene delivery methods such as engineered extracellular vesicles (EVs) are promising due to their low-immunogenicity, biocompatibility, and tuning capabilities. We have previously shown the reprogramming potential of transcription factors *FOXF1* or Brachyury (*T*) in diseased human nucleus pulposus (NP) cells via bulk electroporation [3,4]. Thus, in this study we combined *FOXF1* and *T* to investigate their synergistic effects on diseased human NP cells in a 3D culture system.

METHODS: *FOXF1*, *T* and SHAM (pCMV6) expression plasmids were expanded, isolated, and transfected into autopsy NP cells with engineered EVs produced as previously published[4] to generate allogenic EVs. Diseased human NP cells (N=6) were isolated from male or female patients undergoing spine surgery with IRB approval. Cells were then expanded and encapsulated in 2% agarose constructs, which were subsequently injected with the engineered EVs (~10^8 particles/gel). Dependent variables were assessed at 2- and 8-weeks post treatment and included cell viability, gene expression, and Collagen and Glycosaminoglycan content.

RESULTS: Significant increases in both *FOXF1* and *T* expression was observed in synergy groups (*FOXF1+T*) compared to SHAM and *FOXF1* or *T* treatments alone (Fig1). Catabolic and inflammatory genes were also assessed with differences in individual patient responses varying with both sex and age. Collagen content was increased in all treatment groups compared to SHAM while GAG accumulation was found increased in synergy groups.

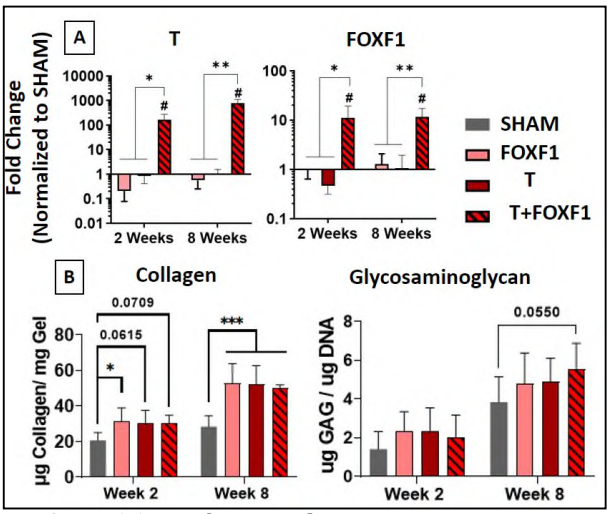

Fig. 1: *(A) Brachyury and FOXF1 gene expression at 2 and 8 weeks post treatment with T+FOXF1-EVs, T-EVs, FOXF1-EVs, and SHAM. B) Collagen and Glycosaminoglycan protein expression in respective groups. (*=p<0.05, **=p<0.01, ***=p<0.001)*

DISCUSSION & CONCLUSIONS: These results demonstrate successful delivery of developmental transcription factors *T* and *FOXF1* via allogenic engineered EVs into diseased human patient NP cells with synergistic effects of *FOXF1+T* and potential sex/age specific responses. This work highlights the potential of *FOXF1+T* in synergy to enhance a healthy NP phenotype as evidenced by increasing phenotypic markers and extracellular matrix content compared to single factors alone. Of clinical significance, the use of engineered EVs to deliver factors in synergy has high potential as a minimally invasive, non-addictive therapy for discogenic back pain.

ACKNOWLEDGEMENTS: G Gunsch for her technical assistance and our funding sources: NIH R61 AR076786, ON/ORS Kickstarter, and OSU CCTS Pilot Award.

REFERENCES: [1]Katz, J. et al, 2006; [2]Freemont A.J.et al, 2009; [3]Tang et al, 2019; [4]Tang et al 2021. *indicates equal contribution. § indicates joint corresponding/senior author

Notochordal Cell Matrix-based Hydrogel Enhances Notochordal Cell Phenotype

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INTRODUCTION: During development, the core of the intervertebral disc (IVD), the *nucleus pulposus* (NP) is populated by notochordal cells (NCs). They are the main producers of extracellular matrix (ECM) components, such as GAGs, allowing high-water intake within the NP, a key factor to support the mechanical loading of the spine. However, NCs eventually evolve towards a chondrocyte-like phenotype with altered ECM composition and decreased swelling capacity of the NP. Hereby, the IVD becomes more prone to degeneration eventually leading to IVD-related lower back pain. Our aim is to develop a therapeutical strategy, by creating a notochordal cell matrix (NCM)-based hydrogel to serve as an injectable vehicle and niche for iPS cells to differentiate towards an NC-like phenotype (iPS-NLCs) and promote IVD regeneration. As such, our primary objective is to fabricate and characterise a NCM-based hydrogel, using poly-ethylene glycol as a crosslinker, that protects and supports the NC phenotype.

METHODS: A NCM-based hydrogel was designed following a chemical crosslinking reaction. Hydrogel formulations (n=18), varying on their NCM and crosslinker concentrations, were initially tested. Those that successfully generated a hydrogel construct were further characterised on their mechanical, gelation and swelling properties, narrowing down to two final hydrogels. Hereafter, to test the maintenance of the NC phenotype, porcine NCs were encapsulated and cultured for 2 weeks in these two formulations. Furthermore, mesendoderm progenitor cells (MEPCs; obtained at day 2 of the iPS-NLC differentiation protocol[1] were encapsulated in the hydrogel and cultured until day 7 and accessed for cell viability (live-dead staining), DNA content (Qubit) and cell morphology (H&E staining). The expression of phenotypic and ECM related markers was evaluated by immuno-histochemistry for pNCs and iPS-NLCs.

RESULTS: All cell types remained viable after being encapsulated within the hydrogel. pNCs showed good survival, maintenance of morphology and phenotype (expression of T, PAX1, Cav-1, FOXF1, panKRT) (Fig) and healthy ECM deposition (Col II and ACAN) after two weeks in culture. Initial viability tests also showed good iPS-NLC survival, and early differentiation towards an NLC phenotype (positive expression of T and FOXA2) was observed at day 7 of the differentiation.

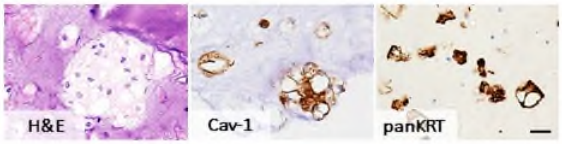

Fig. H&E, Caveolin-1 and pan-cytokeratin stainings of porcine NCs in NCM hydrogel show the presence of vacuoles after 14 days in culture. Scale bar = 20µm.

DISCUSSION & CONCLUSIONS: A naturally inspired hydrogel based on NCM, the ECM isolated from NC-rich NP tissue, has been developed. Tests with pNCs and iPS-NLCs encapsulated in the NCM hydrogel served as a proof of concept that this biomaterial protects the NC phenotype induces healthy ECM production and promotes iPS-NLC differentiation.

ACKNOWLEDGEMENTS: This project (iPSpine) has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 825925. This work was also supported by Science Foundation Ireland (SFI) and the European Regional Development Fund (Grant Number 13/RC/2073_P2).

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Development of a vascularised human synovium-on-a-chip with biomechanical loading

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INTRODUCTION: Synovial inflammation is a common feature of arthritic disease often evident before visible cartilage degradation. Synoviocytes secrete cytokines, inflammatory mediators and degradative enzymes that contribute to cartilage destruction [1]. Targeting the synovium therefore holds great potential for disease prevention, however new, more predictive *in vitro* models are needed for drug testing. Synovial function is regulated by physiological biomechanical loading [2], the response to which is modulated by inflammation [3]. The current study presents a novel vascularised human synovium-on-a-chip model with biomechanical loading and cytokine stimulation.

METHODS: The synovium-on-a-chip was generated using a 2-channel, microfluidic PDMS organ-chip (Emulate Inc). Patient-derived human fibroblast-like synoviocytes (FLS) were cultured in one channel, with human umbilical vein endothelial cells (HUVEC) in a second channel. The two channels were connected by a flexible, porous membrane (Fig1A) and subjected to mechanical stimulation in the form of fluid shear (0.007 dyn/cm^2), and cyclic tensile strain (0-12% strain, 0.2 Hz, 2 h/day). Extracellular matrix coatings were optimized, and cell and cytoskeletal morphology and matrix deposition visualized. Synovial inflammation was initiated by stimulation with interleukin 1β (IL-1β, 1ng/ml) and the effects on barrier function (3 kDa and 70 kDa FITC-Dextran) and interleukin 6 release (ELISA) monitored.

RESULTS: FLS cultured in the organ-chip on collagen type 1 remained viable with a loose actin network (Fig 1B) and significant fibronectin-1 deposition (not shown). HUVEC formed a compact monolayer forming a micro-vessel within the chip and exhibited tight junction formation (Fig 1C). Together a robust barrier was formed which was not significantly altered by stretch (Fig1 C-D). IL-1β treatment increased barrier permeability only in chips subjected to stretch (Fig 1C-D). This was accompanied by increased expression of catabolic enzymes such as MMP-1, 3 and 13 (not shown) and release of IL-6 (Fig 1F).

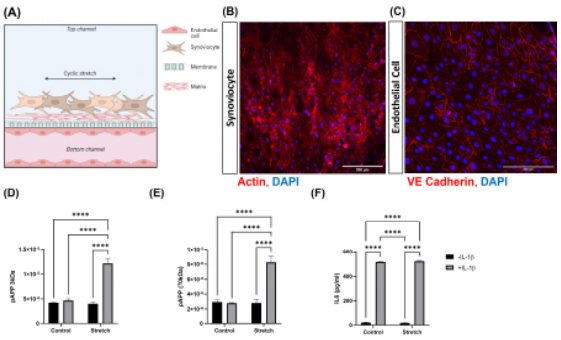

Fig. 1: Synovium-on-a-chip model: (A) Cross sectional schematic. Confocal images of (B) FLS and (C) HUVEC cultured in the chip. Barrier permeability to (D) 3kDa and (E) 70kDa dextran and (F) IL-6 release all -/+IL-1β and -/+ cyclic tensile stretch in the chip.

DISCUSSION & CONCLUSIONS:
Organ-on-a-chip technology represents an opportunity to transform the understanding of human disease processes and to revolutionize the drug development pipeline through more accurately prediction of human physiology in health and disease. This optimised human synovium-on-a-chip model is the first of its kind to integrate essential physiological mechanical stimulation combined with a vascular channel for drug delivery and immune cell recruitment. On-going studies are further characterising the cytokine response and using this model to understand mechanobiology and disease mechanisms.

ACKNOWLEDGEMENTS: TH: Orthopaedic Institute Ltd, MK, HS &CT: Queen Mary+Emulate Organs-on-Chips Centre www.cpm.qmul.ac.uk/emulate/

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"A cell-free therapeutic strategy: the role of Wharton's Jelly MSCs derived exosomes in intervertebral disc regeneration"

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INTRODUCTION:

Intervertebral disc degeneration (IDD) affects more than 80% of the population. Current approaches to treat IDD are based on conservative or surgical procedures relieving the pain[1]. The transplantation of mesenchymal stem cells (MSCs) has arisen as a promising therapy but recent studies have demonstrated as a particularly hostile microenvironment in intervertebral disc (IVD) may compromise survival rate of implanted cells[2]. Interestingly, studies reported as paracrine factors as extracellular vesicles released by MSCs may regenerate IVD[3]. The aim of this study is to investigate the therapeutic effects of Wharton's Jelly MSCs derived exosomes on human nucleus pulposus cells (hNPCs) in an *in vitro* 3D culture model.

METHODS:

Exosomes were isolated by tangent filtration of Wharton's Jelly derived MSCs conditioned media. The exosomes were quantified by BCA, exosomal morphology was characterized by TEM, WB analysis was performed for markers expression and NTA for vesicular size and quantification. Confocal microscopy was used to detect exosomes PKH26-labeled uptake in hNPCs. hNPCs were treated with growth medium and MSCs-exosomes (exos) at 10 μg/ml, 50 μg/ml and 100 μg/ml. Cell proliferation was assessed by Trypan blue assay at different time points. After 24 hours, hNPCs viability were evaluated by an Live/Dead staining. Nitrate and glycosaminoglycan (GAG) quantification were evaluated through Griess and DMBB assays respectively. In addition, hNPCs in alginate beads paraffin-embedded were stained for histological analysis to gauge extracellular matrix (ECM) components. Gene expression levels of catabolic and anabolic genes were analyzed through real time-polymerase chain reaction (qPCR).

RESULTS:

An increase of hNPCs proliferation was reported in all WJ-exos concentrations under study. Live/dead staining suggested that WJ exos at 10 μg/ml was able to attenuate cell death ($p<0.05$). Nitrate production was reduced in a dose dependent-manner with a significant decrease at 100 μg/ml ($p<0.001$). GAG content was enhanced by 10 and 100 μg/ml exos concentrations ($p<0.05$) and confirmed by Alcian blue histological to asses hNPCs' ECM synthesis. WJ-exos modulated gene expression levels compared to control group by increasing ACAN and SOX-9 gene expression and reducing IL-6, MMP-1, MMP-13 and ADAMTS-5 levels.

DISCUSSION & CONCLUSIONS:

Our preliminary results supported the potential use of WJ-exosomes as cell-free treatment of IDD. WJ-exosomes ameliorate hNPCs growth, attenuate ECM degradation and oxidative stress-related IDD progression. These findings offer new opportunities for the potential use of exosomes as an attractive alternative strategy to the effects of cell-therapy. compromised by the harsh microenvironment of the disc.

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Directed differentiation of induced pluripotent stem cells to notochordal-like cells by combinatorial transcription factors activation

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Introduction: Low back pain (LBP) is a leading cause of disability worldwide and intervertebral disc (IVD) degeneration is a major contributor of LBP[1]. IVD degeneration is accompanied and often preceded by the replacement of the large vacuolated notochordal cells (NCs) by non-vacuolated nucleus pulposus cells (NPCs)[2]. The iPSpine project aims to re-populate the degenerated IVD with regenerative iPS-derived NC-like cells (iPS-NLCs). During embryonic development, the determination of cell fates is a result of the combinatorial and concomitant activation of transcription factors. While differentiation of iPS-NLCs can be partially achieved by mRNA transfection of a single gene, such as the notochord-related transcription factor NOTO, such attempts are limited by low and variable differentiation efficiency[3]. This study aims to achieve optimal notochordal lineage commitment by the concomitant and combinatorial activation of multiple key transcription factors via CRISPR activation (CRISPRa) technology. With CRISPRa, transcription activation complexes are recruited to the endogenous promoters of genes to induce expression.

Methodology: Based on a two-step differentiation protocol, we first established iPS-derived mesendodermal progenitor cell by CHIR stimulation to activate the WNT pathway. For notochordal lineage commitment, we focus on NOTO, brachyury (TBXT) and Forkhead Box Protein A2 (FOXA2). Both T and FoxA2 act upstream and are required for the NOTO expression. These three genes were activated at the endogenous gene locus in human iPSCs by CRISPRa technology via the synergistic activation mediator (SAM) system, the most efficient dCas9 gene activator[4].

RESULTS: We show significant activation of all these 3 genes NOTO, Brachyury (T) and FoxA2 by recruitment of CRISPRa to the respective gene promoters. We further focus on using combinations of these transcription factors to induce higher levels of mesendoderm progenitors and improve their commitment toward the notochordal lineage.

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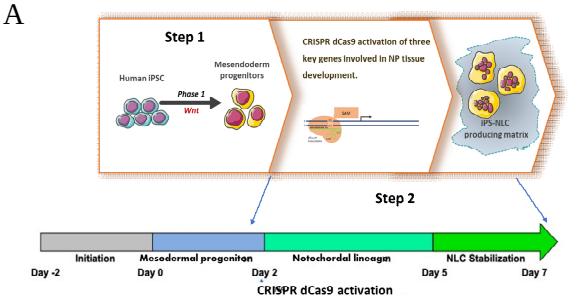

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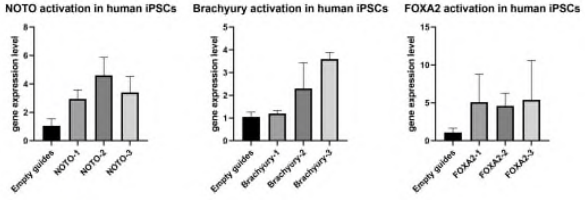

A. Flowchart of CRISPR-mediated gene activation during iPS-NLC differentiation; B. CRISPRa mediated activation of NOTO, brachyury and FOXA2 in human iPSCs.

DISCUSSION & CONCLUSIONS: The study focuses on combinatorial transcription factor activation via CRISPR activation technology to optimize iPS-NPC differentiation. Further work focuses on time-course analysis of lineage specific markers to assess iPS-NLC commitment.

Funding: iPSpine: Horizon2020 (No.825925) and Dutch Arthritis Society (LLP22).

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Characterization of extracellular vesicles from porcine, canine and human notochordal cell-conditioned medium

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INTRODUCTION: Low back pain (LBP) episodes are common and affect everyday life. A major cause of chronic LBP is intervertebral disc degeneration. Notochordal cells (NCs), the juvenile disc cells only present in young individuals and not in degenerated IVDs, possess regenerative potential that could be exploited for therapeutic approaches. Previous work indicated that porcine NCs secrete extracellular vesicles (EVs) that may mediate this effect (1). To ensure that the observed effects in biological studies are associated with EVs and not associated with soluble bioactive molecules, characterization of the EV preparation is essential. This study aims to perform EV characterization of multiple species using a bead-based western technology to identify NC-derived EV-associated protein markers.

METHODS: NC-conditioned medium (NCCM) was generated by culturing NC-rich tissue of porcine, canine, and human origin. EVs were isolated through differential centrifugation followed by size exclusion chromatography (SEC). EV containing fractions were identified based on protein content and pooled for analysis. The SEC fractions from porcine NCCM were pooled in sets of three and subjected to analysis. NC-derived EVs were characterized using DigiWest technology, a high-throughput bead-based multiplex platform.

RESULTS: Using the DigiWest technology, a panel of 33 proteins was determined in NCCM-derived EVs. The analysis of porcine and canine NCCM-derived EVs revealed the presence of 12 EV-associated protein markers in common. In human NCCM-derived EVs, only two proteins (MFGE8 and fibronectin) were identified, most probably due to technical limitations related to low starting protein quantities. These two proteins were also present in porcine/canine NCCM-derived EVs. Possible co-isolated proteins that were detectable in control tissue samples were not detected in the EV-fractions of these tissues.

In addition, the DigiWest platform was used to detect EV-associated proteins in SEC fractions containing relatively small protein quantities (0.9-9 µg input). An enrichment in EV markers (CD9, TSG101, flotillin 1, and HSPA8) was seen in fractions 7-12 compared to later fractions, as expected based on the reported EV-marker profiles.

DISCUSSION & CONCLUSIONS: Altogether, for porcine and canine NCCM-derived EVs, several transmembrane, GPI-anchored, and cytosolic proteins were identified, which is recommended for EV characterization according to the standards of the International Society for Extracellular Vesicles (2). Additionally, the EV samples were devoid of some non-EV-associated tissue proteins that could be co-isolated. Based on these results, a panel of 19 proteins was composed to characterize NC-derived EVs from different species.

The identification of EVs in separate SEC fractions has major challenges with, amongst others, low protein quantities in the fractions and the need for parallel identification of multiple protein markers for proper EV characterization (2). However, the DigiWest technology has been shown to successfully identify multiple EV-associated proteins in a sample containing small protein quantities. It may therefore be used to identify and characterize EVs in SEC fractions.

This project received funding from the European Union's Horizon 2020 program iPSpine (No. 825925).

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Enzyme-induced bovine explant models of intervertebral disc degeneration

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INTRODUCTION: Low back pain affects nearly 75% of the human population at some point in their lives [1] and is associated with degeneration of the intervertebral disc (IVD). Multiple local therapeutic strategies, like growth factors, cell-based therapies and biomaterials have been examined *in vitro* and *in vivo*. However, there is still a need for *ex vivo* models that can be used for testing therapies within the context of clinically-driven outcomes, like tissue structure and biomechanics, without a reliance on *in vivo* studies. The aim of this work was to develop and characterize a set of IVD degeneration models, induced by papain, chondroitinase ABC (ChABC), or collagenase II, hypothesizing that different enzymes could help recapitulate the broad spectrum of changes observed in human degeneration.

METHODS: Bovine caudal IVDs (less than 24 months old) were injected with 100 µL of 65 U/mL papain, 5 U/mL ChABC, 0.5 U/mL collagenase II, or PBS into the center of each explant. IVDs were cultured under free swelling or dynamic physiological loading conditions. At day 7, unfixed IVDs were snap frozen, cryosectioned, and stained with Safranin-O and Fast Green to visualize GAG and collagen, respectively. A 1,9-Dimethyl-methylene blue (DMMB) assay was used to quantify GAG content in tissue samples from the inner and outer AF (iAF and oAF, respectively). DNA content was measured with PicoGreen®. Quantitative measures were analysed with Mann-Whitney U test to detect statistical differences ($p < 0.05$) between the groups.

RESULTS: Histological overviews (Fig. 1A) show that papain and collagenase II, but not ChABC, produced large voids in the nucleus pulposus (NP) region. While all enzymes produced losses of GAG in the NP, areas of concentrated GAG staining were detected in the oAF of the collagenase digested specimens (yellow arrows, Fig. 1A). DMMB assay results correspondingly showed increasing trends in GAG content in the oAF of collagenase-treated specimens compared to PBS, but differences were not significant ($p>0.05$, Fig. 1B).

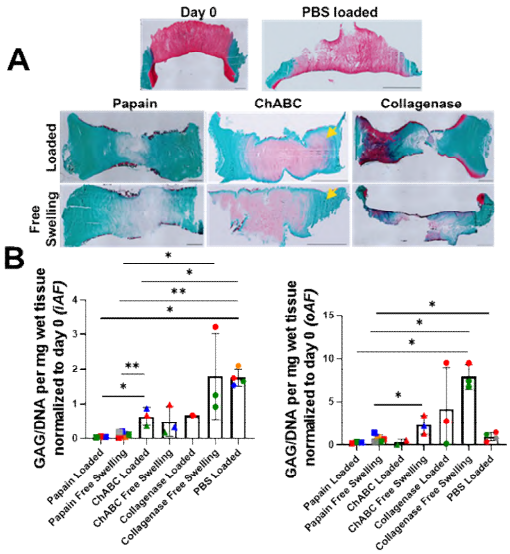

Fig. 1: (A) Representative Safranin O/Fast Green results (scale bars = 5mm). (B) GAG per DNA content in the tissues at day 7 of culture.

DISCUSSION & CONCLUSIONS: The ChABC-digested specimens demonstrated the mildest structural changes, suggesting it may serve as a model for early degeneration. Collagenase digestion induced upregulation of GAG synthesis in the oAF, a repair mechanism that has been observed in humans [2] which could present an interesting timepoint for optimizing efficacy of treatment interventions. Overall, our results suggest that *ex vivo* proteolytic degeneration models can be useful for capturing the broad spectrum of degenerative changes in matrix degradation and remodelling observed in human IVDs.

ACKNOWLEDGEMENTS: The work described is part of the iPSpine project, which has received funding from the European Union's Horizon 2020 Research and Innovation Programme (Grant No 825925).

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MECHANO-RESPONSIVE MOLECULAR EXPRESSION RELATES TO DISEASE STATE IN OSTEOARTHRITIC ARTICULAR CARTILAGE

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INTRODUCTION: Mechanical signals are key factors that contribute to joint homeostasis but are also involved in osteoarthritis (OA) disease progression. Cartilage loading and unloading represent the mechanical stimuli essential for the chondrocytes to maintain cartilage integrity through mechanoadaptation. Although the homeostatic effect of mechanical loading is widely accepted, the molecular mechanisms impairing cartilage anabolic responses following loading during the OA process are still poorly understood. Therefore, we aimed to investigate the mechano-responsiveness of OA human articular cartilage. To gain further insights on whether mechano-responsiveness is affected by the severity of the disease, we related molecular profiling of disease state to chondrogenic potential in patient sample

METHODS: Human articular cartilage explants were harvested from hips of donors with and without OA. In order to establish a physiologically relevant compression protocol, non-OA cartilage explants were subjected to 4 different loading regimes: (i) 1 cycle 10% compression for 1h at 1 Hz followed by 1h free swelling, (ii) 2 cycles 10% compression for 1h at 1 Hz followed by 1h free swelling, (iii) 1 cycle 20% for 1h at 1 Hz followed by 1h free swelling, and (iv) 2 cycles 20% compression for 1h at 1 Hz followed by 1h free swelling. To evaluate OA cartilage mechano-responsiveness, OA cartilage explants were subjected to 2 cycles of 10% compression at 1 Hz for 1h, followed by 1h free swelling. RNA was isolated and mechano-responsiveness was evaluated using real-time quantitative PCR for COL2A1, ACAN, C-FOS, C-JUN, TCF-1 and MMP13. Also, at the harvesting time of the OA samples, human primary articular chondrocytes were isolated by enzymatic digestion and gene expression of the cells (passage 0) was checked to evaluate the disease state based on the expression of COL2A1, ACAN, COL1A1, FN1, FRZB, BMP2 and FGF3[1,2]

RESULTS: All tested loading protocols led to an upregulation of the positive control genes for mechanical stimulation, c-FOS and c-JUN. The loading protocol consisting of 2 cycles of 10% compression for 1h followed by 1h of free-swelling induced the highest upregulation of the chondrogenic markers COL2A1 and ACAN and was thus selected for further experiments. In OA explants, expression levels of anabolic genes COL2A1 and ACAN, and the Wnt inhibitor FRZB were positively correlated with the chondrogenic markers, and thereby associated with healthier OA state. Low/moderate OA states showed better mechano-responsiveness upon loading with increased COL2A1 and ACAN levels and decreased MMP-13. On average, moderate and severe state OA explants showed upregulation of the Wnt signalling pathway upon mechanical stimulation, indicated by increased levels of the TCF-1 gene. Interestingly, this negative effect was not observed in the low OA state.

DISCUSSION & CONCLUSIONS: Human articular cartilage with healthier molecular profile presented with anabolic responses to physiologic mechanical stimulation. On the contrary, more severe profiles were correlated with catabolic responses and showed hyperactivation of the Wnt signalling pathway. These results suggest that Wnt hyperactivation could play a role in the impaired cartilage mechano-responsiveness, thereby contributing to disease progression.

ACKNOWLEDGEMENTS: This work was funded through KU Leuven grants C14/18/077 and G045320N.

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TRANSCRIPTOMIC PROFILE OF HUMAN NOTOCHORDAL CELLS DERIVED FROM INDUCED PLURIPOTENT STEM CELLS

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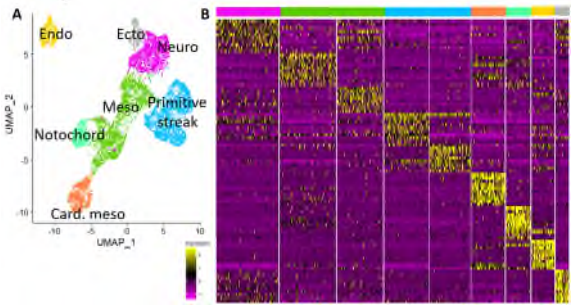
INTRODUCTION: *In vitro* studies have shown that notochordal cells (NC) secretory activities exert anabolic effects on degenerated disc cells. NC are the embryonic progenitors of the nucleus pulposus cells found in the inner core of the intervertebral disc (IVD). Well before skeletal maturity, NC disappear from the IVD. Therefore, there is a need to develop efficient methods to produce *in vitro* this promising cell source for the regeneration of the IVD. Few protocols have described the differentiation of notochordal-like cells (NLC) from human induced pluripotent stem cells (hiPSC).[1] Despite several attempts of optimization, there is still heterogeneity inherent to the differentiation protocols, with off target cell derivatives impacting the differentiation efficiency. New insights on key factors and signalling pathways controlling human NC development would directly contribute to the improvement of the targeted differentiation.

METHODS: Mesendoderm progenitors (MEPC) were differentiated from hiPSC seeded at distinct densities on laminin-coated plates and stimulated for 48 hours with CHIR99021 (WNT pathway activator) in N2B27 media. Next, notochordal differentiation was triggered by the transfection of NOTO mRNA in the MEPC, and culture was pursued for 5 more days in N2B27 media supplemented with CHIR99021 and NOGGIN (BMP inhibitor). The inhibition of the NODAL/TGF-β pathway was performed with addition of 5 or 10µM of SB431542 at different time points during the differentiation. Cell identity along the differentiation process and the efficiency to produce NLC were evaluated by RT-qPCR analysis and immunofluorescence staining. Cells were collected along the differentiation at 2, 3, 5 and 7 days for single-cell RNA sequencing (SC-RNA-seq).

RESULTS: Our results show that lowering the hiPSC seeding density promotes the epithelial-mesenchymal transition (EMT) resulting in high levels of MEPC. Furthermore, following EMT, the supplementation of SB431542 favours the commitment of MEPC with the potency to differentiate toward the notochordal lineage. SC-RNA-seq analysis confirmed the decrease of off-target endoderm and mesoderm derivatives in the presence of the inhibitor. However, despite these treatments, over 40% of the MEPC maintained the potency to differentiate toward ectodermal, endodermal or mesodermal lineages.



A. UMAP of cell populations at day 7 with cluster annotations. B. Heatmap of top differentially expressed genes for clusters shown in A (average log2 fold-change).

DISCUSSION & CONCLUSIONS: The optimization of the differentiation led to the characterization of a cluster displaying a unique signature of human embryonic notochord, including new surface markers. This analysis provides essential insights to design new optimization strategies. Further bioinformatics analysis (WGCNA, Slingshot) will help to identify differentiation trajectories and the gene regulatory networks controlling cell fate to reach high-yield of NLC.

ACKNOWLEDGEMENTS: This project has received funding from EU Horizon 2020 "iPSpine" (grant no. 825925) and French Society of Rheumatology "Spherodisc".

REFERENCES: [1]Colombier et al. *Cells* **2020**, *9*(2), 509.

The role of Noggin in TGF-β1 or TGF-β3 driven chondrogenesis of BM-MSCs

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INTRODUCTION: Noggin (NOG) is an antagonist of BMPs, which regulates development and homeostasis of cartilage. In our previous study, NOG was identified as a new antagonist of TGF-β1[1], but the impact of NOG in TGF-β1-induced chondrogenesis remains unclear. NOG did not appear to influence TGF-β3 driven chondrogenesis[1], so we hypothesised that the role of NOG in chondrogenesis is related to the TGF-β isoform. Therefore, we aimed to investigate the functional significance of NOG in TGF-β1- or TGF-β3-driven chondrogenesis of bone marrow MSCs.

METHODS: MSCs from three donors (Ethik-Kommission der Albert-Ludwigs-Universität Freiburg, EK-326/08) were pelleted to induce chondrogenesis. GAG quantification was done by DMMB assay.

RESULTS: Both TGF-β1 and TGF-β3 upregulated the expression of *NOG* (Fig. 1), which indicates an important role of *NOG* in chondrogenesis. We found 10 or 100 ng/mL NOG reduced both the retention of GAG in pellets (Fig. 2) and release into the medium (Fig. 3) in the TGF-β1 treated pellets over four weeks. NOG (100 ng/mL) also has an inhibitory effect of GAG production in the TGF-β3-treated pellets at day 7, 21 and 28 but to a lesser extent. The inhibitory impact of NOG on TGF-β1-induced chondrogenesis was more pronounced and lower concentrations (10 ng/mL) of NOG reduced GAG production of MSC.

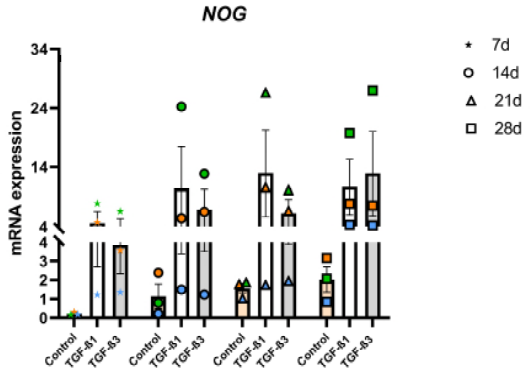

Fig. 1: The expression levels of NOG were shown in the progression of chondrogenesis. Different colors represent different donors.

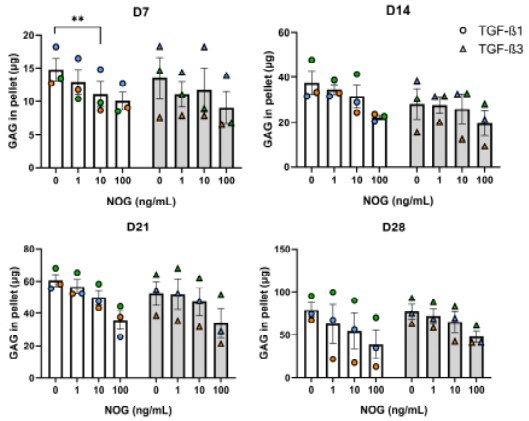

Fig. 2: The effect of different concentrations of NOG (0, 1, 10, 100 ng/mL) on GAG retention in pellets at different time points. ∗∗P < 0.01.

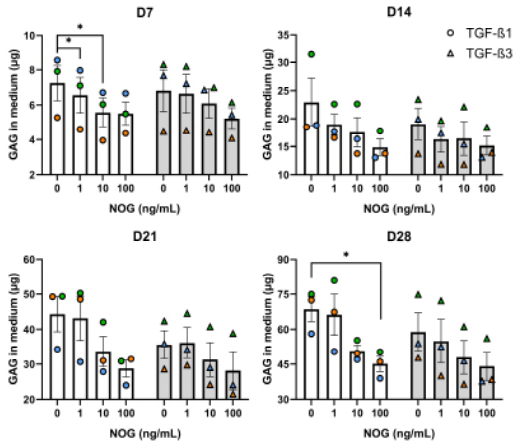

Fig. 3: The impact of NOG on GAG release into medium during chondrogenesis. ∗P < 0.05.

DISCUSSION & CONCLUSIONS: We demonstrated that although endogenous NOG expression increased during TGF-β-induced chondrogenesis, NOG inhibited chondrogenesis induced by both TGF-β1 and TGF-β3.

ACKNOWLEDGEMENTS: This work received funding from the Marie Skłodowska-Curie grant agreement No 955335.

REFERENCES: 1. Wen L, Zhou Z. Biochem Biophys Res Commun. 2021 Sep 17.

Injectable Biomaterials to Maintain Notochordal Cell Phenotype as Potential Therapy for Intervertebral Disc Degeneration

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INTRODUCTION: Low back pain is strongly associated with degeneration of the intervertebral disc (IVD). During degeneration, altered matrix synthesis and increased matrix degradation, together with accompanied cell loss is seen particularly in the nucleus pulposus (NP). It has been proposed that notochordal (NC) cells, embryonic precursors for the NP cells, could be utilized for IVD regeneration. Injectable biomaterials are likely required to support their phenotype and viability within the degenerate IVD. Therefore, viability and phenotype of NC cells were analysed and compared within biomaterial carriers subjected to physiological oxygen conditions over a four-week period.

METHODS: Freshly extracted porcine NC cells were seeded at a cell density of 4 x10^6 cells/ml into three injectable hydrogels: NPgel (a L-pNIPAM-co-DMAc hydrogel), NPgel with decellularized NC-matrix powder (dNCM) and Albugel (an albumin/hyaluronan hydrogel). The NCs and biomaterials constructs were cultured for up to four weeks under 5% oxygen within standard αMEM media (n=3 biological repeats). Cellular and biomaterial constructs were fixed and stained with histological stains: Haematoxylin + Eosin, Alcian blue and Masson trichrome. Immunohistochemical staining was further used to analyse pNC maintenance of notochordal phenotype, using antibodies specific for notochordal phenotypic markers (brachyury and cytokeratin) and extracellular matrix (collagen type II and aggrecan) that healthy NC cells would produce. Further, glycosaminoglycans (GAG) analysis were performed using dimethylmethylene blue assay to quantify and investigate extracellular matrix synthesis and deposition**.**

RESULTS: Histological analysis revealed that NCs survive in the biomaterials after four weeks and maintained cell clustering in NPgel, Albugel and dNCM/NPgel. Immunohistochemical positive staining for brachyury, collagen type II, aggrecan and cytokeratin 8, 18 and 19 was shown in NPgel and Albugel. Matrix staining was also observed in acellular dNCM & NPgel due to the presence of dNCM (Figure 1). GAG analysis demonstrated that NC containing constructs excreted more GAGs over the four weeks than the acellular controls.

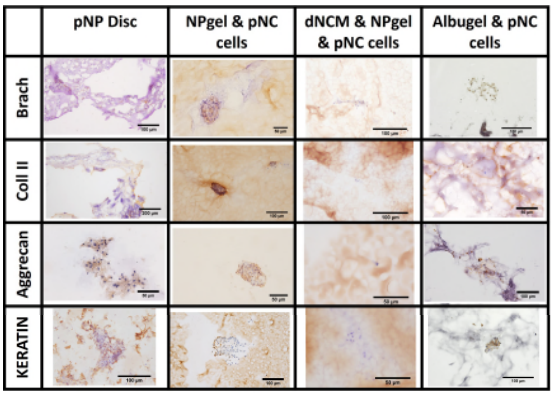

Fig. 1: Immunohistochemistry staining of porcine notochordal cells (NC cells) cultured in NP gel, dNCM + NPgel and Albugel for up to 4 weeks in a hypoxia unit of 5% oxygen at 37ºC. Antibodies brachyury (Brach), Collagen type II (Coll II), Aggrecan and cytokeratin 8, 18 and 19 (Keratin) were used to distinguish the cell phenotype.

DISCUSSION & CONCLUSIONS: NC cells maintain their phenotype and characteristic features *in vitro* when encapsulated into biomaterials, especially NPgel and Albugel. NC cells and biomaterial constructs could potentially become a therapy to treat and regenerate the IVD.

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Effects of different glucose supplementation on bovine intervertebral disc organ cultures under physiological loading

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INTRODUCTION: Ex vivo intervertebral disc (IVD) organ culture has become a well-accepted model in the field of IVD research because it may provide valuable information on pathologies and development of new treatments. Often, *ex vivo* culture protocols employ the use of high concentrations of glucose (25 mM), which do not reflect the IVD physiology.

METHODS: In the present study, we investigated how different concentrations of glucose could affect bovine IVD cells in bioreactor loaded organ culture. Whole IVDs were cultured under physiological condition of loading (0.02-0.20 MPa, 0.2 Hz, 2h or 8h/day) and varying glucose supplementation (25, 11 and 5.5 mM) for one week. After culture, the effects of glucose supplementation on the IVD cell viability (lactate dehydrogenase/ ethidium homodimer staining), expression of the main anabolic and catabolic markers and histological morphology were investigated.

RESULTS: Results showed that decreasing glucose concentration during organ culture dramatically influenced the viability of bovine IVD cells. In 25 mM glucose media a high cell viability was maintained after 7 days, both in annulus fibrosus (AF) and nucleus pulposus (NP), which was found to be ~90% and ~95%, respectively. In the lower concentrations of glucose media (11 and 5.5 mM), cell viability dropped significantly in the inner AF (~20% and 0%) and NP (~30% and 0%) (Fig. 1). Preliminary data revealed no significant differences between glucose concentrations for relative gene expression and histology.

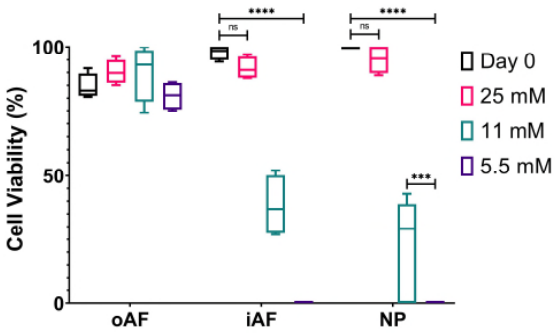

*Fig. 1: Cell viability in the outer AF (oAF), inner AF (iAF) and in the NP at different concentrations of glucose:25 mM, 11 mM and 5.5 mM. Data are expressed as mean ± SD; significant difference is marked by black asterisks (**** p ≤0.0001, *** p =0.0001).*

DISCUSSION & CONCLUSIONS: This study provides a better understanding of bovine disc cells metabolism during organ culturing. It will be necessary to further investigate the physiological role of nutrients, metabolites and loading in order to refine our *ex vivo* protocols to close the gap with *in vivo* models.

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