ANNUMECH: Experiences and insights from the AO collaborative research program for annulus fibrosus repair

Prof. Stephen Ferguson, Ph.D.

The direct repair of lesions of the annulus fibrosus (AF) has been limited to investigational studies of the use of suturing methods, membrane barriers or injectable sealants. The optimal biomechanical properties for such a repair remain an open question. The overall aim of the "ANNUMECH" project, within the AO Collaborative Research Program for annulus fibrosus repair, was to establish the functional requirements for next-generation annulus repair methods through iterative, parametric experimental and simulation studies, to develop methods for the fabrication of novel biomaterial-based repair devices, and to validate their mechanical and biological performance in organ models.

Electrospinning has established itself as a promising and versatile method for the creation of fibrous membranes with applications in tissue reconstruction surgery. However, conventional electrospinning, by nature of the process, creates fibre mats with random fibre orientation. Annulus fibrosus tissue is characterised by lamellar sheets with highly-oriented collagen fibre bundles. Orientation of fibres in electrospinning is possible using a rotating drum collector, however the resulting fibre mats often prove to have an insufficient inter-fibre spacing to promote cell and tissue growth. In our work, we developed a novel method based on the application of a secondary electric field to directly control fibre alignment during deposition, allowing the creation of strong and stiff oriented fibre structures, however with a high porosity and excellent cell and tissue ingrowth [1]. These methods were further extended to enhance cell and tissue adhesion on one side of the membrane, while preventing tissue adhesion on the other, ideal characteristics for an annulus repair strategy to be applied directly adjacent to sensitive neural structures.

In vitro biomechanical experiments performed in collaboration with several project partners established robust methods for the validation of annulus repair strategies [2] and defined the boundaries of the complex physiological loading that such a repair should be expected to endure. In our own simulation studies, a parametric spinal segment model was used to explore the range of local stresses and strains experienced by the annulus fibrosus, in response to global bending and compressive loads applied to the spine. Based on these simulations, which covered a broad range of normal intervertebral disc geometries, target design parameters were defined for a final membrane repair device, and the durability of such membranes was proven in long-term degradation studies [3].

In a final series of validation studies, the ability of the electrospun membrane to effectively seal complex lesions in the mid-substance of the disc and also in a challenging new endplate delamination model was proven [4]. The detrimental effect of disc injury and herniation was shown with this model, and the effectiveness of the repair strategy to promote a persistent restoration of mechanical integrity was also demonstrated. In subsequent trials, tissue growth onto and into the membrane was shown, incorporating the degradable membrane into de novo tissue. Finally, the entire consortium contributed to a highly relevant and promising in vivo validation of different repair strategies for the intervertebral disc, exploring the effects of injury and the potential for long-term repair, based on biochemical, biomechanical and histological outcome parameters [5].

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Converged Biofabrication Technologies for Osteochondral Implants

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A major challenge in the application of regenerative medicine strategies is the replication of the complexity and functionality of living tissues. Biofabrication techniques, such as inkjet, extrusion, or light-assisted printing, can result in living structures that further replicate the 3D architecture and organization of native tissue.

Recent developments have resulted in the availability of a plethora of bioinks, new printing approaches, and the technological advancement of established techniques However, it remains largely unknown which materials and technical parameters are essential for the fabrication of intrinsically hierarchical cell-material constructs that truly mimic biological function of tissues. Moreover, it is unlikely that any of these technologies used individually would be able to replicate this complexity and functionality. Hence, we here present an orthopaedic example, in particular the treatment of osteochondral defects in a large (equine) animal model. Driven by the observations that the collagen structure provides articular cartilage with its extraordinary mechanical properties and that this structure does not recover once damaged, we evaluated the performance of a biofabricated composite osteochondral implant designed to provide long-term mechanical resistance, comparing implants cell-seeded with cell-free implants.

The implants were composed of a gelatin methacryloyl cartilage phase, reinforced with precisely patterned melt electro-written polycaprolactone micrometer-scale fibers and displaying a zonal, cartilage-mimetic architecture. This structure was firmly integrated with a room temperature-setting calcium phosphate cement-based bone anchor via a converged melt electrowriting-extrusion-based printing approach.

Cell-containing implants were pre-cultured *in vitro* and samples were then implanted orthotopically in ponies for 6 months. The reinforcement of the cartilage phase enhanced both the compressive and shear properties of the implants considerably, closer to native values than achieved before.

Abundant deposition of glycosaminoglycan- and type II collagen-rich tissue was observed *in vitro*, which was maintained during the 6-month *in vivo* implantation period, showing that the composite architecture, comprising multi-scale printing of hydrogel, microfibers and ceramic materials had provided the required mechanical stability and integration of the implant within the subchondral bone to allow survival of the implant in the mechanically challenging environment of the equine knee joint over an extensive period of time while retaining its mechanical characteristics.

Our findings underscore the hypothesis that the mechanical stability is more determining for the success of the implant than the presence of transplanted cells. This observation is of great translational importance and highlights the aptness of advanced (bio)fabrication and 3D printing technologies for functional tissue restoration in the harsh articular environment.

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3D Printing and Tissue Engineering for Skeletal Regeneration – Achievements from RAPIDOS Consortia Project –

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INTRODUCTION: The RAPIDOS research project was from the first co-ordinated call for research proposals in Biomaterials launched by the European Union Commission and the National Natural Science Foundation of China in 2013 for facilitating bilateral translational research. We formed the RAPIDOS European and Chinese consortium with the aim to apply technologies creating custom-made tissue engineered constructs made of resorbable polymer and calcium phosphate ceramic composites specifically designed by integrating: 1) imaging and information technologies, 2) biomaterials and process engineering, and 3) biological and biomedical engineering for novel and truly translational bone repair solutions [1].

METHODS: 3D printing developed or used included advanced solid free form fabrication technologies, precise stereolithography, and low temperature rapid prototyping (LTRP). For China task force, we focused on modification of LTRP towards R&D of Class III bioactive porous composite scaffold materials, incorporating herbal or mineral based bioactive compounds, such as Chinese Medicine derived-molecule bone anabolic factor icaritin and icariin [2-3] and biodegradable magnesium powder for repair of challenging bone defects in osteoporotic fracture, SAON, and OCD [4-7]. Biosafety of these degradable bone substitutes have been tested using internal protocols and by regulatory body authorized testing centers.

RESULTS: Major findings have been summarized in a significant number of relevant papers dominantly using animal experimental models, including quadrupedal rabbits [3,4] and bipedal emus [2] for treatment efficacy and *in vitro* tests for postulated biological mechanism. Results of ISO-based biosafety tests or modified ISO testing protocols for Mg-based biodegradable implants have also published and made available to public [8].

DISCUSSION & CONCLUSIONS: Our preclinical study results are encouraging, and our joint efforts also laid down a solid foundation towards multi-center clinical trials. This fulfilled our initial goal of our founded EU-China collaboration where we can advance therapeutic solutions for population suffering from bone non-healing in the future and help to achieve faster patient recovery through the development of custom-made implant and patient-specific therapy and/or 3D based bone substitutes for general applications to serve large patient pool. However, in order to realize from bench-to-bedside, establishing GMP for facilitating medical implants production and clinical trials is essential. How to find the industrial collaborators or create spin-off with investment are beyond academia yet we do make efforts to explore such opportunities.

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Engineered nasal cartilage for osteoarthritis?

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INTRODUCTION: We previously showed that Tissue Engineered Cartilage generated with autologous Nasal chondrocytes (N-TEC) is suitable for the repair of focal articular cartilage defects (1,2). We then evaluated the compatibility of N-TEC in the more challenging OA environment where chronical inflammation is present (3)

METHODS: N-TEC were generated with expanded human nasal chondrocytes (NC) and exposed *in vitro* to an inflammatory cytokine cocktail (IL1β, IL6 and TNFα) or conditioned medium from OA-synoviocytes. Additionally, inflammatory factors secreted by synoviocytes and chondrocytes isolated from OA joints were quantified upon culture with N-TEC conditioned medium.

N-TEC generated with GFP-labelled human NC were combined with a bone-like tissue engineered with osteoblasts or osteochondral tissue explants from patients with OA and subcutaneously implanted in mice for 8 weeks.

OA was induced in an orthotopic sheep model by generation of full thickness cartilage defects in the femoral condyles. Two months after OA induction, N-TEC generated with GFP-labelled autologous sheep NC (sN-TEC) were implanted into the degenerated cartilage defects for up to 12 months and the repair tissue quality assessed immuno-/histochemically. In addition, inflammatory factors were quantified in the synovial fluids harvested from joints of healthy (before OA induction), OA (at the time of treatment) and treated sheep (at the time of explantation).

Safety of autologous N-TEC transplantation was tested in 2 patients with radiological signs of medial compartment OA (Kellgren and Lawrence grades 3 and 4, age 34 and 36) who were otherwise considered for unicondylar knee arthroplasty. N-TEC implantation was combined with corrective high tibial osteotomy to reduce abnormal mechanical loading due to varus malalignment.

RESULTS: We demonstrated that N-TEC are able to maintain cartilaginous properties in different inflammatory *in vitro* models simulating conditions of an osteoarthritic joint. Importantly, factors secreted by N-TEC significantly reduced the secretion of several specific inflammatory cytokines by OA-synoviocytes, including IL6 and TNFα. These effects were at least partially mediated by WNT signaling, a pathway that is chronically upregulated in OA.

When N-TEC were combined with OA bone tissues in different ectopic *in vivo* models, we demonstrated cell survival, N-TEC integration and maintenance of the cartilaginous properties. Successful engraftment of N-TEC was also observed in a weight bearing orthotopic sheep OA model.

No adverse reactions occurred in our first treated patients. In a patient´s self-reported Knee Injury and Osteoarthritis Outcome Score (KOOS) both patients stated improvements in all the categories, including reduced pain, improved joint function and life quality until 14 months after the treatment. KOOS scores generally further increased with time until our last follow-up time points at 24 or 30 months.

DISCUSSION & CONCLUSIONS: Our findings demonstrated the compatibility of N-TEC with an OA environment, as it not only resisted, but also seemed to positively modulate the chronically inflamed joint environment. Our pre-clinical results indicated that implanted NC directly contribute to cartilage repair and engraftment in OA cartilage defects. To verify the regenerative capacity of N-TEC in patients, a suitably powered phase II clinical trial in a larger cohort of patients with OA is now required

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Beneficial structural impact of Liraglutide, a GLP1 Receptor agonist, in inflammatory and post-traumatic OA animal models

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INTRODUCTION: Osteoarthritis (OA) is a whole-joint disease characterized by synovial inflammation, cartilage degradation and bone changes leading to swelling, pain, and loss-of-function. To date, only symptomatic anti-inflammatory treatments are available, however there is an urgent unmet need for a disease-modifying OA drug that would alleviate both pain and ameliorate structural damages in order to change the course of the disease. Liraglutide is a Glucagon-Like-Peptide 1 Receptor (GLP-1R) agonist widely prescribed for the treatment of type 2 diabetes. Liraglutide possesses anti-inflammatory, anti-degradative effects in vitro on murine chondrocytes and analgesic effects in vivo. Here, we used animal models of inflammatory and post-traumatic OA to evaluate the impact of Liraglutide on the whole diseased joint tissues by quantifying synovial membrane inflammation, surrogate markers of cartilage matrix proteolysis along with knee joint structure both at the cartilage and bone levels

METHODS: The impact of intra-articularly (IA) administered Liraglutide on synovial membrane inflammation and catabolic markers was evaluated by histology and RT-qPCR, respectively, in a short-term inflammatory model of OA, namely the mono-iodo-acetate mouse model. The structural effect of IA-delivered Liraglutide on cartilage damages and knee joint structure were evaluated in the destabilization of the medial meniscus (DMM) model of OA in rat as well as in the type II collagenase rat model. Histopathological analyses and scoring were performed by independent investigator

RESULTS: Single IA administration of Liraglutide into the knee joint resulted in a significant dose-dependent decrease in particular on ADAMTS-5 gene expression 8 days following treatment (2.32± 0.73 for vehicle vs 2.01±0.45, 1.57±0.27 and 0.76±0.26 for Liraglutide 10, 20 and 30 µg, respectively, p=0,023). As well as an improved synovitis score inflammation (liraglutide 20 µg $p = 0.0099$, compared to vehicle-treated group). Histological analyses following 6 weeks of IA administratered Liraglutide into the knee joint in the rat DMM-induced OA model resulted in a significant reduction (-13%, p=0.028 in Liraglutide-treated vs vehicle) in total cartilage degeneration width. Interestingly, zonal analysis of damaged tibial cartilage revealed that Liraglutide preferentially reduces lesions in the most inner zone of the tibial cartilage (zone 3) which accounts for proteoglycan loss (0.53±0.03 vs 0.40±0.07 for vehicle and Lirgalutide-treated, respectively), this targeted action on the most inner zone of the tibial plateau was also confirmed in Collagenase type II model. Furthermore, along with reducing cartilage degeneration, Liraglutide-treated animals presented a decreased osteophyte score (3.14±0.35 vs 2.47±0.32 for vehicle and liraglutide, respectively).

DISCUSSION & CONCLUSIONS: In vivo, in OA animal models, Liraglutide has anti-degradative effects whereby the reduction in cartilage degradation width is sustained by less proteoglycan loss and in keeping with decreased ADAMTS-5 gene expression, this is accompanied by a reduction in osteophytes score. In addition, liraglutide reduces synovial membrane inflammation. The integrative action on synovial membrane, bone and cartilage could have a dual symptomatic/structural positive impact and thus would places Liraglutide as a potential candidate DMOAD.

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NUCLEUS PULPOSUS REPLACEMENT MITIGATES IVDD IN A CAPRINE MODEL – A 3 YEAR FOLLOW-UP

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INTRODUCTION: Lumbar intervertebral disc degeneration (IVDD) impacts up to 90% of the aged population, with > 25% of Americans reporting low back pain. Early degenerative changes typically occur in the central nucleus pulposus (NP), where progressive dehydration from proteoglycan loss compromises mechanical function. We have developed a non-crosslinked injectable NP replacement hydrogel based upon a polyvinyl alcohol and polyvinyl pyrrolidine co-polymer, treated with polyethylene glycol to yield a thermally processible hydrogel (HY) with 59-66% water. The hydrogel incorporates barium sulphate (BaS) for imaging contrast. The objective of this work was to utilize clinical exam, serial radiography, contrast-enhanced imaging techniques, μCT and histology to evaluate hydrogel disc replacements for IVDD over a three year follow up. **METHODS:** Three adult goats (50-80kg) underwent chemonucleolysis with 1.5U Chondroitinase ABC (CABC) at levels L1-2, L2-3, and L4-5 [1]. Two weeks later, discs L2-3 and L4-5 were injected with HY under fluoroscopic control via a 17g spinal needle. Animals were maintained for up to 3 years with unrestricted exercise and daily evaluation for pain, gait symmetry, lameness, and signs of neurologic deficits. Monthly standing lateral radiographs were used to evaluate implant placement, disc space geometry and disc height index changes. After 2.5-3 years, 3T MRI was performed to evaluate disc hydration (correlated with T_2 relaxation time) and disc diffusion (correlated with ΔT_1 time). A T_1 map was acquired for the pre-contrast condition, then gadodiamide was delivered intravenously at 0.1 mmol/kg. A post-contrast T_1 map was acquired 30 minutes post-administration, the difference between the pre- and post- T_1 maps (ΔT_1) indicating the uptake of gadodiamide into the disc. The T_1 maps were quantified in ImageJ and the T_2 images were analyzed by fitting the intensity in the nucleus pulposus regions of interest (ROIs) to noise-corrected exponentials, as previously described [2]. **RESULTS:** No lameness or neurologic deficits were observed in any of the animals. Longitudinal disc height index (DHI) analysis showed that HY-treated disc maintained the same height as non-degenerated discs. The T_2 signal, was significantly reduced for the HY-treated discs. The magnitude of T_1 times were similarly reduced in the HY implants; however, the diffusion assessment is quantified as the difference between two image sets and the reduced overall signal did not impede the diffusion assessment, which showed similar rates in the HY-implanted and native control discs. HY-implanted discs exhibited improved overall histological grade compared to untreated discs. For discs treated with HY, cortical endplate comparison of BV/TV ratio showed slightly decreased bone volume fraction (BV/TV) compared to controls. Adjacent trabecular bone comparison of BV/TV ratio revealed increased bone volume fraction (BV/TV) for treated discs. **DISCUSSION & CONCLUSIONS:** Our long-term clinical and *ex-vivo* findings demonstrate that minimally invasive, percutaneous nucleoplasty in CABC treated discs mitigates disc degeneration and end-plate changes in this large animal model of IVDD. BaS-enhancement of the HY enabled visibility on radiographs and fluoroscopy, which affords adequate intraoperative visualization during delivery and allows for long-term device monitoring. Collectively, these data indicate that for BaS-enhanced replacement discs, the HY can be readily tracked with clinically relevant imaging methods and the disc can be assessed using MRI with changes imposed by secondary contrast agents, though other MRI measures such as raw T_1 and T_2 measures will be confounded by the BaS contrast. Ongoing work is focused on elucidating changes in the cellular / inflammatory signalling milieu and the development of imaging techniques to improve assessment of HY-treated and untreated degenerating discs.

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Cell senescence and senolytic drugs to treat back-pain in mice and men

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INTRODUCTION: Low back pain is a global health problem that is experienced by ~ 80% of individuals at some time in their lifespan. Despite its prevalence, little is known about the mechanisms leading to painful IVD degeneration, leaving surgical removal and vertebral fusion at end-stage disease as the most common treatment. There is growing recognition that senescent cells accumulate during tissue degeneration, where they contribute directly to disorders like heart-disease, cancer and osteoarthritis. The **overall objective** of this research is to determining if senolytic drugs, can selectively remove senescent IVD cells, reduce inflammatory mediators, and relieve pain in degenerating human IVDs and in SPARC null mice with back pain *in vivo.*

METHODS: Human IVD cells: Isolated IVD cells were treated with combinations of o-Vanillin and RG-7112. p16, ki-67, caspase3 and inflammatory cytokines were measured. SPARC-null Mice: 5-6-month-old SPARC-null mice with signs of IVD degeneration and low back pain and age-matched wild-type C57BL6 female and male animals were treated once a week for 8 weeks with o-Vanillin and RG-7112. Pain behavior, tissue homeostasis and senescence-associated secretory phenotype was evaluated.

RESULTS: o-Vanillin and RG-7112 significantly reduced the presence of senescencent cells when applied separately. Combining the drugs significantly reduced the presence of senescencent cells when compared to the senolytic drugs alone both in degenerating human IVDs and in SPARC null animals. Pain behavior was significantly reduced at 4 weeks and further improved by the 8-week time point when treated and non-treated animals were compared. The greatest effect was from a combination of o-Vanillin and RG7112.

DISCUSSION & CONCLUSIONS: The overall objective of this research is to evaluate a synergistic effect of combining senolytics targeting multiple senescent anti-apoptotic pathways as potential treatment for low back pain. We use a combination of a human *in vitro* models and in an *in vivo* mouse model (SPARC-null). There are many shared mechanisms in SPARC-null mice and human patients with IVD degeneration and pain symptoms, including age-related disease onset, degeneration severity, loss of SPARC expression, pain symptoms and an accumulation of senescent cells in degenerating IVDs. The use of similar strategies in both human and rodent tissue, ensures that we identify targets with clear translational value to the clinic. Senescent cells are heterogeneous and a drawback of using a single senolytic agent is the failure to target multiple senescent anti-apoptotic pathways in the same cell type, or different cell populations within a target tissue. Our research demonstrates that RG7112 and o-Vanillin individually significantly reduce the presence of senescent IVD cells and significantly improve tissue homeostasis. However, 30- 50% remain after either treatment, which may be due to the heterogenous nature of senescent cells. Initial experiments evaluating senescence and apoptotic pathways indicated that the o-Vanillin and RG-7112 targets different molecular pathways suggesting that a combination may significantly increase clearance. In fact, combining the drugs further reduced inflammatory mediators, improved tissue homeostasis in human and mouse IVDs and reduced pain behavior in SPARC-null mice. Senolytic combination therapies could revolutionize treatment of back pain and be one step closer to offering a preventative treatment for individuals at risk of lower back pain or avoid/prolong the need for invasive surgery.

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Mitochondrial DNA variants influence the risk of Knee Osteoarthritis

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INTRODUCTION: The study of the mitochondria in the context of osteoarthritis (OA) attracted much attention. In the present work, we analyze the role of the mtDNA haplogroups in the risk of incident knee OA in well-characterized study cohorts and the influence of mitochondrial genome variation on the DNA methylome of articular cartilage.

METHODS: The influence of the haplogroups in the rate of incident knee OA at eight years in 2579 subjects from the Osteoarthritis Initiative (OAI) and 635 subjects from the Cohort hip and Cohort knee (CHECK) was assessed. A subsequent meta-analysis was conducted to synthetize results. Transmitochondrial cybrids carrying the haplogroups J and H were constructed to study the influence of the mitochondrial background in different OA-related features using an extracellular flux analyzer. DNA methylation profiling was performed from data deposited in the NCBI Gene Expression Omnibus (GEO) database (accession number GSE43269) consisted in the data of 14 haplogroup J cartilages and 20 H. Subsequent validation was performed in an independent cohort of 7 haplogroup J cartilages and 9 H by RNA-seq. Correlated genes were validated by real-time PCR in an independent cohort of 12 J cartilages and 12 H.

RESULTS: The haplogroup J associates with a decreased risk of incident knee OA in subjects from the OAI. The subsequent meta-analysis including 3214 cases showed that the haplogroup J associates with a lower risk of incident knee OA. Cybrids with the haplogroup H show higher mitochondrial respiration and glycolysis leading to an increased ATP production. On the contrary, haplogroup J shows a significantly lower free radical production, higher cell survival under oxidative stress conditions and lower grade of apoptosis as well as lower expression of the mitochondrially-related pro-apoptotic gene BBC3. DNA methylation profiling revealed 538 differentially methylated loci (DML) between H and J cartilages. 17 genes showed an inverse correlation between methylation and expression. In terms of gene ontology, negative correlations between methylation and expression were also detected between H and J cartilages; highlighting a significantly enhanced and repressed apoptotic process in H and J cartilages respectively.

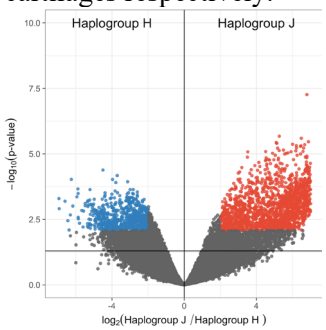

Fig. 1: Volcano-plot: Differentially expressed genes between haplogroup H and haplogroup J.

DISCUSSION & CONCLUSIONS: The haplogroup J reduces the risk of incident knee OA. This mitochondrial variant constitutes a protective genotype against the development of OA, emerging as a powerful OA biomarker and Mitochondrial DNA variation modulates the methylation status of articular cartilage by acting on key mechanisms involved in OA, such as apoptosis.

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USE OF INTERVERTEBRAL DISC CELLS FROM SHEEP TO EVALUATE BIOTHERAPIES IN VITRO

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INTRODUCTION: Intervertebral disc (IVD) degeneration is among the leading cause of low back pain. Given the lack of etiological treatments, IVD regeneration is a major public health challenge. Human IVD cells are difficult to obtain, especially healthy ones, and murine IVDs present numerous differences with human ones (size, mechanical loading, presence of notochordal cells). The sheep spine exhibits biological and biomechanical similarities with the human one and is thus recognized as a relevant model for translational applications[1]. With the goal of reducing our reliance on animal models, and for economic, regulatory, and ethical reasons, we have set up an in vitro platform based on sheep annulus fibrosus (AF) and nucleus pulposus (NP) cells to evaluate cell and extracellular vesicle (EVs) therapies.

METHODS: Cells from both the AF and the NP were isolated from the IVDs of five young sheep (≈ 3 months old) and three older ones (≈ 4 years old). The expression of the major genes involved in matrix synthesis, matrix degradation and inflammation were compared between AF and NP cells, from young or old sheep. To simulate a degenerative IVD microenvironment in cells from young animals, they were treated with either recombinant IL-1β (10 ng/mL) or H_2O_2 (500 µM) or maintained in culture for over 10 passages. EVs from human adipose-derived mesenchymal stromal cells (hASCs) were produced in a turbulent flow as previously described[2] and their effect on degenerative-like NP and AF cells was evaluated. In a separate experiment, hASCs cells were co-cultured with NP or AF cells, in direct or indirect (Transwell®) contact. The use of two species allowed us to analyze RNA expression from disc cells in direct co-culture with hASCs by using sheep-specific primers.

RESULTS: Sheep AF and NP cells exhibited differential RNA expression, notably a higher expression of *COL1A1* in AF cells while NP cells favored *COL2A1*. Cells from older sheep also displayed those differences but with an overall lower expression of matrix proteins and higher expression of metalloproteinases (MMPs) and inflammatory cytokines (*IL6*, *CXCL8*) compared to the cells from young sheep. Prolonged culture of young cells or treatments with IL-1β or H_2O_2 led to a rather similar expression profile as for older cells. While EVs consistently increased basal metabolic activity of both AF and NP cells at early and late passages, they had little effect on gene expression. On the other hand, direct cocultures with human ASCs profoundly affected the transcriptional profile of disc cells. Notably, both types of cocultures led to a drastic downregulation of *CXCL8* in disc cells, reduced by over 60% in indirect coculture and even undetectable in direct coculture, while we observed an upregulation of *COL1A1* but also *MMP1*. Surprisingly, *IL6* expression by disc cells slightly increased with hASCs on Transwells but sharply decreased by over 80% when hASCs were in direct contact.

DISCUSSION & CONCLUSIONS: We demonstrated that healthy sheep cells expressed markers of degeneration after IL-1β and H_2O_2 treatment, or after numerous passages in culture. They showed biological responses to hASCs and, to a lesser extent, to hASC-derived extracellular vesicles. These results confirm the suitability of sheep disc cells to model IVD degeneration in vitro and assess biotherapies.

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Effect of entire MSC secretome versus extracellular vesicles in pro-inflammatory annulus fibrosus cell cultures

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INTRODUCTION: Extracellular vesicles (EV) secreted by mesenchymal stem cells (MSC) cultured under basal conditions were shown to reduce cell apoptosis and inflammation in intervertebral disc (IVD) degeneration models and to promote extracellular matrix synthesis and proliferation of IVD cells.[1] MSC priming with interleukin (IL)-1β improved the pro-anabolic effect of the entire MSC secretome on IVD.[2,3] Taking this, we investigated the impact of EV *versus* entire secretome from IL-1β-primed and non-primed MSC on annulus fibrosus (AF) cells.

METHODS: Human MSC were cultured with or without 1 ng/mL IL-1β for 48h (n=3). EV were purified from the entire secretome by differential (ultra)-centrifugation. EV morphology, particle diameter and size distribution/polydispersity were assessed by transmission electron microscopy (TEM) and dynamic light scattering. Biochemical characterization included western-blot detection of CD63 and calnexin. Human AF cells isolated from IVD biopsies of disc degeneration patients were cultured with IL-1β for 48h (n=8). Subgroups of IL-1β-stimulated AF cells were treated with entire secretome or EV from non-primed or IL-1β-primed MSC (n=4). Unstimulated AF cells were used as control. Cell metabolic activity, gene expression of *BAX*, *IL6, IL8*, matrix metalloproteinase *MMP1*, MMP inhibitor *TIMP1* and collagen type I (*COL1A1*) were evaluated. Statistics: one-way ANOVA (significance, $p<0.05$).

RESULTS: Both EV from primed and non-primed MSC displayed typical size distribution (diameter of 105±53 nm, PdI of 0.30), expressed the positive EV marker CD63 and did not express calnexin, a negative EV marker. IL-1β stimulation of AF cells increased cell proliferation and metabolic activity/cell, *IL6*, *IL8*, *MMP1* (Fig.1A) and *TIMP1* (Fig.1B) expression ($p<0.05$) versus control, while downregulating *BAX* and *COL2A1* (Fig.1C) ($p<0.0001$). All treatment groups decreased *BAX*, *IL6* and *IL8* in AF cells compared to IL-1β stimulation alone; but the secretome from primed and non-primed MSC also downregulated *TIMP1* and *COL1A1* ($p<0.05$). Interestingly, the treatment with EV from IL-1β-primed MSC upregulated *TIMP1* ($p<0.0001$) and did not further decrease *COL1A1*.

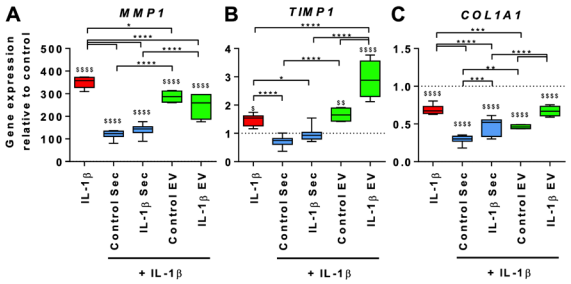

*Fig. 1: Relative mRNA expression of **A)** matrix degrading enzyme MMP1, **B)** its inhibitor TIMP1 and **C)** COL1A1 for IL-1β stimulated AF cells and subsequently treated with secretome (Sec) or EV from control or IL-1β-primed MSC. Results were normalized to GAPDH and control cells. n=4-8; $, comparison to control; *, comparison between treated groups and IL-1β stimulation alone.*

DISCUSSION & CONCLUSIONS: Overall, the results demonstrate a potent anti-inflammatory effect of the entire secretome and EV on human AF cells. However, EV from primed MSC may have a stronger effect on AF matrix metabolism, representing a promising therapeutic approach. Investigations of the proteomic content of the EV and secretome to identify potential effector molecules/subsequent pathways is ongoing.

ACKNOWLEDGEMENTS: *Deutsche Wirbelsäulenstiftung*.

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Extracellular Vesicles (MSC-EV) - influence on cartilage regeneration in osteoarthritic surroundings

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INTRODUCTION: Osteoarthritis (OA) is a complex immunologic disease. Current surgical and non-surgical concepts to treat OA only result in symptom-modifying effects. However, there is no disease-modifying therapy available. Extracellular vesicles released by mesenchymal stem/stromal cells (MSC-EV) are promising agents to positively influence joint homeostasis in osteoarthritic surroundings. Nevertheless, functional phenotypic differences of different MSC populations can influence the therapeutic potential of specific EV preparations. The aim of this study was to investigate the influence of characterized MSC-EVs on the chondrogenesis in a 3D chondrocyte pellet culture model. To analyze the therapeutic potential of MSC-EVs in a standardized inflammation model with the pro-inflammatory cytokine tumor necrosis factor alpha (TNFα) was used.

METHODS: Bovine articular chondrocytes were cultured and transferred into pellet culture at passage 3. TNFα (20ng/ml), human MSC-EV preparations (MSC-EV batches 41.5, 84; 2x 10^5 cell equivalent), EVs from human platelet lysate (hPL) or the combination of cytokine and EVs were supplemented. To assess the effect of MSC-EVs in the chondrocyte inflammation model after 14 days, DNA, glycosaminoglycan (GAG), total collagen, IL-6 and NO release were quantified, and gene expression of anabolic (collagen 2, aggrecan, COMP, PRG 4), catabolic (MMP 3, MMP 13), dedifferentiation (collagen 1), hypertrophy (collagen 10) and inflammation (IL-8) markers were analyzed; histological evaluation was performed using safraninO/ fast green staining and immunohistochemistry of collagen 1 and 2. Apoptosis was analyzed by immunolabeling of anti-active caspase 3. For statistical evaluation non-parametric tests were chosen with a significance level of $p<0.05$.

RESULTS: TNFα supplementation resulted in catabolic stimulation with increased levels of NO and IL-6, upregulation of catabolic gene expression and downregulation of anabolic markers. These findings were supported by a decrease of matrix differentiation (collagen 2) and increased apoptosis. Supplementation of EVs resulted in an upregulation of the chondrogenic marker PRG 4. All EV preparations significantly enhanced GAG retention per pellet (fig. 1).

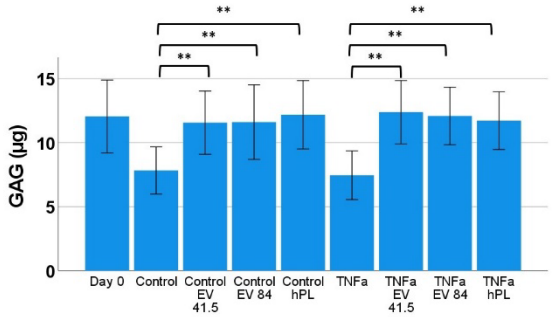

*Fig. 1 GAG quantification in pellet culture (day 0: 7 days preculture without treatment; control: no TNFα; TNFa: TNFα 20ng/ml; EV 41.5 and EV 84: MSC-EV preparations; hPL: EVs from human platelet lysate);n =12. ** = $p<0.05$.*

On the other hand, catabolic markers and IL-8 were upregulated by MSC-EV 41.5. On protein level, IL-6 and NO release was increased by MSC-EV 41.5. Histologic and immunohistochemical evaluation demonstrated a higher differentiation potential of MSC-EV 84.

DISCUSSION & CONCLUSIONS:

TNFα has a negative influence on chondrogenesis of chondrocyte pellets. MSC-EVs can positively influence chondrocyte matrix production in pro-inflammatory surroundings, but can also stimulate inflammation. In this study MSC-EV 41.5 enhanced inflammation, whereas MSC-EV 84 had a higher chondrogenic potential. MSC-EVs present heterogenous inter-donor and intra-preparation properties. Further investigation to identify a MSC-EV preparation for OA treatment needs to be addressed.

ACKNOWLEDGEMENTS:

We are thankful to Werner Masson[1], Lisa Grede[1], Robin Dittrich[2], Nora Goudsouzian[3] and Mauro Bluvol[3] for excellent technical support.

Terminal complement complex is a potential inducer of premature senescence in trauma- and age-associated osteoarthritis

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INTRODUCTION: Sublytic deposition of the terminal complement complex (TCC) has been associated with enhanced cytokine release and phenotypical alteration of chondrocytes.[1] Since oxidative stress and inflammation are main drivers of chondrosenescence in osteoarthritis (OA),[2] we investigated whether TCC deposition contributes to stress-induced premature senescence (SISP) after ex vivo injury or during in vivo aging.

METHODS: To study age-dependent OA, femoral condyles of 80-week-old CD59-KO mice (lack of TCC inhibitor CD59) and C6-deficient mice (insufficient TCC formation) were analysed by qRT-PCR. OA scoring was assessed by Safranin-O stained sections. Human cartilage explants were isolated from macroscopically intact (OARSI grade ≤1) or highly degenerated cartilage (OARSI grade ≥3). TCC was detected by means of IHC. After mechanical impact (0.59 J; applied by a drop tower), OARSI grade ≤1 explants were cultured with/ without 30% human serum (HS) and treated either with the TCC-inhibitor clusterin (CLU) or antioxidant N-acetylcysteine (NAC) for 4d. mRNA level of CDKN1A/ CDKN2A and SOD2 as well as senescence-associated secretory phenotype (SASP) markers (IL6, MMP13) were assessed by qRT-PCR. Unimpacted cartilage served as control. Data sets (n ≥5 donors per group) were analysed by means of one-way ANOVA or t-test.

RESULTS: In the murine aging model, we found significantly lower gene expression of *Cdkn1a* (-1,8-fold, p= 0.04), *Cdkn2a* (-2.3-fold, p= 0.005), *Il1b* (-1.7-fold, p= 0.02) and *Il6* (-1.8-fold, p= 0.02) in femoral condyles of C6-deficient mice as compared to the CD59-KO group, while no differences were found for *Mmp13* or *Sod2*. Overall, aged CD59-KO mice displayed a higher OA score than the C6-deficient group (p= 0.03).
In human cartilage, TCC deposition and the gene expression of CDKN1A and CDKN2A were significantly higher in OARSI grade ≥3 tissue than in macroscopically intact samples (3.1-fold, p= 0.002 and 4.7-fold, p= 0.02). Exposure to HS after trauma revealed additive effects on the mRNA levels of *CDKN1A, CDKN2A* and *IL6* (Fig. 1). IL-6 release was confirmed via ELISA. HS-mediated effects could be significantly reduced by CLU (Fig. 1). Although, NAC treatment significantly increased *SOD2*, it reduced *CDKN1A/ CDKN2A* expression only by trend and had no effect on IL6 mRNA levels (Fig.1) or secretion.

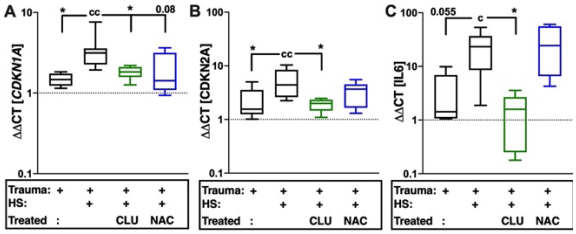

*Fig. 1: Relative mRNA levels of (A) CDKN1A, (B) CDKN2A and (C) IL6 in human cartilage 4d after trauma (T) and serum (HS) exposition w/ or w/o addition of CLU or NAC. c= [vs ctrl]; * = [vs T+HS], n ≥5.*

DISCUSSION & CONCLUSIONS: The present results imply a clear association between TCC deposition and a SISP-like phenotype of chondrocytes in the human ex vivo cartilage trauma model as well as in aged CD59-KO mice. We suggest that sublytic TCC deposition and subsequent cytokine release triggers chondrosenescence rather than the oxidative stress itself. However, further studies are required to clarify the underlying mechanisms in more detail. Overall, TCC might represent a potential target, in order to reduce phenotypical alteration of surviving chondrocytes after joint injuries.

ACKNOWLEDGEMENTS: This study was supported by the CRC1149 (DFG), the European Social Fund and Ministry of Science, Research, and Arts Baden-Württemberg

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What do mouse studies teach us about osteoarthritis?

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INTRODUCTION: In recent years our view of osteoarthritis (OA) has changed markedly. Instead of considering OA as a passive 'wear and tear' disease, we now know that OA initiation and progression are active processes, driven by molecular activities in a variety of cells within and outside the joint. While 'omics' studies have identified many molecules that become de-regulated in OA, the specific roles of many of these molecules in joint homeostasis and pathogenesis are not known. Moreover, functional studies are challenging to perform in humans, in particular for early stages of the disease that might be most amenable to therapeutic intervention. Thus, animal studies are commonly used to determine the mechanisms driving OA in vivo. In particular, the large variety of genetic tools to address the roles of specific genes and pathways during skeletal development, homeostasis, aging, and disease makes the mouse an important model to help understanding OA pathogenesis. This presentation will review progress in the field and provide insight from one example from our laboratory.

METHODS: We will review genetic tools available to manipulate gene function in mice. We will then review the various models used to induce OA in mice, as well as outcome measures employed by us and others. We will provide specific examples from our lab where the *Panx3* gene (encoding the channel protein Pannexin 3) was deleted either specifically in cartilage or globally in all cells (1,2).

RESULTS: We will discuss how mouse models of OA have contributed to better understanding of OA pathogenesis in multiple tissues, including cartilage, bone, synovium, and nervous system. Where possible, we will compare results from mouse studies to those from clinical studies, to highlight translational relevance.

One particular focus will be on how we can use reductionist approaches to examine one specific risk factor for OA at a time, while keeping others constant. For example, we can induce joint injury using surgery or other approaches and determine the role of a specific gene or pathway in this specific form of OA. We will provide several examples of how the same gene plays different roles in different subtypes of OA, contributing to the ongoing discussion of OA phenotypes.

In particular, we will present the example of our *Panx3* KO mice. These mutant mice are protected in a post-traumatic model of OA (destabilization of the medial meniscus/DMM model) (1) but show vastly accelerated OA development during aging (2). These highlights opposite roles of the same gene in different forms of OA, with implications for our understanding of this complex disease as well as for potential therapeutic approaches.

DISCUSSION & CONCLUSIONS: Mice have proven to contribute strongly to our understanding of pathogenesis and will play an important role in the design of new therapeutic strategies. However, there are also several limitations to the use of mice in OA research that will be discussed as well.

ACKNOWLEDGEMENTS: We thank all members of our lab that have contributed to these studies and our ongoing discussions. Similarly, we are very grateful for the strong contributions of numerous colleagues across the world to establishing the mouse as a model system for OA. We acknowledge funding of research by the CIHR, NIH, and Canada Research Chairs.

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Immunometabolism in articular cartilage and intervertebral disc in health and disease

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Metabolism plays a key role in the regulation of inflammatory and catabolic processes. Under adverse microenvironmental conditions, mammalian cells undergo a switch in metabolism from a resting regulatory state to a highly metabolically and activated state in order to maintain the energy levels required for physiological homeostasis. This metabolic shift occurs when oxygen levels are low (i.e. in physiological normoxia), limiting the metabolism of pyruvate by the tricarboxylic acid (TCA) cycle in mitochondria during oxidative phosphorylation. However, in some instances this occurs under aerobic conditions. Recent research suggests that this metabolic shift occurs in many degenerative and inflammatory conditions, thus representing a potential threat to cell function and survival. This phenomenon also leads to an increase in metabolic intermediates for the biosynthesis of inflammatory mediators and proteins involved in catabolic and degradative proteins. These metabolic intermediates can be pro-inflammatory and can activate key transcription factors and inflammatory signalling pathways involved in catabolic processes and the persistent perpetuation of drivers of disease pathogenesis. Environmental cues such as the availability of nutrients (i.e. glucose) and oxygen are sensed by the mechanistic target of rapamycin (mTOR), AMP-activated protein kinase (AMPK) and hypoxia-inducible factor 1 □ (HIF-1□) which comprise a series of circuits that can determine cell activation and differentiation. Immunometabolism is an emerging field that focuses on the role of cellular metabolism in the regulation of immune cell function. The interconnection between specific metabolic components and inflammatory pathways creates new opportunities for developing novel and targeted therapeutics in immune related diseases. This presentation will discuss aberrant immunometabolism in osteoarthritis (OA) and intervertebral disc (IVD) degeneration with a particular focus on metabolic dysfunction in chondrocytes and IVD cells. The role of impaired metabolism will be reviewed in the broader context of arthritis therapeutics, highlighting areas for future research, such as the potential to target immunometabolic pathways and mediators therapeutically.

Human organ culture model demonstrates potential regeneration of naturally degenerate discs with an injectable hydrogel system.

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INTRODUCTION: Low back pain resulting from disc degeneration is a leading cause for disability worldwide. However, to date few therapies target the cause and fail to tackle the degenerate intervertebral disc (IVD). We have previously reported an injectable hydrogel (NPgel), which could deliver patients own stem cells, via small bore needles, decreasing damage to the annulus fibrosus. NPgel drives differentiation to NP cells, and can inhibit the degenerate niche. However, clinical success of NPgel is dependent on the capacity to inject it into naturally degenerate human discs, restore mechanical function to the IVD, prevent extrusion during loading and induce regeneration. This study assessed injectability of NPgel into human IVD, influence on mechanical properties, regeneration ability in an *ex vivo* organ culture system and retention under failure testing.

METHODS: Cadaveric human discs were used to calculate disc height and to determine Youngs Modulus during simulated walking pre and post injection of NPgel, failure testing was then performed to investigate potential extrusion. To evaluate tissue repair strategies, whole human IVDs were injected with NPgel +/- human BMPCs and maintained in culture under physiological loading regime for 4 weeks. Pre and post culture MRI imaging was performed and in line biomechanical characteristics determined. Following culture discs were fixed, EDTA decalcified and paraffin embedded for histology and immunochemistry for matrix proteins, matrix degrading enzymes, cytokines and vascular and angiogenic factors.

RESULTS: NPgel injection significantly increased disc height and Youngs modulus (Figure 1A) with no extrusion observed during failure testing. T1ρ intensity was increased during culture in those injected with NPgel +/- cells compared to non-injected discs (Figure 1B), and biomechanical restoration. Histological analysis demonstrated excellent tissue attachment to the injected gel (Figure 1C), and cellular migration into acellular gel systems. With increased matrix production and decreased catabolic factor expression (Figure 1D).

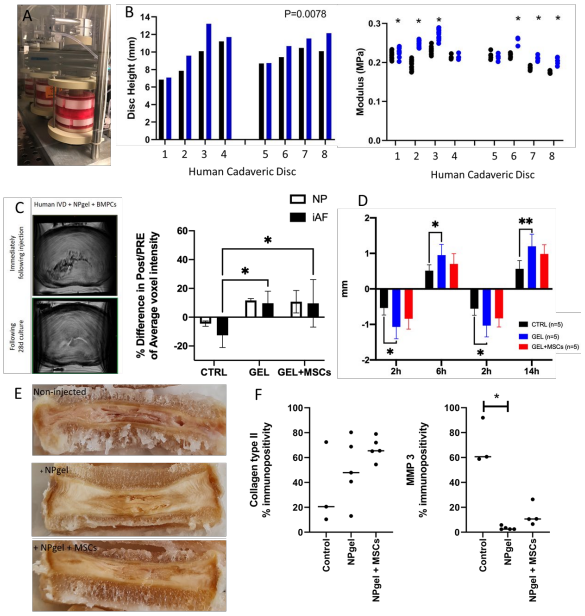

Figure 1: *a) Organ culture system, b) NPgel injection into cadaveric human IVDs induced a significant increase in disc height and youngs modulus. c) Mid axial T1r showed increased signal intensity in discs injected with NPgel +/- MSCs following 4 weeks of culture under physiological loading. d) Disc height change during daily loading. Compression of discs seen during high loading cycle (2hrs), followed by reswelling during low load periods (6hr & 14hr). Increased compression and swelling seen with discs increased with NPgel restoring normal biomechanical properties of the native human IVD. e) IVDs following EDTA decalcification, paraffin embedding, discs were sectioned into three transverse slices and macroscopic images captured. Naturally degenerate discs fissures could be clearly seen. NPgel filled cracks and fissures and appeared to show signs of regeneration. f) Immunohistochemical staining showed increased immunopositivity for collagen type II and decreased MMP3 following injection of NPgel into cadaveric discs following 4 weeks culture under physiological load. *=P<0.05.*

DISCUSSION & CONCLUSIONS: Injection of NPgel into naturally degenerate human IVDs increased disc height and Youngs modulus and was retained during extrusion testing. Injection into cadaveric discs followed by culture under physiological loading increased MRI signal intensity, restored natural biomechanical properties and showed evidence of increased anabolism and decreased catabolism with excellent integration observed. Providing essential proof of concept data supporting the use of NPgel as an injectable therapy for disc regeneration.

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Image-based Analysis of Mass Transport Through Cartilage Endplates

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INTRODUCTION: The etiology of lower-back pain (LBP) is multifactorial. The cartilage endplate (CEP) is the spinal tissue receiving the least attention in scientific literature. Yet it plays a crucial role in keeping the intervertebral disc (IVD) healthy since it is the main gateway to nutrients and waste in and out of the nucleus pulposus in the avascular IVD[1]. It is also suggested that imperfections and weaknesses in the CEP can be a better anticipator of pain than IVD degeneration[2] since chemical changes to the CEP are directly related to the intervertebral disc degeneration[3]. Immunohisto-chemical research showed that the decline of CEP permeability due to calcification causes blood vessels and nerve fibers to infiltrate the IVD from the CEP and subchondral bone in an attempt to repair the damage and this infiltration leads to LBP[3]. In this study we aim to investigate the mass transport through the CEP in and out of the IVD starting from CT images.

METHODS: Given the scarcity of human CEP, a fresh bovine tail was obtained from the local slaughterhouse and 6 CEPs were harvested from it. 8 mm punches were taken from the CEPs. The samples were incubated with 20% Hexabrix, a contrast agent to visualize sulfated glycosaminoglycans. The samples were imaged on a Nanotom® M (GE nanoCT©) using in-house optimized settings for osteochondral tissues.

The CT images were exported to Salome and manually curated to generate a high-quality volume mesh of the CEP. OpenFOAM® was used to perform Computational Fluid Dynamics (CFD) Simulations to quantify the mass transport through the CEP.

The CEP samples were fitted into the 8 mm silicon tubes of a custom-made mesofluidic setup and fixed in place using clamps. physiological fluid (PBS) and standard growth medium were passed through the specimens separately. The specimens were left to fill then the time to pass 10 ml of solution was recorded for each specimen in the forward and reverse flows to calculate the volumetric flow rate and the permeability using Darcy's law.

RESULTS: The nCT imaging settings used resulted in a good quality image of the scanned samples. The 20% Hexabrix was unable to fully stain the CEP and a higher concentration is currently being tested. Both the CFD simulations and the in vitro diffusion tests are ongoing.

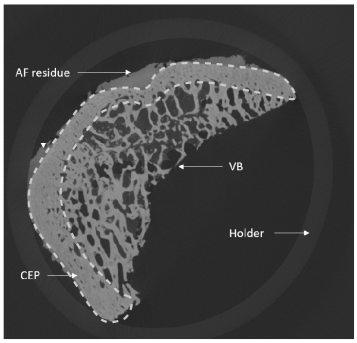

Figure 1: nCT-scan of CEP with vertebrate bone attached (sagittal view)

DISCUSSION & CONCLUSIONS: Combining image-based in silico modelling with in vitro experiments, as proposed in this study, will allow to quantify the fluid dynamics across the CEP, which is the gateway controlling mass transport in and out of the IVD.

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EXCESSIVE WNT SIGNALLING ALTERS CARTILAGE'S MECHANO-RESPONSE IN A HUMAN EXPLANT *IN VITRO* MODEL

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INTRODUCTION: Regulation of articular cartilage homeostasis is a complex process in which biologic and mechanical factors are involved. Tissue loading and unloading cycles represent mechanical stimuli essential for the chondrocytes to maintain cartilage integrity by mechanotransduction[1]. Hyperactivation of Wnt signalling, associated with osteoarthritis (OA), could jeopardize the protective anabolic effect of physiological loading[2]. Therefore, in this study we investigated the role of excessive Wnt signalling in cartilage molecular responses to cyclic compressive loading using a cartilage explant *in vitro* model.

METHODS: Human cartilage explants were harvested from hips of donors without OA (N=5). The Wnt agonist CHIR99021 was used to activate Wnt signalling 24 hours before cartilage explants were subjected to a loading protocol, representative for a physiologic stimulation by mechanical loading, consisting of 2 cycles of 1 hour of 10% compression at 1 Hz, followed by 1-hour free swelling in the BioDynamic® 5200 bioreactor (TA Instruments). After loading, RNA was isolated and mechano-responsiveness was evaluated using real-time quantitative PCR for type II Collagen, Sox9, Aggrecan and MMP-13. Expression of known target genes TCF-1 and c-JUN was evaluated as positive control for Wnt and mechanical stimulation, respectively. Furthermore, effects of mechanical stimulation and Wnt hyperactivation on the pericellular matrix (PCM) was also evaluated by analysing the expression of type XI collagen and Perlecan.

RESULTS: In the absence of loading, CHIR99021 decreased the expression of the cartilage anabolic genes collagen type II, Sox9 and Aggrecan, and increased the levels of MMP-13, corroborating that Wnt hyperactivation disrupts cartilage homeostasis. In the absence of Wnt hyperactivation, the applied loading protocol led to an increase in type II collagen and Sox9 expression. However, when cartilage explants were subjected to mechanical stimulation in the presence of CHIR99021, the expression of cartilage anabolic genes was decreased and the catabolic enzyme MMP-13 was upregulated, indicating changes to the cells' mechano-responsiveness following Wnt hyperactivation. Interestingly, this condition also inhibited the loading-induced expression of the PCM markers type XI collagen and Perlecan (Fig. 1).

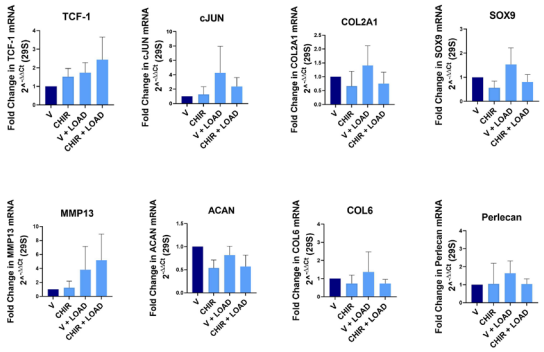

Fig. 1: Altered cartilage's response to physiologic loading upon Wnt hyperactivation under CHIR stimulation. Graphs represent the fold change in TCF-1, c-JUN, COL2A1, SOX9, MMP12, ACAN, COL6 and Perlecan mRNA levels compared to the control. Data are expressed as Mean ± SD (N=5); V, vehicle.

DISCUSSION & CONCLUSIONS: Hyperactivation of Wnt signalling alters cartilage's mechano-responsiveness by reversing the anabolic effect of physiologic compressive loading towards a more catabolic effect, which could contribute to the development and further progression of OA.

ACKNOWLEDGEMENTS: This work was funded through KU Leuven/FWO grants C14/18/077, G045320N and 12Y7422N.

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Activation of YAP suppresses cartilage inflammatory signalling and matrix degradation

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INTRODUCTION: In articular cartilage, Interleukin-1β (IL-1β) triggers inflammatory signalling leading to matrix degradation [1]. In vivo studies have shown that activation of mechanosensitive Yes-associated protein (YAP) can supress degradation of cartilage [2]. However, the exact role of YAP in osteoarthritis (OA) progression remains unclear, with other studies suggesting YAP activation promotes disease progression in OA [3]. Here we test the hypothesis that activation of YAP supresses chondrocyte inflammatory response to the cytokine, IL-1β.

METHODS: Isolated bovine chondrocytes were cultured on PDMS substrates with stiffnesses of 6 and 400kPa and YAP activation was measured by immunofluorescence. Cells transfected with YAP siRNA or scrambled control were treated with IL-1β (10ng/ml, 24hrs) and inflammatory signalling was measured by release of nitric oxide (NO) and prostaglandin E2 (PGE2). Further studies were conducted with isolated chondrocytes and cartilage explants treated with the YAP agonist LPA. Inflammatory signalling was measured as above, along with cartilage matrix degradation quantified by release of sGAG and uniaxial unconfined compression after 12 days culture.

RESULTS: On stiffer substrates, chondrocyte YAP activation was increased, as shown by nuclear translocation (Fig 1A). This was associated with a reduction in inflammatory signalling measured by NO release (Fig 1B). Inhibition of YAP with siRNA increased NO release and blocked the regulation by substrate stiffness (Fig 1B). Similar responses were observed for PGE2 release (data not shown). Similarly, activation of YAP by the agonist LPA, blocked IL-1β induced inflammatory signalling in isolated chondrocytes (data not shown) and in cartilage explants (Fig 1C). Treatment with LPA also blocked cartilage matrix degradation caused by IL-1β as quantified by reduced sGAG release (data not shown) and inhibition of the reduction in tangent modulus (Fig1 D)

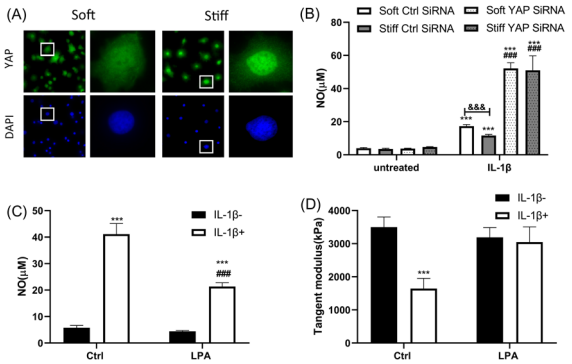

Figure 1 Activation of YAP by increasing substrate stiffness or pharmaceutical regulation suppresses inflammatory response and matrix degradation

*(A) YAP immunofluorescence on soft (6kPa) or stiff (400kPa) substrates and (B) corresponding NO release +/-IL-1β for YAP siRNA and scrambled control. (C) Cumulative NO release from cartilage explants treated for 12 days +/-IL-1β +/-LPA and (D) corresponding tangent modulus (D). ***p<0.001*

DISCUSSION & CONCLUSIONS: YAP activation as a result of increased substrate stiffness suppressed IL-1β signalling. The YAP agonist, LPA also blocked IL-1β signalling in isolated cells and cartilage explants and prevented matrix catabolism and loss of biomechanical integrity. Hence, cartilage degradation may exacerbate inflammatory signalling by reducing chondrocyte YAP activity. Conversely, targeted pharmaceutical activation of YAP may provide a novel treatment for inflammatory joint diseases such as OA.

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Generation of intervertebral disc progenitor cells from human induced pluripotent stem cells

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INTRODUCTION: Low back pain is one of the most common musculoskeletal disorders. Although aetiology is complex and not fully understood it is often associated with degeneration of the intervertebral disc (IVD). There is currently no effective treatment for disc degeneration. This is largely due to a lack of basic knowledge of the molecular and cellular controls of disc development, growth, maturation, and homeostasis, during embryogenesis and at different stages of life[1].

The notochordal cells (NCs) are the founder cells of the centre of the disc, the nucleus pulposus (NP). They originate from an axial mesoderm-derived embryonic structure with signalling function called the notochord. After birth, they have matured and reside within the NP where they behave as key regulators to keep the disc healthy. With ageing process, the observation is made that NCs disappear leaving room for anabolic/catabolic imbalance and tissue degeneration. Increasing research studies have demonstrated that native NCs exert rejuvenating effects on degenerated disc. Consequently, it has been broadly considered that NCs may be prime targets for cell-based therapeutic interventions to delay degeneration or mediate regeneration of damaged disc. Since NCs' number dramatically decreases within the disc as smaller NP cells replaced them, no robust cell model exists to further study NCs and their maturation into functional cells with regenerative properties.

By translating fundamental knowledge from mouse developmental biology to human induced pluripotent stem cells (iPSCs) research, we recently demonstrated that WNT signalling pathway activation and the expression of notochord-related transcription factor NOTO are sufficient to trigger the emergence of a stable population of cells with specific molecular features of embryonic notochord (RNA-Sequencing DGE-seq) [2].
Research efforts are pursued to identify the trajectories and key molecular mechanisms governing cell fate decision to reduce cellular heterogeneity observed during iPSCs differentiation process toward notochord-like cells (NLCs), notably with the presence of off-target endoderm and mesoderm derivatives. In addition, the identification of NC-specific surface markers will allow straightforward purification of the NLCs population for further use in basic or translational research.
To address these issues we performed a transcriptomic analysis at the single cell level at distinct differentiation time points. Overview of the research work and finding advancing our understanding of the basic regulatory network controlling human notochord differentiation program will be presented.
Phenotypic maturation of these embryonic NLCs should result in a regenerative-cell source of mature NCs with great potential in disc degenerative disease. Investigations on the influence of high-density 3D micromass culture (compared to classical 2D culture) on notochord identity maintenance and phenotypic maturation (appearance of cytoplasmic vacuolated phenotype in association with the synthesis of collagen type II- and aggrecan-rich extracellular matrix) will be presented. Mature NCs should express relevant adult notochord-specific factors that may also be essential players for healthy adult disc maintenance. This work will contribute to the imperial need to better characterise the precise phenotype of cells with the therapeutic potential to regulate disc homeostasis and develop innovative clinical approaches for IVD repair [3].

ACKNOWLEDGEMENTS: Financial support was received from EU Horizon 2020 "iPSpine" (grant no. 825925) and French Society of Rheumatology "Spherodisc".

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Induced pluripotent stem cells for cartilage regeneration

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BACKGROUND:

Chondrocytes are the central elements of cartilage regeneration not only when directly implanted into cartilage defects during clinical therapy. Chondrocytes are also essential for basic research studies that continuously increase our understanding of embryonic cartilage development, further illuminate mechanisms of cartilage pathology, and develop novel cartilage therapies. Moreover, we need chondrocytes to test the effectiveness and tolerability of new cartilage therapeutic drugs. For all these variable applications, different cell types with chondrogenic capacities, ranging from committed chondrocytes to mesenchymal stromal cells (MSCs) and induced pluripotent stem cells (iPSCs) are currently being used. With increasing immaturity of the originating cells, the challenges to obtain cartilage of good quality using cell differentiation and tissue engineering-based approaches intensify. Although growing in number, convincing reports of successful iPSC chondrogenesis remain rare.

IPSC-DERIVED CHONDROCYTES:

The attractiveness of iPSCs for cartilage regeneration roots from three major beneficial features. 1) Their ability to undergo chondral development and give rise to articular chondrocyte (AC)-like non-hypertrophic cells that do not mineralize and do not induce ectopic bone formation in vivo. Thereby, iPSCs overcome MSCs which inevitably undergo endochondral development during in vitro chondrogenesis. 2) iPSCs can be reprogrammed from any nucleated body cell and are thus non-invasively available. By contrast, non-regenerating cartilage defects need to be created to harvest ACs. 3) Their high capacity for cell expansion makes iPSCs available in virtually unlimited amounts, while both, ACs and MSCs loose cartilage formation potential during excessive expansion in vitro. Their clonal expansion capacity allows genetic cell engineering of iPSCs, which is inapplicable in MSCs and ACs, and which is a highly capable tool to illuminate the function of individual genes and transcripts in cartilage development, homeostasis and pathology.

On the downside, iPSC generation, culture and differentiation are extremely laborious. The high variability of iPSC lines and significant gaps of knowledge regarding the cellular events triggered by individual differentiation signals currently limit our ability to transfer protocols between independent groups and reproduce key results. Filling in these gaps and defining reproducible standard protocols for iPSC chondrogenesis is now necessary to harness the full potential of iPSCs for cartilage regeneration.

This presentation will focus on the status and recent progress in chondrogenic in vitro differentiation of human iPSCs. The most relevant differentiation strategies will be summarized and major insights and achievements will be highlighted. Limitations of our current understanding will be discussed along with future perspectives.

HYPEROSMOLAR EXPANSION MEDIUM IMPROVES CANINE NUCLEUS PULPOSUS CELL PHENOTYPE

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INTRODUCTION: Lower back pain due to intervertebral disc (IVD) degeneration is a major health problem and often starts in the centre of the IVD, the nucleus pulposus (NP). Repopulating the degenerated IVD with tissue-specific NP cells has already been shown to promote regeneration in various species. Yet the applicability of NP cells as cell-based therapy has been hampered by the low cell numbers extracted from donor IVDs and their potentially limited regenerative capacity due to their degenerated phenotype. A promising strategy to optimize NP cell expansion is to employ physiological stimuli *e.g.* osmolarity[1], which is known to decrease during the degenerative process *in vivo*[2]. Therefore, the aim of this study was to investigated the effect of increasing culture medium osmolarity during expansion on the phenotype of dog NP cells and their regenerative capacity in a 3D culture model.

METHODS: NP cells of 6 Beagle donors (mildly degenerated IVDs) were expanded for 2 passages in expansion medium with a standard osmolarity (300 mOsm/L) or adjusted to 400 or 500 mOsm/L mimicking increased osmolarity of the healthy IVD. Following expansion, cells from each condition were re-differentiated for 14 days in 3D re-differentiation culture in chondrogenic medium with a standard osmolarity of 300 mOsm/L to mimic placing them back in the degenerated IVD environment. Readout parameters during expansion were focussed on cell morphology, phenotype and proliferation rate. After re-differentiation we assessed the phenotype and regenerative capacity of the NP cells.

RESULTS: NP cells expanded in medium with increased osmolarity (500 mOsm/L) were able to maintain an *in vivo*-like, rounded (less fibroblast-like) cell morphology at the expense of a lower cell proliferation rate (Fig. 1). Further, expansion in 500 mOsm/L lead to a significant increase of several healthy NP cell and progenitor markers at gene (*KRT18, ACAN, COL2, CD73, CD90*) and protein level (ACAN, PAX1, CD24, TEK, CD73) compared to NP cells expanded in standard medium osmolarity (300 mOsm/L). Immunohistochemical stainings for ACAN, PAX1, CD24 and TEK and qualitative and quantitative assessment of extracellular matrix deposition showed that NP cells that had been expanded at 500 mOsm/L were able to retain their NP cell phenotypic markers and regenerative capacity in the 3D re-differentiation culture model.

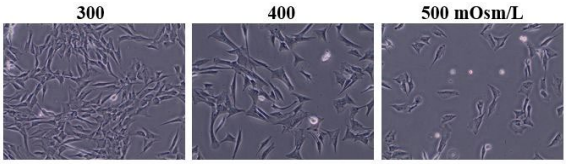

Fig 1: Brightfield images of NP cell morphology at day 3 of expansion in the different media osmolarities.

DISCUSSION & CONCLUSIONS: Our data show that expansion of dog NP cells in higher osmolarity promotes the expression of healthy NP cell and progenitor markers at gene and protein level. This population with improved NP cell phenotype could have a beneficial potential for cell-based therapies. Altogether, we expect that future studies on mitigating the effects of higher osmolarity on cell proliferation rates will make NP cells a powerful addition to the cell-based regenerative toolbox for IVD degeneration.

ACKNOWLEDGEMENTS: The European Union's Horizon 2020 program (iPSpine; #825925) and the Dutch Arthritis Society (LLP22) supported this work.

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Could Notochordal Cells be the future of cell-based treatment for Disc Degeneration?

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INTRODUCTION: Low back pain resulting from Intervertebral disc (IVD) degeneration is a serious worldwide problem, with ineffective treatment options available thus, establishing a cell-based treatment is a necessity. Notochordal cells (NC), could be an attractive cell source displaying anti-catabolic and regenerative effect. However, their behaviour in the harsh degenerate environment is unknown. Thus, we firstly aimed to investigate and compare their physiological behaviour in an *in vitro* niche that mimics the healthy and degenerated IVD environment. Secondly, we aimed to set up co-culture of these cells with degenerated human NP cells to further understand their anti-catabolic effect on degeneration process.

METHODS: Porcine NCs, isolated from porcine spine encapsulated in 3D alginate beads to maintain their phenotype. Initially, pNCs were cultured in media to mimic the healthy and degenerate disc environment (+/- 100pg/ml IL-1β), together with control NC media for 1 week. Following which viability using PI and Calcein AM, RNA extraction and RT-PCR for NC cell markers, anabolic and catabolic genes analysed. Proteomic analysis was also performed using Digiwest technology. Next, human NP cells (hNP) isolated from NP tissue expanded in monolayer prior to encapsulation in alginate beads for two weeks before being cultured in media to mimic the healthy and degenerated disc environment (+/- 100pg/ml IL-1β. The culture of human NP cells was established either alone or in co-culture with pNCs or stimulated with notochordal cell condition media (NCCM). Cells were sampled after 48 and 72 hrs for gene expression, and after 72hr and 1 week for protein analysis (Luminex and Digiwest respectively).

RESULTS: A small increase in NC death was observed in degenerated media compared to standard and healthy media, with a further decrease seen when cultured with IL-1β (Figure 1). Whilst no significant differences were seen in phenotypic marker expression in NCs cultured in any media at gene level (ACAN, KRT8, KRT18, FOXA2, COL1A1 and Brachyury). Preliminary Digiwest analysis showed increased protein production for Cytokeratin 18, src and phosphorylated PKC but a decrease in fibronectin in degenerated media compared to standard media (Figure 1). Luminex analysis of cytokine expression showed significant increases in cytokine production by human NP cells cultured in degenerated media + IL-1β as compared to other treatment conditions (Figure 2).

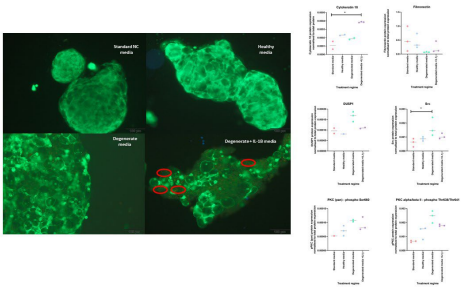

Fig. 1: Viability staining of porcine NC cells in 4 different media (left) Digiwest analysis of protein expression in NC cells (right).

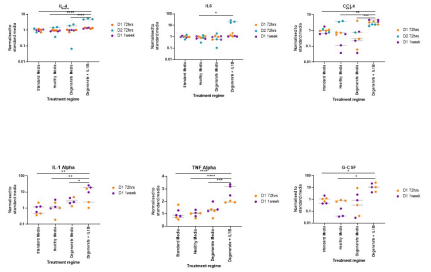

Fig. 2: Luminex analysis of cytokines expression by human NP cells cultured in 4 different media treatments.

DISCUSSION & CONCLUSIONS: Studying the behavior of the NCs in *in vitro* conditions that mimic the *in vivo* healthy or degenerate niche will help us to better understand their potential for therapeutic approaches. This work is being translated to investigate the potential use of iPSCs differentiated into notochordal like cells as potential regenerative cell sources.

ACKNOWLEDGEMENTS: The European Union's Horizon 2020 program (iPSpine; #825925) and (H2020-MSCA-ITN-ETN-2020 GA: 955735) supported this work.

Identifying a unique gene profile for the meniscus cells in tissue and *in vitro* monolayer and pellet cultures

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INTRODUCTION: Adult meniscus contains distinct zones that have been shaped during development due to different biomechanical loading within tissue regions. Compressive loads on the inner meniscus leads to an avascular zone with an articular cartilage-like matrix, whilst tensile loads on the outer meniscus results in a more vascularized zone with a predominantly collagen I-rich matrix, similar to tendon (1). Due to these developmental features, cartilage and tendon genes have been used to characterise the meniscus phenotype (e.g. SOX9, MHWK). Recent studies have also proposed meniscus-specific genes (e.g. IGF-BP3, R-Spondin-2, CILP2, CHAD, CSRP2, ID2, Dermatopontin, Collagen XV) (2, 3). Here, using a combination of multiple gene candidates, we investigated their specificity on meniscus tissue, as well as on avascular and vascular meniscus cells in *in vitro* 2D and 3D cultures.

METHODS: Portion of human meniscus tissue biopsies (n = 6) was snap frozen. The remaining tissue was split into avascular and vascular regions, then sequentially digested in pronase (70U/mL) and collagenase (200U/mL) at 37^{0}C. Avascular and vascular meniscus cells were isolated and then cultured in either a 20% oxygen (hyperoxia) or 2% oxygen (physioxia) incubator. At passage 1, QIAzol was applied to monolayer cells at each oxygen tension and frozen. At passage 2, avascular and vascular meniscus pellets were created and cultured for 21 days at 2 or 21% oxygen tension using previously described protocol (4). RNA was isolated from tissue, cells and pellets and subsequent qPCR analysis for the eight genes was performed (4).

RESULTS: Meniscus tissue showed elevated expression levels of all selected genes when compared to cartilage tissue control, with a significant upregulation in CLIP2, CSRP2, Dermatopontin and collagen XV (*p < 0.05; Figure 1a). In monolayer cells, all genes were downregulated apart from IGF-BP3. Upon pellet redifferentiation, only IGF-BP3, CHAD and Dermatopontin were upregulated in avascular and vascular meniscus cells with no significant difference with respect to oxygen tension (Figure 1b).

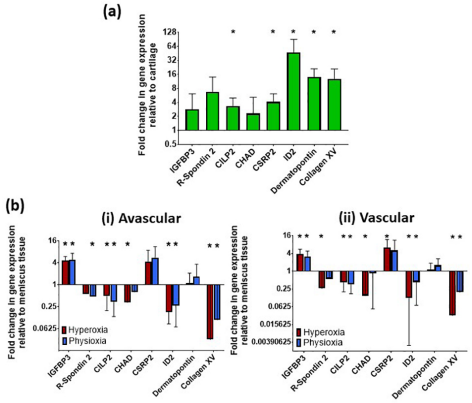

*Fig. 1. Gene expression in (a) meniscus tissue relative to cartilage and in (b) pellet cultures of (i) avascular and (ii) vascular meniscus under hyperoxia and physioxia relative to meniscus tissue for stated gene (n = 6; *p < 0.05)*

DISCUSSION & CONCLUSIONS: All selected genes were upregulated in meniscus tissue. Monolayer expansion led to downregulation of all genes, whilst subsequent pellet redifferentiation resulted in upregulation of only IGF-BP3, CHAD and Dermatopontin. Future studies will identify optimal *in vitro* conditions to maintain expression of meniscus-specific genes, thus preventing phenotype drift during cell expansion.

ACKNOWLEDGEMENTS: This project was funded by HORIZON 2020: 814444 (MEFISTO)

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Pro-inflammatory Priming of Mesenchymal Stem Cells Increases their Immunosuppressive Properties only short-term

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INTRODUCTION: Mesenchymal stem cells (MSCs) have been studied in numerous clinical trials due to their exceptional regenerative and immunomodulatory potential on a wide variety of conditions, including degenerative disc disease and osteoarthritis. Various priming strategies including pro-inflammatory, chemical, hypoxic, and 3D culture priming have been shown to enhance the therapeutic potential of MSCs. To consider a priming strategy for clinical application, MSCs should maintain their primed phenotype after administration. We investigated the temporal response of MSCs to pro-inflammatory priming *in vitro*.

METHODS: Bone marrow-derived MSCs were obtained from vertebral aspirates through plastic adherence. MSCs were primed for 72 h with tumor necrosis factor alpha (TNF-α) (20 ng/mL), interferon gamma (IFN-γ) (20 ng/mL), or a combination of TNF-α and IFN-γ (both 20 ng/mL) in minimum essential medium α. The effect of pro-inflammatory priming on the expression of indoleamine 2,3 dioxygenase 1 (IDO1) and programmed death-ligand 1 (PD-L1) was measured by real-time quantitative PCR (RT-qPCR) and compared to unprimed MSCs. Transcriptional changes of key pro-inflammatory and immunomodulatory factors of TNF-α and IFN-γ primed MSCs were assessed by RT-qPCR over nine days.

RESULTS: First, we compared different priming media compositions to achieve a strong pro-inflammatory priming of MSCs. The combination of TNF-α and IFN-γ showed the strongest upregulation of IDO1 and PD-L1 (Fig. 1) and was therefore chosen for the subsequent time course experiment. Pro-inflammatory priming of MSCs led to a strong upregulation of genes associated with immunomodulation and cell migration (Fig. 2). Upregulation faded within one week.

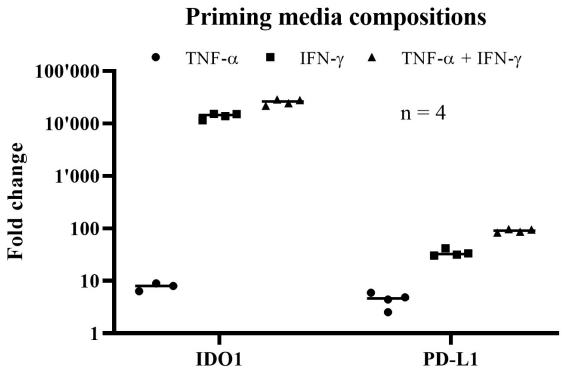

Fig. 1: Comparison of fold change gene expression between primed and unprimed MSCs as measured by RT-qPCR. All differences are considered highly significant (P-value < 0.001) as tested with an ordinary one-way ANOVA with Tukey's multiple comparison test for IDO1 and PD-L1 individually.

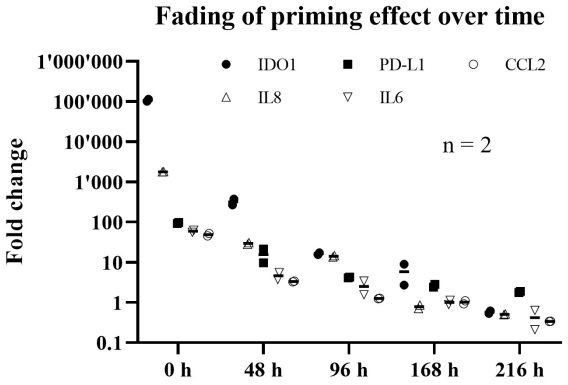

Fig. 2: Comparison of fold change gene expression between primed and unprimed MSCs over nine days as measured by RT-qPCR. Interleukin 6 (IL6), interleukin 8 (IL8), C-C motif chemokine ligand 2 (CCL2).

DISCUSSION & CONCLUSIONS: The therapeutically desired cytokine profile of pro-inflammatory primed MSCs is rapidly lost after removing the priming agent. A continuous inflammatory stimulus might be needed to maintain the immunomodulatory phenotype. Administration of MSCs in inflammatory tissues might ensure a continuous stimulus, thereby possibly decelerate the fading of the priming effect.

Computational modeling and artificial intelligence in spine research

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INTRODUCTION: In the last decades, the importance of computational modeling for the investigation of research questions in the field of orthopedics has been continuously increasing. The available literature currently contains thousands of papers and covers all the major joints and anatomical districts, with the spine playing a prominent role. More recent years have seen the rise of machine learning and artificial intelligence (AI) in several fields, including spine research. Such technologies are increasingly used for several applications such as object detection in images and videos, speech recognition, autonomous driving, and natural language processing, and have recently started to dramatically impact spine research as well.

COMPUTATIONAL MODELING: Computational models used in spine research can be classified into: (1) multibody models, based on dynamics of rigid bodies and (2) finite element (FE) models, based on the equations of continuous mechanics. Multibody models are typically used to estimate physiological loads in vivo based on inverse dynamics simulations, and provide a valuable tool to study the biomechanical functioning of the musculoskeletal system. However, these models have some limitations: the body segments are assumed to be rigid, thus neglecting deformations, and muscles are commonly simplified as single line actuators. On the other side, FE models are based on the division of complex mechanical structures into finite numbers of separate components with simple geometry, called elements. In this way, complex nonlinear problems are converted in problems that can be solved numerically. FE models can be employed to investigate issues such as local stresses and strains, contact between tissues and implants, as well as material nonlinearity, damage and failure.

The application of computational modeling to research questions regarding the spine goes back to the 1980s, when it was used to investigate the stress-distribution on a vertebra after laminectomy and Harrington rod procedures. Currently, computational models of the spine are used in various fields, such as: implant development and design optimization, surgical treatment analysis, e.g. scoliosis, conservative treatment, e.g. bracing, investigation of spinal pathologies, such as disc degeneration, investigation of the aetiology of diseases, bone remodelling after spinal fusion, prediction of vertebral fractures. Computational models are typically built based on medical imaging such as CT scans (Fig. 1).

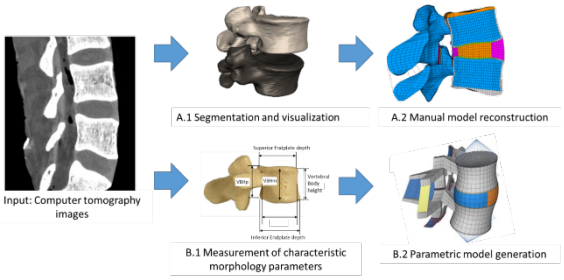

Fig. 1: Development of a computational model based on a CT scan, either anatomically detailed (top) or simplified (bottom).

ARTIFICIAL INTELLIGENCE: Machine learning and AI methods have also been used for several applications such as the evaluation of the severity of scoliosis on X-rays, to classify the pattern of curvature of adolescent scoliotic patients, to predict the outcome of treatments as well as to process MRI scans. One of the most well-known solutions for this task is SpineNet (http://zeus.robots.ox.ac.uk/spinenet2), a freely available tool developed by researchers at the University of Oxford able to perform the automated extraction and classification of degenerative features in lumbar sagittal MRI scans, e.g. disc degeneration grading, disc narrowing, endplate alterations, localization of the so-called "evidence hotspots" which justify the model predictions, as well as automated localization and segmentation of intervertebral discs in three dimensional datasets. Many other applications are being developed and new papers are appearing continuously.

CONCLUSIONS: Computational models of the spine and AI tools have shown to be very powerful instruments and are used for a wide range of research and clinical applications, having a strong potential to improve diagnosis and patient-specific treatment outcomes.

AN INTEGRATED, MULTI-SCALE APPROACH TO UNDERSTAND THE ROLE OF MECHANICAL LOADING IN CARTILAGE HOMEOSTASIS AND DISEASE: BRIDGING FROM JOINT TO CHONDROCYTE.

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INTRODUCTION: To design and customize therapeutic approaches that successfully support cartilage health, arrest cartilage damage, and even promote cartilage regeneration, it is indispensable to understand how articular cartilage is loaded during movement. It is within the context of this local cartilage loading environment that biological responses of chondrocytes need to be evaluated. To this end, my group is using a multi-scale *in silico* approach to integrate experimental insights on the role of mechanical loading in degenerative joint diseases, such as osteoarthritis (OA).

METHODS: We use dedicated integrated 3D motion capture techniques in human OA patients, as well as biplanar fluoroscopy in rodent models of OA, to capture dedicated kinematic and kinetic characteristics of locomotion. Using personalized or generic multi-body musculoskeletal models, we then identify biomarkers of (early) OA during locomotion at both joint and tissue level using multi-scale models. Integration of mechano-regulatory algorithms allows us to predict cartilage tissue response in terms of constituent damage and loss. Furthermore, information on disease-state specific, mechano-responsiveness at molecular level as observed during multi-axial bioreactor experiments, are integrated to predict both anabolic and catabolic chondrocyte responses to loading. For evaluating OA-related changes in mechanical characteristics at cell and tissue level, 3D traction force microscopy and high-field MRI techniques are explored.

RESULTS: Using this multi-scale modelling approach, we have been able to identify elevated contact pressures and altered loading locations as biomarkers of early knee OA during gait that discriminate progressing and non-progressing patients. Furthermore, collagen loss was identified as the point of no-return in primary and secondary OA, whereas collagen fibril reorientation protected against excessive local cartilage deformation and consequent constituent damage and depletion. Additionally, Wnt upregulation as documented in early OA, was found to impact anabolic mechano-responsiveness, thereby further impairing the inherent regenerative potential of OA-implicated chondrocytes.

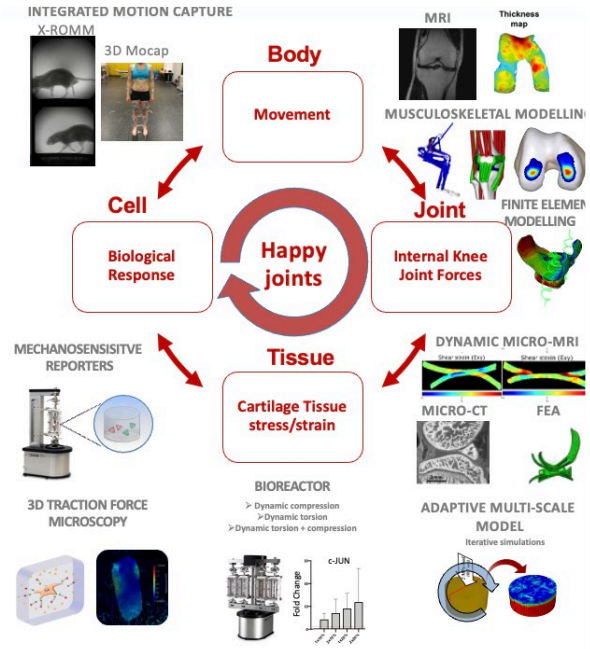

Fig. 1: Conceptual multi-scale framework to understand degenerative joint disease.

DISCUSSION & CONCLUSIONS: Multi-scale insights will be the basis of innovative, regenerative mechano-therapy that provides an optimally-tuned combination of biological and mechanical stimulation, with the unique potential to prevent, slow down, or one day reverse early structural cartilage changes that would otherwise progress to end-stage OA.

ACKNOWLEDGEMENTS: This work was funded through MSCA-IF-2019-893771; KU Leuven/FWO grants C14/18/077 and G045320N and EOS excellence funding G0F8218N.

The mechano-regulation of phenotypic plasticity studied in a developmental engineering model of endochondral ossification

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INTRODUCTION: Biological processes are guided by a genetic instruction manual and shaped by biological and physical cues. How mechanical cues are integrated into the coordinated formation of bone from a cartilage template in the epiphyseal plate is not well understood. How adolescent growth and controlled ossification is tolerant to the heterogenic forces in the growing limb is poorly understood. We have found that primary cilia proteins (IFT88) play a role in safeguarding phenotypic transitions governing endochondral ossification from physiological loading (1). We hypothesise ciliary proteins act as a mechanodampener in chondrocytes to help control coordination of change and resilience of phenotype. **METHODS:** To follow-up and compliment *in vivo* studies in the mouse we are using a developmental engineering approach to create a murine and humanisable gradient model of endochondral ossification. Mouse primary and human bone-marrow derived stem cells are seeded in a bulk gel (gelatin methacryloyl, functionalization assessed by NMR and stiffness controlled) with a controllable gradient of heparin methacrylate to carry affinity bound BMP-2. Over a 28-day period these constructs are differentiated (2). This chondro-osseous model is being characterized using uCT, confocal and light sheet microscopy, by IHC and RNA scope initially before bulk, single nuclei and spatial transcriptomics. Exogenous loading is applied using a cell culture loading rig. Tamoxifen is used *in vitro* to delete IFT88 in differentiated chondrocytes isolated as BMDMSC from ACAN_IFT88$^{fl/fl}$ mice (3). now crossed to ARL13bmcherry/CentrinGFP (4) to visualize cilia both *in situ* and *in vitro*.**RESULTS:** *In vivo* deletion of IFT88 (KO) or increased loading uncouples trans-differentiation of epiphyseal hypertrophic chondrocytes. In these regions IHC reveals increased pSMAD2/3 activity and sustained SOX9 expression. VEGF expression is lost along with vascular invasion and recruitment of osteoclasts. This is rescued in KO mice by off-loading. *In vitro*, BMP-driven differentiation leads to presumptive hypertrophic chondrocytes and controlled bone differentiation. Within constructs differential morphologies of chondrocytes are clear from phalloidin staining, hypertrophy from histology and differentiation from SOX9 and RUNX2 staining. Preliminary data suggests the *in vitro* model can be used to track and understand chondro-osseous transitions *in vitro***DISCUSSION & CONCLUSIONS:** *In vivo* data, suggest IFT88 dampens force-induced TGFβ signalling, and SOX9 expression, protecting chondrocyte trans-differentiation and VEGF-mediated vascular invasion. An *in vitro* model of endochondral ossification can track these processes in human and murine cells in 3D. Confetti lineage tracing is being used to track cell fates *in vivo* and *in vitro*. Studying the mechanism for apparent ciliary-regulated tolerance of physiological forces have relevance to morphogenesis, homeostasis, pathology and repair.

ACKNOWLEDGEMENTS: We acknowledge support of the KIR BSU staff, Jadwiga Zarebska, Histology and the CfOAPath. Funding: Kennedy Trust for Rheumatology.

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How to create artificial disc degeneration by enzyme digestion for biomechanical testing of biomaterials

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INTRODUCTION: Biomaterials have an increasingly important role in the development of regenerative approaches for the intervertebral disc (IVD) [1]. Since native human discs are limited for research and animal models often do not mimic human disc degeneration, testing the biomechanical performance of a biomaterial after implantation remains difficult. The aim of the present study was to adapt and optimize an in vitro organ culture model of bovine tail disc for biomechanical testing. The discs were artificially degenerated with different enzymes. The distribution and biomechanical effects of a hydrogel after injection were studied.

METHODS: 24 fresh bovine motion segments (CY3/4), assigned to four groups (n = 6), were prepared and embedded. In three groups either 5 U/ml chondroitinase ABC (ChABC), 65 U/ml papain, or PBS was injected into the discs. After culturing (7 days, 6% O_2, 37°C), complex loading was applied to diminish disc swelling. The maximum possible amount of hydrogel was injected into all four groups and measured. After injection, µCT scans were performed to view the material distribution within the discs. The tissue was collected for Safranin-O/Fast-Green staining and glycosaminoglycan (GAG) quantification. Before and after enzyme treatment, after complex loading and after injection, range of motion (ROM) and disc height were obtained. Statistics: Mann-Whitney-U, Friedman, Benjamini-step-up ($p \leq 0.05$).

RESULTS: Within 7 days, papain-digested discs developed a cavity in the nucleus, while all other groups appeared macroscopically intact. After incubation and subsequent loading, disc height significantly decreased and ROM increased in all groups ($p \leq 0.026$, Fig 1B). Significantly more hydrogel could be injected into enzyme treated discs than into the controls ($p = 0.002$). µCT reconstructions and dissections of the IVDs showed one large sphere for papain and a more inhomogeneous "fluffy-cloud-like" distribution of the hydrogel for ChABC (Fig. 1A/C). Injection of the hydrogel restored the initial disc height in all digested groups, and also increased the height of non-treated discs ($p \leq 0.015$). For ChABC and papain, ROM decreased to the intact state ($p \leq 0.037$).

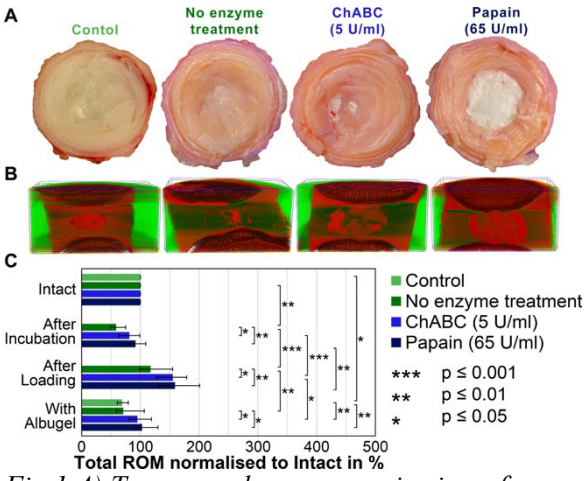

Fig.1 A) Transversal, macroscopic view of one disc per group B) Reconstruction of µCTs of -disc with injected radiopaque hydrogel (one example per group) C) Total ROM in Flexion-Extension normalized to the Intact condition.

DISCUSSION & CONCLUSIONS: Using matrix degrading enzymes, we were able to artificially degenerate IVDs. Papain creates cavities leading to increased ROM and loss of disc height, similar to herniation or nucleotomy [2]. ChABC, in contrast, induces rather age-related degeneration without large cavities. Both enzymes lead to similar biomechanical changes but differences in the hydrogel distribution could be noticed, which we hypothetically relate to the specific digestion of GAGs by ChABC, resulting in different structural defects than papain. These results have improved our overall understanding of the biomechanical effects of IVD tissue digestion with ChABC and papain and indicate that biomaterials, like hydrogels, can be injected standardized and studied with both models based on the research question.

ACKNOWLEDGEMENTS: iPSpine (825925)

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Dynamic loading leads to increased metabolic activity and spatial redistribution of viable cell density in nucleus pulposus explants

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INTRODUCTION: Nucleus pulposus (NP) cell density is orchestrated by an interplay between nutrient supply and metabolite accumulation[1]. Dynamic axial loading is important for tissue homeostasis and is believed to promote a healthy tissue. However, dynamic loading may increase metabolic activity and could thereby hypothetically interfere with regenerative strategies. Therefore, the aim of this study was to determine whether dynamic loading can increase cell metabolism and if this is associated with changes in the viable cell density (VCD) of the NP.

METHODS: NP explants were cultured in a previously developed controlled swelling bioreactor chamber[2]. Explants were subjected to either dynamic or non-dynamic loading for 7 days in milieus mimicking the healthy (HM) or degenerated (DM) intervertebral disc environment. The treatment groups were compared to native tissue. The extracellular matrix content was evaluated biochemically and by Alcian Blue staining. Metabolic activity was measured by glucose and lactate assays of tissue and of medium supernatants. In order to determine the VCD, a lactate dehydrogenase staining was performed in the peripheral and core regions of the NP explants.

RESULTS: The tissue composition of NP explants did not change in any of the groups. However, glucose levels in the tissue reached critical values for cell survival (≤ 0.5 mM)[3] in all groups. Lactate release was increased in the dynamically loaded compared to the non-dynamically loaded groups (Fig 1A). The VCD remained unchanged in the peripheral regions but was significantly reduced in the dynamically loaded groups on Day 7 ($p \leq 0.01$) in the NP core (Fig. 1B). This led to a significant gradient formation of VCD in the dynamically loaded group cultured in a degenerative milieu ($p \leq 0.05$).

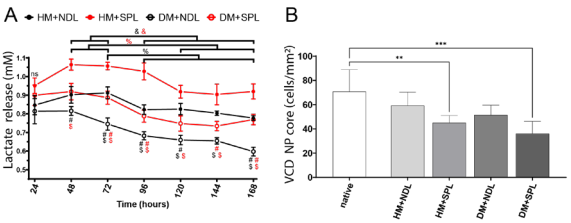

Fig. 1: (A) Dynamically loaded explants (SPL) released more lactate than unloaded explants (NDL) in both, degenerative milieu (DM) and healthy milieu (HM). (B) The viable cell density (VCD) in the NP core was reduced in the dynamically loaded explants.

DISCUSSION & CONCLUSIONS: We demonstrated that dynamic loading in a nutrient deprived environment can affect cell metabolism to the extent that it was associated with changes in cell viability. This led to a new equilibrium in the tissue core when explants were cultured in a degenerative milieu. Physiological levels of loading lead to metabolically more active NP cells, which can be an additional cause of the previously reported low cell density in the intervertebral disc[4]. This should be considered for therapeutic strategies that involve cell injections or that lead to proliferation of resident NP cells.

ACKNOWLEDGEMENTS: This project received funding from the European Union's Horizon 2020 research and innovation program iPSpine under the grant agreement #825925 (www.ipspine.eu).

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A novel viscoelastic bovine intervertebral disc finite element model

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INTRODUCTION: Intervertebral disc (IVD) degeneration and regeneration are commonly studied using bovine tail tissues in a long-term organ culture in a bioreactor. Predictions of global and local tissue mechanical responses are needed in the design and use of a novel bioreactor system, equipped with six degrees-of-freedom mechanical stimulation. Finite element (FE) models can be efficiently used to make such predictions, especially under complex loading situations. Therefore, the aim of this study was to create and validate an FE model of the bovine IVD to be able to predict the global response of the disc, as well as to estimate tissue internal responses in a novel bioreactor application.

METHODS: The annulus fibrosus (AF) lamellae were modelled as a fiber-reinforced viscoelastic material, with a matrix material represented with a Neo-Hookean model, and the collagen fibre network with a viscoelastic model (Fig. 1a) [1]. Elastic and viscoelastic material properties of inner and outer lamellae were determined with a stress-relaxation experiment. The material properties within the AF were assumed to be distributed according to collagen type I content. The AF consisted of 10 lamella layers with alternating orientation of ±30° with respect to the circumferential axis. The nucleus pulposus was modelled as a Neo-Hookean material with Young's modulus of 0.02 MPa and Poisson's ratio of 0.49995, making the behaviour close to a fluid-filled cavity. The bovine IVD model geometry was a cylinder with a diameter of 23.7 mm and height of 6.83 mm, replicating a bovine IVD extracted from a tail (Fig. 1b). The FE-model and the extracted IVD were subjected to a four-step compressive stress-relaxation experiment (4-8-12-16 % of strain), and the force-time output of the model and the experiment were compared to validate the model.

RESULTS: The global response of the model in compression corresponds well to the experimental behaviour (R^2=0.90, Fig 1c). The model is able to capture the force peaks and relaxation phenomena. The initial rates of relaxation of the model and experiment at the third step were -1.76 N/s and -1.97 N/s, respectively. The model under-predicts the total relaxation at higher strain.

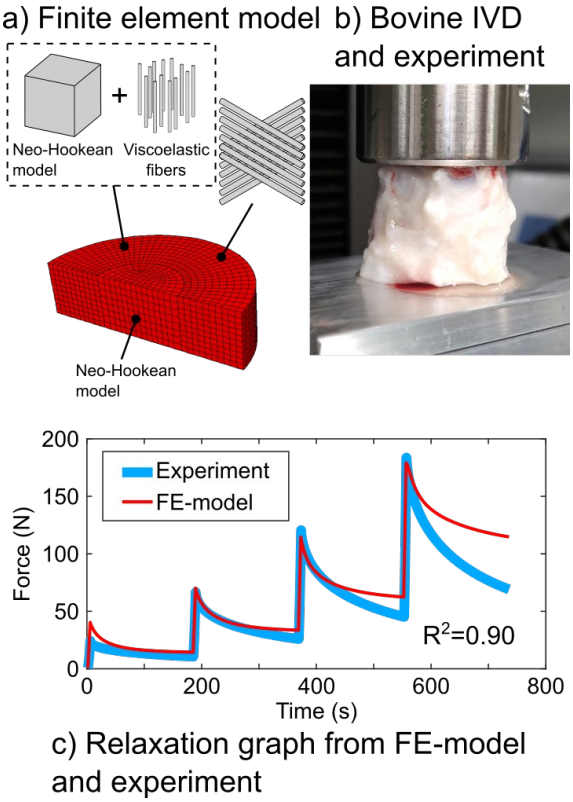

Fig. 1: a) Finite element (FE) model of bovine IVD, b) bovine IVD extracted from a tail and subjected to a compressive relaxation experiment and c) force-time graph of experiment and FE-model.

DISCUSSION & CONCLUSIONS: The proposed model can predict the global viscoelastic response in compression, although the cause of discrepancies at higher strain must be explored. The model validity for internal tissue responses and in other loading types will be explored.

ACKNOWLEDGEMENTS: This work received funding from the Swiss National Science Foundation (grant no. 189915).

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Extracellular cartilage matrix as biomaterial for cartilage regeneration – more than a scaffold.

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INTRODUCTION: After more than two decades of cartilage defect treatment, long term clinical studies reveal incomplete recovery and late deterioration as well as tissue structures that often do not provide all the native properties of hyaline cartilage and are rather fibrous, even when collagen type II is present. Due to the variety of biomaterials that have already been used and the similarity of outcomes, a change in the concept might be indicated. We therefore developed a high-density-scaffold that provides an authentic environment for the cells and is also appropriate to remain in the defect as long replacement material: decellularized and perforated cartilage matrix.

METHODS: Human articular cartilage was decellularized and laser engraved (CO_2 and femtosecond laser) and bovine auricular cartilage enzymatically treated with different protocols; in a second approach auricular cartilage was also laser engraved. All materials were morphologically, biochemically and mechanically characterized and their performance assessed in combination with cells bearing chondrogenic potential (chondrocytes, mesenchymal stromal cells) in vitro, in an experimental defect model (osteochondral plug) as well as in vivo (ectopic nude mouse model).

RESULTS: The decellularized laser incised articular cartilage (CartiScaff) and enzymatically perforated auricular cartilage (AuriScaff) were both several times stiffer than frequently used commercial scaffolds. The incision and channels guided matrix alignment, leading to physiologically (vertically) arranged collagen as in the scaffold and the native cartilage when implanted into the defect. Deep inside the incisions, but also in the channels (of AuriScaff) and the remaining lacuna of the removed chondrocytes, chondrogenesis was superior in comparison to areas outside the scaffolds. The degree of scaffold turnover was dependent on the cell type present inside the incision, with bone marrow strongly degrading the matrix at the incision edges and replacing it with endogenous matrix. Different treatment protocols leading to a graded series of matrix stiffness of AuriScaff revealed that matrix stiffness or composition influence cell invasion into the matrix channels. Combination of the two technologies leading to lasered auricular cartilage (L-AuriScaff) did further facilitate the all over matrix repopulation for less invasive cells such as chondrocytes.

Fig. 1: Decellularized, laser-engraved cartilage scaffold surrounded by neo-tissue in an artificial defect of an osteochondral plug.

DISCUSSION & CONCLUSIONS: Decellularized cartilage matrix is a promising biomaterial, providing the cells not only a scaffold but also a chondrogenic environment, which itself serves as defect filler, relieving the burden from the cells to fill the whole defect with endogenous matrix. With the choice of porosity, the material can be accorded with the clinical strategies and the turn over activity of the involved cell types. Treatment-tailored scaffold materials could help to phase the degradation and regeneration time for more physiological regeneration and long term durability.

ACKNOWLEDGEMENTS: FFG Bridge project #842455 and #874759, Lorenz Böhler Fonds (16/13 and 5/19) and the City of Vienna Competence Team Project Signaltissue (MA23, #18-08), Evercyte GmbH for kindly providing the ASC/TERT1 cell line, Gerjo van Osch Erasmus Medical Center Rotterdam, Johann Zehetner FH Vorarlberg for their cooperation.

Developing Novel Treatments for Joint Diseases

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Degenerative joint disease, also known as osteoarthritis (OA), is a common "wear and tear" disease mostly affecting the elderly. One of its predisposing factors is joint injury. Meniscal tears are the most common injuries of the knee affecting both the young and aged. Beyond the short-term pain, disability, time lost from desired activities including work, meniscal injuries are important early events in the initiation and later propagation of OA. Currently, there is no disease modifying treatment available for these devastating joint diseases.

Our group has a long-term interest in identifying skeletal mesenchymal stem and progenitor cells and understanding mechanisms regulating these progenitors under physiological, pathological and treatment conditions. In joints, our research focuses on articular cartilage and meniscus. Our early research identified the chondrogenic EGFR signaling pathway as an essential regulator of cartilage matrix degradation during growth plate development. By studying the formation of articular cartilage and its OA progression, we discovered that EGFR signaling is a novel and important growth factor pathway that maintains chondroprogenitors in the superficial layer of articular cartilage, promotes cartilage surface lubrication, and stimulates mechanical strength of articular cartilage (1). In collaboration with bioengineers, we recently developed a nanoparticle approach to deliver TGFα, an EGFR ligand, into mouse knee joints as an efficient OA therapy (Fig. 1) (2).

Meniscus-specific mesenchymal progenitors are still largely unknown. Our recent studies find that Gli1+ cells at the superficial layer of mouse meniscal horns express known mesenchymal progenitor markers. In culture, meniscal Gli1+ cells possessed high progenitor activities under the control of Hedgehog signal. Meniscus injury at the anterior horn induced a quick expansion of Gli1-lineage cells. Normally, meniscal tissue healed slowly, leading to cartilage degeneration. Ablation of Gli1+ cells further hindered this repair process. Strikingly, intra-articular injection of Gli1+ meniscal cells or a Hedgehog agonist right after injury accelerated the bridging of the interrupted ends and attenuated signs of osteoarthritis. Taken together, our work identified a novel progenitor population in meniscus and proposes a new treatment for repairing injured meniscus and preventing OA (3).

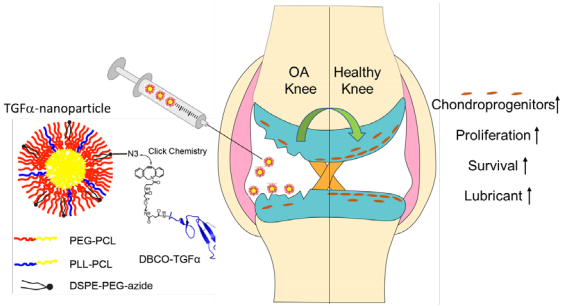

Figure 1. Targeting cartilage EGFR pathway for OA treatment.

ACKNOWLEDGEMENTS: The author wishes to acknowledge the funding support from NIH (R01AG067698 and P30AR069619).

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Injectable Microscaffolds for IVD Regeneration

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INTRODUCTION: Injectable biomaterials for drug and cell delivery into the degenerated intervertebral disc are of high clinical need. Here we report on the fabrication of ECM-like, injectable nanofibrous microscaffolds (MS). The MS were cultured with Mesenchymal Stem Cells (MSC) and Nucleus Pulposus cells (NPC) and their injectability was tested with the use of 23-26G needles.

METHODS: PLLA, PLGA and PCL nano- and microfibers were electrospun on glass collectors and then structured into microscaffolds with a picosecond laser. MS were functionalised with natural polymers: chitosan and chondroitin sulfate. We used Scanning Electron Microscopy (SEM) for morphological characterisation of MS with and without cells. NPC and MSC cells were used to assess the biocompatibility of produced MS and the possibility to inject them as a biomaterial-cell construct. In vivo study in the pig model of intervertebral disc degeneration was conducted to assess the safety and potential of MSC-laden MS in IVD regeneration.

RESULTS: With the use of biomaterials of high porosity, cell-protective MS can be formed which could lead to an increase in the survival rate of injected cells. Any shape of microscaffolds can be created when using laser processing, as shown in Fig. 1a. However, the cube-like shape is preferential due to low structurization time. The cytocompatibility assays show an increase in cell number with culture time. The MSC cells attached well to the fibres (Fig. 1b,c) and populated MS at each side, resulting in the formation of MS agglomerates. The injectability studies through 26G and 24G needles showed that the ejection rate was 92% and 97%, respectively. In the in vivo study, after nucleus pulposus vaporization, MSC-laden MS labelled with superparamagnetic iron oxide nanoparticles were injected into the tissue. Suitable deposition of the construct was observed without leaking through the needle path.

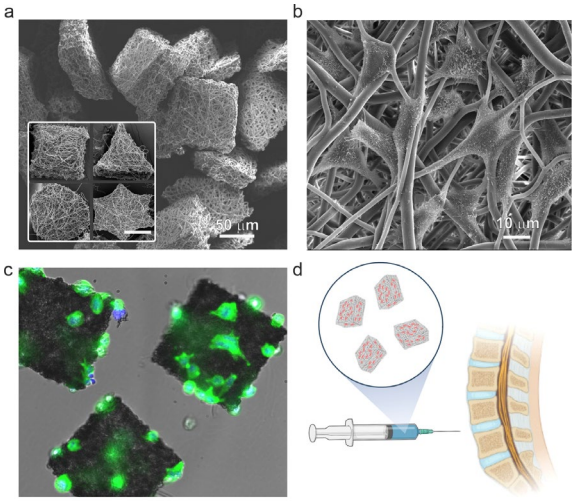

Fig. 1: MS fabrication and biocompatibility a) SEM micrograph presenting cut MS b) MSC cells spreading filopodia between fibres c) morphology of NPC cells on the MS's surface d) scheme presenting proposed use of the cell-laden microscaffolds.

DISCUSSION & CONCLUSIONS: MSs are compatible with living tissues and readily populated with cells. By functionalising the surface of nanofibers, the physical and chemical structure of MSs can be customised to improve cell-MS interaction. The surface modification could provide additional functionality like neuroinhibitory properties with the use of chondroitin sulfate. The injectability studies show that polymer-based MSs are injectable through the tested range of needle sizes and improve disc height when minimally invasively injected into degenerated IVD.

ACKNOWLEDGEMENTS: This work was supported by the National Centre for Research and Development grant no. LIDER/14/0053/L-9/17/NCBR/2018, and Kosciuszko Foundation (the American Centre of Polish Culture).

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Glycomic enhancement of hyaluronic acid-based hydrogel with notochordal cell-derived matrix for disc regeneration

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INTRODUCTION: Notochordal cells (NC) in the nucleus pulposus (NP) disappear in adulthood in humans, unlike other species like pigs. Porcine derived NC-secreted matrix possesses regenerative properties and outperforms that of matrix derived from bovine NPs, which are devoid of NCs [1]. We hypothesized that the NC-derived matrix (NCM) will have a distinct *N*-glycosylation modification to the proteins, and that the glycosylation modifications can be harnessed for its bioactivity by incorporating NCM within a hyaluronic acid (HA)-based hydrogel, which is a therapeutic biomaterial for the treatment of intervertebral disc degeneration.

METHODS: The *N*-glycome of NCM, lyophilized and pulverized from porcine NP, was analyzed by hydrophilic interaction ultra-performance liquid chromatography in combination with exoglycosidase digestions, and mass spectrometry. HA-NCM mixture was crosslinked by 8-arm polyethylene glycol (PEG), and characterized by rheology, swelling studies in degenerative media, and cell injectability and viability. Porcine NCs were encapsulated in HA-NCM hydrogel and stained for hematoxylin/eosin, aggrecan, and caveolin-1.

RESULTS: NCM *N*-glycome was found to be highly glycosylated. In over 300 individual glycans identified, fucosylated (60%), sialylated (66%) and oligomannose type (13%) glycans were the most abundant (Fig.). NCM, along with its glycans was incorporated in HA and an injectable, fast polymerizing (12 min), high swelling (168%), and soft (168 Pa) hydrogel was developed. HA-NCM hydrogel was also shown to accommodate viable cells through injection when encapsulated, and to support the vacuolated NC phenotype at least until day 14, as indicated by positive aggrecan staining for extracellular matrix production and caveolin-1 for vacuole presence.

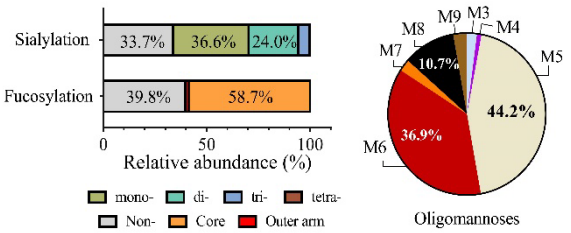

Fig. Major N-glycan motifs in NCM. Sialylation and fucosylation relative to total N-glycans (left), normalized oligomannose type N-glycans (right).

DISCUSSION & CONCLUSIONS: HA has already been proven to be anti-inflammatory in the disc in the form of a hydrogel [2]. Here we combined it with NCM to enhance its potential for regeneration. HA-NCM is shown to be an injectable cell carrier and NC phenotype was maintained. Our findings indicate high abundance of sialylation and fucosylation in NCM. Since sialylation is reported to increase with inflammation [3], our data suggests that matrix produced by NCs may influence other processes than degeneration. In light of these data, we conclude that NCM has a distinct *N*-glycome and that it can be incorporated into a hydrogel to be used in a therapeutic application. Further studies will focus on the effect of these two motifs in terms of the bioactivity of NCM and its regenerative potential together with HA.

ACKNOWLEDGEMENTS: This project received funding from the European Region Development Fund and Science Foundation Ireland (13/RC/2073_P2), and the European Union's Horizon 2020 research and innovation programme (825925) as a part of the iPSpine project. MAT is financially supported by the Dutch Arthritis Society (LLP22).

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SELF-ASSEMBLING PEPTIDE GELS FOR ARTICULAR PATELLA CARTILAGE REPAIR

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INTRODUCTION: Osteoarthritic cartilage is associated with a loss of glycosaminoglycans (GAGs) and reduced biotribological function [1]. There is a clinical need for early intervention treatments to restore cartilage function and delay the progression of degeneration. Chondromalacia patella is softening of the cartilage surface of the patella and is associated with patellofemoral pain.

A self-assembling peptide (SAP) hydrogel, combined with chondroitin sulfate (CS), has shown early promise in restoring GAG content and biomechanical function in degenerate cartilage samples [2].

The aim of this study was to develop an *in vitro* GAG-depleted patella model and assess the biomechanical effects following treatment with a SAP:CS self-assembling hydrogel.

METHODS: Porcine patellae (4-6 month old) were harvested and subject to surfactant washes to remove GAGs. Histological and quantitative biochemical analyses were carried out (n=6).

Patellae were GAG depleted and then treated by injection with SAP (~ 6 mM) and CS (10 mg) solution through a needle. Native, GAG-depleted and treated groups (n=6/group) were characterised mechanically through indentation. The depleted and treated groups were compared to the native group (Kruskal-Wallis, post-hoc Dunn-Bonferronni, $p<0.05$)

Native and GAG depleted paired patellae and femurs were characterized tribologically through whole joint experimental wear simulation (n=6 per group). The cartilage surfaces were assessed and compared (Mann-Whitney, $p<0.05$) using the ICRS scoring system.

RESULTS: The GAG depletion process removed 56±12% of the GAGs within porcine patella cartilage (mean ± 95% C.I.). Histological analysis of GAG depleted samples showed GAG loss with the remaining architecture unaffected.

Mechanically, a significant increase in deformation was seen in the GAG depleted group (n=6, $p<0.001$) compared to native. Treated samples showed significant reduction in normalized deformation (n=6, $p<0.05$) compared to depleted. The injected treatment into GAG depleted cartilage restored the deformation to a level similar to the native group, as shown in Figure 1.

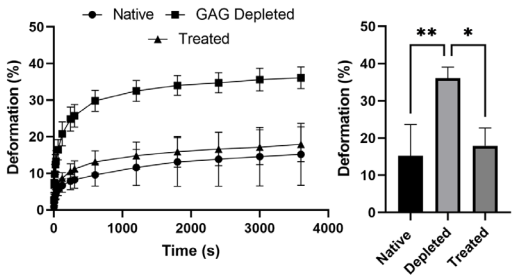

*Figure 1. A) Percentage deformation of different patella cartilage states over 1h. B) .Normalised for each sample's cartilage thickness. (n=6, error bars = mean ± S.D) * = $p<0.05$, **=$p<0.001$*

Wear simulation indicated a significant increase in the cartilage damage on the femoral surface of the patellofemoral joint in the GAG depleted group (n=6, $p<0.001$), localised to the medial and lateral regions of the femoral surface with no significant damage on associated patella surface.

DISCUSSION & CONCLUSIONS: The ~50% reduction of GAGs represented a moderate osteoarthritic patella cartilage model and did not cause observable changes in the tissue architecture or collagen orientation. This loss showed a significant change in the cartilage stiffness response under indentation loading. This same loss transferred to the dynamic wear studies with significant changes in the damage on the femoral counter face associated with the GAG loss. SAP:CS treatment showed promise in restoring cartilage stiffness to treat Chondromalacia patella.

ACKNOWLEDGEMENTS:

The authors would like to thank the EPSRC (Grant no: EP/P001076/1) for providing financial support to this project.

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Bioprinting and characterization of a sheep intervertebral disc model

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INTRODUCTION: Intervertebral disc (IVD), a fibro-cartilaginous structure, contains a central gelatinous core (*Nucleus pulposus, NP*) and an outer network of collagen fibers (*Annulus fibrosus, AF*). IVD degeneration is a debilitating disorder with a considerable socio-economic impact. Conventional treatments are symptomatic and new bio-inspired therapies are necessary to overcome IVD degeneration. Recent approaches to engineer a functional IVD model, as an alternative to animal experiments, are very limited and fail to recapitulate the IVD complex organization. Thus, the goal of the project is to generate an IVD model by 3D bioprinting.

METHODS: An in-house extrusion bioprinter specially designed for this project was developed. A 3D model was designed from a histological section of sheep lumbar IVD. The biomaterial ink (gelatin (7% (w/v)), hyaluronic acid (0.2% (w/v)), alginate (0.6% (w/v))) printability was optimized by varying the printing pressure (0.4-1.1 Bar), flow rate (2-10 $mm.s^{-1}$) and nozzle type (needle or tip). Then, NP and AF cells were harvested from 4-month-old lamb (N=3), mixed with the biomaterial ink ($5*10^5$ cells/mL) and bioprinted. Cell viability (Live/Dead assay), shape (actin immunostaining) and cell distribution (confocal microscopy) were evaluated for up to 21 days of culture.

RESULTS: We first established an analytical model to predict the width of the bioink filament obtained after extrusion, as a function of the printing conditions. This was used to determine the optimal printing parameters (Table 1) to print the IVD model.

Table 1. Selected printing conditions

Parameter	Value
Nozzle	Needle – ⌀ : 0.25mm
Pressure	0.7 Bar
Flow Rate	10 $mm.s^{-1}$
Filament Width	0.73±0.02 mm

The IVD model with fluorescently labelled NP and AF cells was then printed (Fig. 1, left). Spatial distribution of the cells was maintained for up to 21 days of culture (Fig. 1, right). A 25% decrease of the construct height was observed after 21 days of culture modifying cell repartition: more cells at the bottom of the model. The cell density was maintained for 21 days (Fig. 2), with most cells exhibiting a round shape.

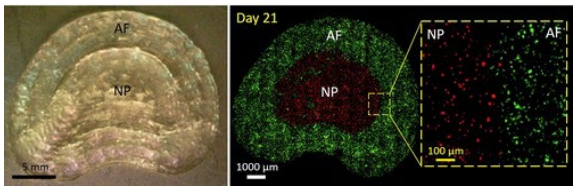

Fig. 1: Macroscopic and microscopic observation of a bioprinted IVD model with Nucleus Pulposus (NP) and Annulus Fibrosus (AF) fluorescent cells.

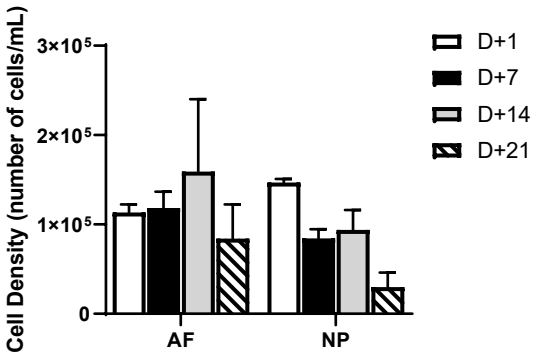

Fig. 2: Cell density in the bioprinted IVD model as a function of time.

DISCUSSION & CONCLUSIONS: We demonstrated the printability of the biomaterial ink used and the fidelity of the bioprinting process, with the spatial distribution of the cells mimicking an IVD architecture. Future studies will include the deposition of *de novo* extracellular matrix components then *ex vivo* validation of the IVD model.

ACKNOWLEDGEMENTS: We thank the financial support from RFI Bioregate ”4DBio-DIV", Région Pays de la Loire.

Regeneration of the intervertebral disc for back pain targeting the degenerate niche

Keynote Presentation: Professor Christine Lyn Le Maitre
Sheffield Hallam University, Sheffield, UK, c.lemaitre@shu.ac.uk

Low back pain is the leading cause of morbidity worldwide and yet most therapies fail to target the cause and are purely symptomatic or end stage surgical options. Intervertebral disc degeneration is associated with approximately 40% of low back pain cases and thus a target for potential regeneration. Intervertebral disc degeneration is a catabolic process caused by altered cell behaviour and tissue biomechanics, leading to a harsh environment for potential cell therapies. Including low oxygen, low nutritional supply, altered osmolarity and mechanical environment together with increased catabolic cytokines which further drive degeneration. Thus, to generate a successful regeneration strategy for the intervertebral disc this harsh environment must be considered, and therapies assessed within conditions which mimic this degenerate niche.

The utilization of cells alone for regenerative therapies are unlikely to be successful if the degenerative cascade and mechanical environment are not restored, hence the combination of cells with biomaterials offers advanced therapeutic approaches. Injectable biomaterials which can restore the mechanical properties of the disc, inhibit catabolic processes of disc degeneration whilst delivering a regenerative cell source hold the most promise to halt disc degeneration and enabling regeneration (Figure 1).

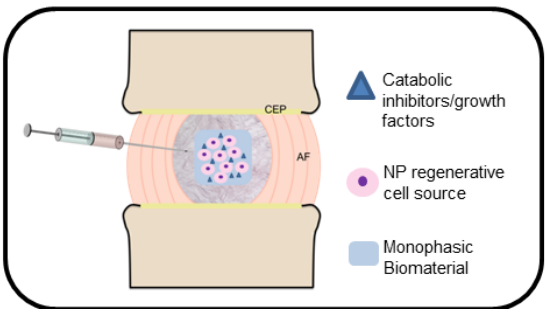

Figure 1: Injectable strategies combining smart biomaterials, regenerative cell sources and mechanisms to inhibit catabolic disc environment and promote anabolism holds the most hope for successful regeneration of the IVD.

Here, the development of novel injectable hydrogel systems which show potential to deliver a three-pronged attack to regeneration of the disc will be discussed. The application of differential cell sources including mesenchymal stromal cells from adipose and bone marrow, notochordal cells and induced pluripotent stem cells will be discussed (Figure 2).

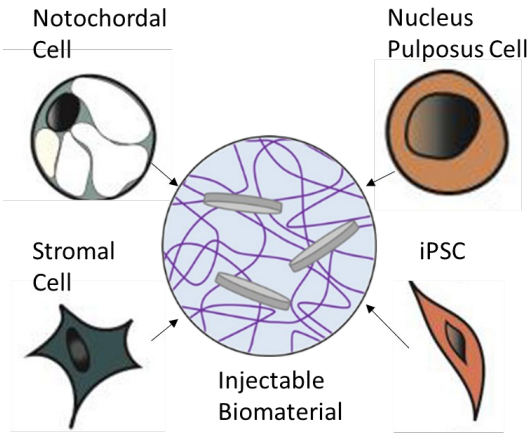

Figure 2: Potential regenerative cell sources to promote regeneration when combined with injectable biomaterials.

Importantly model systems which can recapitulate the degenerate disc environment as testing platforms for potential regenerative approaches for the disc will be introduced including specialist culture media, explants and organ culture models.

ACKNOWLEDGEMENTS: I would like to thank my research group and collaborators for the various contributions to the work to be presented. Funding support for the research presented includes, Versus Arthritis, MRC (Grant number: MR/P026796/1), The European Union's Horizon 2020 program (iPSpine; #825925) and Horizon 2020 (H2020-MSCA-ITN-ETN-2020 GA: 955735).

Vertebral endplate changes and back pain: are there any clinically relevant phenotypes?

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Vertebral endplate (VEP) plays a key role in the regulation of nutrient exchange to and from the intervertebral disc, and also acts as a physical barrier to maintain the functional status. Changes in the VEP appear in the form of bone marrow changes and structural defects, and are closely associated with disc degeneration and back pain. These changes are believed to be densely innervated, hence considered as significant pain generators with reported greater frequency and longer duration of painful episodes. They are also linked with poorer outcomes on conservative treatment and surgery. Few studies, however, contradict and suggest that not all VEP changes are source of pain. The controversial reports might be due to lack of proper classification scheme of VEP changes and/or limitations associated with imaging modalities. The co-existence of VEP and disc changes also make it difficult to identify the actual source of pain in these patients.

The talk will review the existing knowledge regarding the area and will highlight some of the significant VEP phenotypes. Furthermore, the importance to understand the clinically relevant VEP phenotypes in order to focus on targeted interventions and therapies for pain management will be discussed.

Diurnal Variations in Extracellular and Pericellular Sodium Content of Nucleus Pulposus Tissue

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[1]*Department of Biomedical Engineering,* [2]*School of Earth Sciences Trace Element Research Laboratory,*
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INTRODUCTION: Diurnal loading of the intervertebral disc (IVD) causes cyclic changes in tissue osmolarity that result from the daily compression and relaxation of the charged and hydrated extracellular matrix (ECM). These cycles regulate cellular metabolism and are dependent on the fixed charge density (FCD) of the tissue. However, cells exist within a highly specialized ECM called the pericellular matrix (PCM) which has been suggested to have a different FCD than the ECM [4]. The objective of this study is to use a novel application of laser ablation inductively coupled plasma mass spectroscopy (LA-ICP-MS) to quantify the variations in sodium content around the cell and determine how they vary during a daily cycle.

METHODS: Two caudal (c1/c2) motion segments (bone-disc-bone) were isolated from bovine tails, potted and loaded under simulated diurnal loading (16hr of 0.6MPa compression / 8hrs of 0.2MPa compression) for 40 or 48hrs to obtain tissue deformations representative of those that occur at the end of the day-time and night-time, respectively. Loading occurred within a 0.15M NaCl 37C heated waterbath on an Instron ElectroPulsTM E3000. After loading, the motion segment was frozen at -20C. Frozen IVDs were isolated, cut along the mid-sagittal plane and 10µm thick cryo-sections taken from the exposed face. (Fig 1A). Sodium content was then measured along six spots (25µm^2) orientated radially from a cell using a LA-ICP-MS system (Fig 1B). The total sodium signal from the spot nearest (PCM) and farthest (ECM) from the cell were compared for each line. A total of 7 ablation lines were taken around four cells in the daytime tissue and 14 lines around seven cells in the night-time tissue. The ECM and PCM values from each line were analysed via a repeated measures two-way ANOVA in Graphpad Prism with $p<0.05$ considered significant.

RESULTS: There was a significant effect of both diurnal time (i.e., Night vs Day, $p<0.001$) and ablation region (i.e., PCM vs ECM $p<0.02$) in sodium content within the tissue. The relative sodium content in the PCM region was ~11% higher than the ECM region in the night-time tissue and ~7% higher than the ECM region in the daytime tissue. The relative sodium content within the daytime measurements were significantly greater than the night-time measurements. The average sodium content within the daytime ECM was 1.96x greater than the night-time ECM. The daytime PCM was 1.87x greater than the night-time PCM.

DISCUSSION & CONCLUSIONS: Results demonstrate the sodium content of the PCM is greater than the ECM, a finding consistent with articular cartilage [4]. These results are the first measurements of the relative sodium content of the PCM in nucleus pulposus cells. Overall, this study developed a technique to quantify the spatial and diurnal variations in sodium content within IVD tissue which can be used to better understand osmotic mechanotransduction and how it changes with disease.

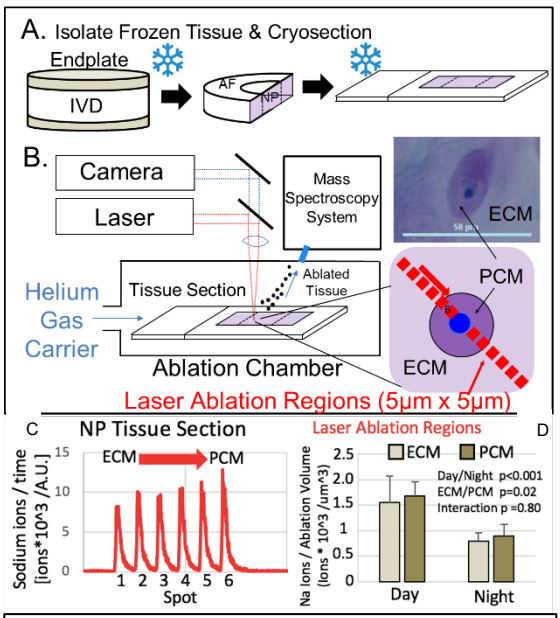

Figure 1: A. Frozen IVD was extracted, cut mid-sagitally and cryo-sectioned. B. Cryo-sections were loaded into LA-ICP-MS system tissue ablated C. Mass spec results of sodium content in each ablated region. D. Sodium content within the PCM and ECM regions after diurnal loading.

ACKNOWLEDGEMENTS: This work was funded by NIH NIAMS R21AR076611 and the Department of Biomedical Engineering.

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Microextrusion-based anisotropic cell patterning within temperature-sensitive hydrogel matrices for annulus fibrosus regeneration

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INTRODUCTION: Low back pain affects nearly 75% of the population at some point in their lives [1] and is associated with degeneration of the intervertebral disc (IVD). Towards IVD restoration, multiple approaches have focused on scaffolds for annulus fibrosus (AF) regeneration. For example, nanofiber laminates have been produced mimicking the angle-ply AF architecture [2], but they must be stacked to form 3D structures and exhibit limited porosity. Alternatively, injectable hydrogels can provide a space filling, porous microenvironment for regenerating cells, but their isotropic structure provides insufficient organizational cues for regenerating functional AF tissue. Here we present a novel injectable hydrogel composed of poly(N-isopropylacrylamide)-graft-chondroitin sulfate (pNIPAAm-CS) combined with Carbopol 940® (Carbopol). The composite hydrogel supports freeform microextrusion of living bioinks within its hydrogel structure, making it possible to capture the cellular microarchitecture of the AF within a space-filling 3D structure. In this study, we characterize the hydrogel rheological properties and its capacity to maintain a patterned configuration of bioprinted cells *in vitro*.

METHODS: Aqueous solutions of pNIPAAm-CS were prepared at a concentration of 3 % w/v and combined with 0.8 % w/v Carbopol. Rheological tests were carried out to evaluate flow point (amplitude sweep test), shear thinning behaviour (rotational test), shear recovery (step strain test), and gelation (temperature ramp test). In parallel, L929 murine fibroblasts (P14) were suspended in 6 % w/v porcine gelatin in Dulbecco's Modified Eagle's Medium (DMEM) at a density of 1 Mio. cells/mL. This bioink was microextruded through a 300 µm (inner diameter) needle into 1 mL of composite hydrogel at 25°C. After printing, constructs were incubated at 37°C for 30 minutes to fully gelate and then covered with medium. Cell morphology was evaluated with TRITC-phalloidin and DAPI counterstain at days 0, 1, and 3 of culture.

RESULTS: At 25ºC, hydrogels comprised of pNIPAAm-CS + Carbopol behaved as a shear thinning, self-healing, yield stress fluid. We demonstrated the capability of pNIPAAm-CS + Carbopol to support extrusion of floating ring patterns (Fig. 1A). The injectable PNIPAAm-CS + Carbopol composites exhibited a nearly 400% increase in storage modulus upon heating from 25°C to 37°C, in contrast to Carbopol alone, which exhibited no temperature sensitivity (Fig. 1B). A patterned configuration of fibroblasts was maintained at days 0, 1 and 3 of culture (Fig. 1C).

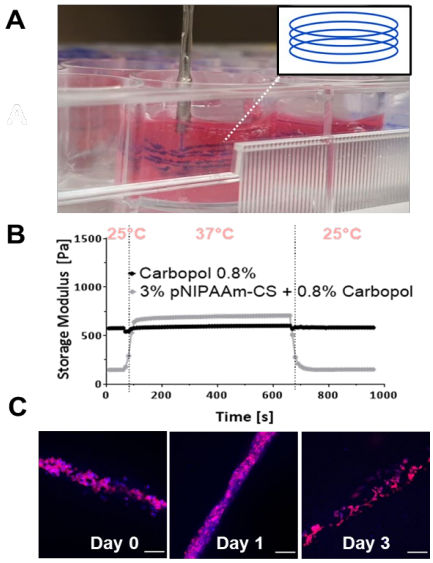

Fig. 1: (A) Freeform printing of unsupported rings within the patternable hydrogels at 25°C. (B) Temperature ramp test of pNIPAAm-CS + Carbopol. (C) Phalloidin/DAPI staining of patterned fibroblasts cultured at 37°C after printing. Scale bars = 100 µm.

DISCUSSION & CONCLUSIONS: PNIPAAm-CS + Carbopol hydrogels were shown to support *in vitro* culture of patterned embedded cells. The long-term aim of this work is to use the injectable hydrogels to fill AF defects *in situ* and subsequently embed cellular patterns within the structure via intraoperative bioprinting.

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IMMUNE CELLS IN IVD THERAPIES: CAUSE, CONSEQUENCE OR TOOL?

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INTRODUCTION: Inflammation correlates with the onset of Intervertebral Disc (IVD) degeneration. However, the proper balance of the inflammatory response is also critical to the success of IVD regenerative therapies. Macrophages are immune cells which may be key targets for the development of IVD therapies. A proof-of-concept of macrophage-based therapy for IVD hernia regression is proposed here.

METHODS: Bone marrow monocyte-derived macrophages were administered intradiscally in vivo into a rat model of IVD herniation (Group Mac6w). The tissue herniated area was quantified by histology in consecutive proteoglycan/collagen sections throughout the IVD, at 2 and 6 weeks post-lesion. The systemic effects were evaluated in blood and spleen by flow cytometry analysis and in the plasma by cytokine array and proteomics.

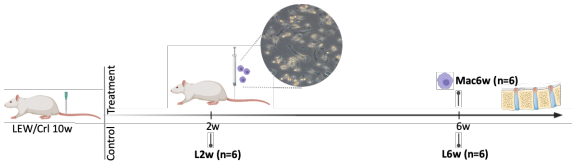

Figure 1. Experimental setup of macrophage local delivery for IVD hernia regression. One group of animals received macrophage administration at 2 weeks post-lesion and was sacrificed at 6 weeks post-lesion (Group Mac6w, n=6). Control groups consisted in lesioned animals sacrificed at 2w (L2w, n=6) and 6w (L6w, n=6) post-lesion, without macrophage administration.

RESULTS: Monocyte-derived macrophages were successfully administered into rat IVD hernias resulting in a 44% hernia size decrease. No relevant systemic immune reaction was identified by flow cytometry and proteomic analysis.

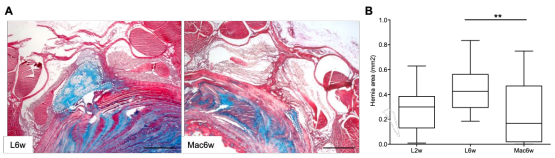

*Figure 2. Hernia histopathological analysis 2 and 6 weeks post-lesion. A) Representative images of the hernia by Alcian blue/Picrosirius red staining. Dashed line outlines the hernia. Scale bar: 500µm. B) Quantification of the hernia area (mm2) across the depth of all sections of an IVD with visible herniation. Results are presented as box and whiskers plots. **p<0.001.*

DISCUSSION & CONCLUSIONS: This study represents the first preclinical proof-of-concept of macrophage-based therapy for IVD hernia regression. This therapy relies in the potentiation of the physiological hernia regression mechanisms. Further development of this macrophage-based therapy for clinical application is warranted.

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Disc-bone marrow cross-talk with Modic changes

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Chronic Low Back Pain is a leading cause of disability. Vertebral bone marrow oedema, known as Modic changes (MC), are common in patients with low back pain and a specific source of pain (*1*). Patients with MC have been suggested to be *"an entity on its own"* that *"deserve to be diagnosed as having specific LBP"* (*2*). Yet, no targeted treatment for MC exists.

MC lesion size and the amount of affected bone marrow is critical, because larger lesions are more likely painful and less likely to resolve (*3*, *4*). Unfortunately, the pathomechanisms of MC are poorly understood. MC, disc degeneration, and endplate damages strongly associate and progress synchronously. Endplate defects enable a hydraulic coupling of the disc and trigger disc degeneration (*5*). Upon endplate damage, the immune privileged disc is exposed to the immune cells of the bone marrow and trigger an autoinflammation with accumulation and activation of T-cells and neutrophils (*6*, *7*). Activated neutrophils can resorb endplates and marrow-sided leukocytes enhance inflammation and catabolism in the disc even without infiltrating the disc (*8*). Hence, MC discs degenerate at an increased rate and have a pro-inflammatory profile (*9*). Disc inflammation is critical because inflamed discs themselves can cause endplate damages and trigger MC (*6*).

The exact pathomechanisms depends on the aetiology of MC. Accumulating evidence for two aetiologies exist (*7*). First, an occult infection of the disc with bacteria, e.g. *Cutibacterium acnes*, and second, an autoinflammatory reaction of the bone marrow against the disc. While an autoinflammation may be treated with steroids, this would be contraindicated with an infected disc, which might be better treated with antibiotics. Unfortunately, no biomarkers exist that could distinguish the two aetiologies and support the physician's decision. Understanding the disc/bone marrow crosstalk in MC could support the identification of aetiology specific biomarkers and targeted treatment approaches.

In conclusion, MC are not just reactive changes to degenerating discs but are important contributors to progressive segmental degeneration and pain. Understanding the underlying mechanisms are imperative for targeted treatments and regenerative approaches.

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Link N as a Modulator of IL-1β-Induced DRG Neuronal Hypersensitivity in Osteoarthritis

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INTRODUCTION: Osteoarthritis (OA) is a painful and disabling chronic condition that constitutes a major challenge to health care worldwide. One of the main factors implicated in OA pain is abnormal sensory input due to aberrant activation of dorsal root ganglion (DRG) nerve endings in the joint. IL-1β is a key proinflammatory cytokine involved in the progression of OA. The canonical IL-1β signalling pathway involves the activation of the transcription factor NFκB, important in the expression of catabolic enzymes, inflammatory cytokines and pain factors.

We and others have demonstrated that LN, a 16-residue peptide derived from link protein, can act as an anabolic agent *in vitro* and *in vivo* in OA animal models. In addition, we have discovered that LN can suppress the upregulation of catabolic enzymes and inflammatory and pain factors in human chondrocytes. We have also demonstrated that LN could modulate pain behavior in a murine model with advanced OA. It remains unclear as to the mechanism(s) of LN on pain behavior. We hypothesize that LN plays a direct role in regulating IL-1β signaling in joint and DRG neuronal cells to alter sensory pain in OA.

METHODS: ***Western Blotting*** Chondrocytes and synovial fibroblasts were isolated from OA donors undergoing knee arthroplasty (ages 45–65 yrs). Cells were treated with LN with or without IL-1β [1 ng/mL] for up to 60 min and measured for changes in P-NFκB. ***Ca^{2+}-mobilization-*** DRG neurons from lumbar regions L2-5 were isolated from 15-week-old C57BL/6 mice and cultured in glass chamber slides for 7 days with IL-1β [1 ng/mL] with or without LN [1μg/mL]. Imaging was done with Fura-4, AM for changes in intracellular Ca^{2+} either at resting state or following stimulation with capsaicin.

RESULTS: In both chondrocytes and synovial fibroblasts, LN significantly suppressed the activation of P-NFκB to levels comparably to control for up to 60 min (ANOVA, posthoc Tukey; $p < 0.01$; $n = 4$). When DRG neurons were incubated with IL-1β, basal intracellular Ca^{2+} levels were elevated when compared to controls ($p < 0.001$; $n = 4$). LN significantly decreased basal Ca^{2+} levels when compared to IL-1β ($p < 0.0001$; $n = 4$). When DRG neurons were stimulated with capsaicin, IL-1β preconditioned neurons demonstrated a sustained increase in intracellular Ca^{2+}. Co-treatment with LN blunted the sustained Ca^{2+} increase induced by IL-1β (Fig 1).

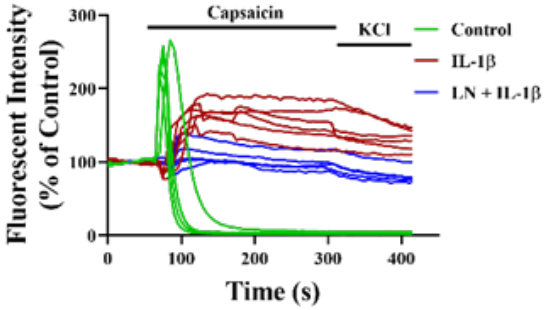

Fig 1. Effect of LN on IL-1β-induced hyperalgesia in isolated murine DRG neurons. *DRG neurons were stimulated with capsaicin [100 nM] for 240 s followed by KCl (100 mM) for 120 s. N = 6.*

DISCUSSION & CONCLUSIONS: In summary, LN inhibits IL-1β signalling and regulates DRG neuronal-induced hypersensitivity. These results may support the use of LN in the treatment of OA pain.

ACKNOWLEDGEMENTS: This work was supported by grants from the Canadian Institutes of Health Research

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Irisin stimulates anabolism and matrix synthesis in human nucleopulpocytes in vitro: new insights into a cross-talk between the muscle and the intervertebral disc.

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INTRODUCTION: Exercise has showed to reduce pain and improve function in patients with discogenic low back pain (LBP). Although there is currently no biologic evidence that the intervertebral disc (IVD) can respond to physical exercise in humans, a recent study has shown that chronic running exercise is associated with increased IVD hydration and hypertrophy[1]. Irisin, a myokine released upon muscle contraction, has demonstrated to yield anabolic effects on different cell types, including chondrocytes[2]. This study aimed to investigate the effect of irisin on human nucleopulpocytes (hNPCs) *in vitro*. Our hypothesis was that irisin would improve hNPC metabolism and proliferation.

METHODS: hNPCs were isolated from IVD samples obtained during spine surgery and cultured in alginate beads. hNPCs were then exposed to either phosphate-buffered saline (PBS) or human recombinant irisin (r-irisin) for 7 days at a concentration of 5, 10 and 25 ng/mL (n = 4). Each experiment was performed in triplicate. Cell proliferation was assessed with trypan blue staining-automated cell counting and PicoGreen assay at 4, 10 and 14 days of culture. Glycosaminoglycan (GAG) content was measured using the dimethylmethylene blue (DMMB) assay and normalized to the DNA ratio at 7 days of culture. Metabolic activity was assessed with the MTT assay and the Griess Reagent System at 4 days of culture. Gene expression of collagen type II (COL2), matrix metalloproteinase (MMP)-13, tissue inhibitor of matrix metalloproteinase (TIMP)-1 and -3, aggrecan, interleukin (IL)-1β, a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-5 was measured by real time-polymerase chain reaction (RT-PCR) at 7 days of culture. In addition, MTT assay and ADAMTS-5, COL2, TIMP-1 and IL-1β gene expression were evaluated following incubation with 5, 10 and 25 ng/mL r-irisin for 24 hours and subsequent culture with 10 ng/ml IL-1β for 7 days and vice versa (incubation for 24 hours with IL-1β and subsequent culture with r-irisin for 7 days).

RESULTS: Irisin increased hNPCs proliferation ($p<0.001$), metabolic activity ($p<0.05$), and GAG content ($p<0.01$), as well as COL2 ($p<0.01$), ACAN ($p<0.05$), TIMP-1 and -3 ($p<0.01$) gene expression, while decreasing mRNA levels of MMP-13 ($p<0.05$) and IL-1β (Fig. 4, $p<0.001$). r-irisin pretreatment of hNPCs cultured in pro-inflammatory conditions resulted in a rescue of metabolic activity ($p<0.001$), as well as a decrease of IL-1b ($p<0.05$) levels. Similarly, incubation of hNPCs with IL-1β and subsequent exposure to r-irisin led to an increment of hNPC metabolic activity ($p<0.001$), COL2 gene expression ($p<0.05$) and a reduction of IL-1β ($p<0.05$) and ADAMTS-5 levels ($p<0.01$).

DISCUSSION & CONCLUSIONS: The present study suggested that irisin may stimulate hNPCs proliferation, metabolic activity, and anabolism by reducing the expression of IL-1β and catabolic enzymes while promoting the synthesis of extracellular matrix components. Furthermore, such myokine was able to blunt the catabolic effect of *in vitro* inflammation. Our results indicate that irisin may be one of the mediators by which physical exercise and muscle tissues modulate IVD metabolism, thus suggesting the existence of a biological cross-talk mechanism between the muscle and the IVD.

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Fibroblast-like synoviocytes modulate the inflammatory response of chondrocytes in a mechanosensitive manner

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INTRODUCTION: The synovium is implicated in inflammatory arthritis and cartilage degradation. However, the contribution of fibroblast-like synoviocytes (FLS) is unclear. Here we test the hypothesis that FLS regulate chondrocyte inflammatory response to interleukin 1 beta (IL-1β) and that this behaviour is modulated by loading of the synovium. We utilise this understanding to develop a cartilage-synovium organ-chip model to investigate joint disease and therapeutics.

METHODS: Primary bovine FLS were cultured in monolayer and treated with ±IL-1β (10ng/ml) for 24h and ±cyclic tensile strain (CTS; 12%, 0.2Hz, 6h using Flexcell®) to generate conditioned media (CM). CM was applied to primary bovine chondrocytes in monolayer and within 2% agarose gels. Separate studies were conducted with cartilage explants and FLS in co-culture over 12 days. Nitrite release was assessed by Griess assay. Further studies developed an organ-chip model of human synovium and cartilage.

RESULTS: IL-1β stimulated the release of the pro-inflammatory mediator, nitric oxide (NO) in chondrocytes but not in FLS. The addition of CM from FLS treated with IL-1β increased NO release in chondrocytes in monolayer and in agarose compared to none-CM with IL-1β (Fig 1A, B). Similarly, co-culture of cartilage explants with FLS showed higher IL-1β-induced NO release compared to explants alone (Fig 1C). The application of CTS to FLS further increased the upregulation of inflammatory signaling in chondrocytes by CM (Fig 1D). Similar results were demonstrated in the release of PGE2 (data not shown).

A two-channel microfluidic chip (Chip-S1®, Emulate Inc) was used to establish the organ-chip model. Primary human FLS were seeded in 10% Matrigel in the top channel and primary human chondrocytes in 2% agarose in the bottom channel, with channels separated by a semi-permeable membrane (Fig 2A). Organ-chips were cultured for 10 days under flow, then subjected to CTS for 5 days (12%, 0.2Hz, 2h/day). Both cell populations demonstrated good viability and appropriate morphology (Fig 2A). The FLS responded to CTS, evidenced by the upregulation of hyaluronic acid synthase genes HAS-1, -2 and -3, compared to the static and flow-only conditions (Fig 2B).

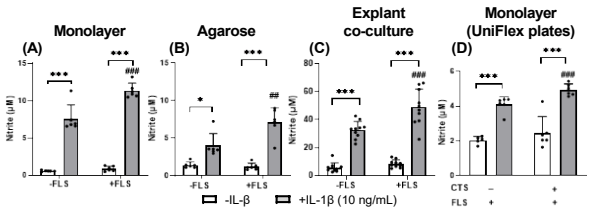

Fig. 1: NO release from chondrocytes in (A) monoculture and (B) agarose +/- CM from FLS. (C) NO from cartilage explants +/- co-culture with FLS. (D) NO from chondrocytes treated with FLS CM +/- CTS. All +/- IL-1β.

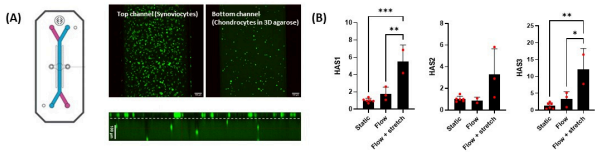

Fig 2: (A) Emulate Chip-S1 schematic and calcein-AM stained FLS and chondrocytes in the organ-chip. (B) Expression of HAS-1, -2 & -3 by FLS in organ-chip.

DISCUSSION & CONCLUSIONS: FLS increase chondrocyte inflammatory response to IL-1β which is further upregulated by loading of FLS. Hence, organ-chip models of inflammatory joint disease need to incorporate both FLS and chondrocytes and mechanical loading. We have developed a human organ-chip model of cartilage-synovium interaction using the Emulate platform. Further work will examine the paracrine cross talk mechanism.

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Biological Barriers and Delivery Solutions for Nucleic Acid Therapeutics

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Nucleic acid-based drugs such as gene silencing molecules are susceptible to rapid renal clearance, degradation, non-specific accumulation and poor cellular uptake. Enabling technologies are, therefore, required to overcome both extracellular and intracellular delivery challenges. This talk will first describe work from our laboratory on nanoparticle-based local [1] and systemic [2] delivery of small interfering RNA (siRNA). Barrier-specific design criteria will be discussed, with challenges for nucleic acid delivery to articular cartilage highlighted and the approach of local delivery using a hydrogel with the dual property of chondrocyte binding and gapmer antisense oligonucleotide (ASO) release for modulation of gene expression in osteoarthritis [3]. The second part of the talk will focus on our recent work on the application of serum albumin as an alternative delivery technology to nanoparticles, motivated by its neonatal Fc receptor (FcRn)-driven long circulatory half-life [4] and tissue accumulation properties. I will describe cholesterol [5] and fatty acid [6] modifications that mediate nucleic acid binding with the endogenous serum albumin pool for tuning siRNA and gapmer ASO pharmacokinetics and gene silencing. Furthermore, an alternative albumin biomolecular assembly [7] that combines recombinant human albumins engineered for tunable FcRn binding with nucleic acids modules for site-selective incorporation of nucleic acid therapeutics will be introduced. The potential application of these albumin-based designs for treatment of osteoarthritis will be discussed.

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FOXF1 delivery via Engineered Extracellular Vesicles in an *in vivo* mouse model of discogenic back pain

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INTRODUCTION: Intervertebral disc degeneration (IVDD) is a major contributor to low back pain (LBP), yet there are currently no clinical treatments to target the underlying pathology or promote tissue repair[1-2]. Viral gene delivery and stem cell treatments have shown promise, however, they are clinically hampered due to unwarranted mutagenesis, immunogenicity, and cell survivability. Engineered extracellular vesicles (EVs) are promising as a mode of non-viral gene delivery due to their naturally derived, minimally invasive nature. We have previously shown the potential of transcription factors such as Brachyury or *FOXF1*, to reprogram degenerate human nucleus pulposus (NP) cells to a healthy phenotype[3, 4]. Therefore, this study assessed the efficacy of *FOXF1* delivery via engineered EVs in a mouse lumbar IVD puncture model.

METHODS: *FOXF1* and SHAM vector (pCMV6) plasmid were expanded and bulk electroporated into mouse fibroblasts (PMEF). EVs containing FOXF1 and SHAM were isolated from the culture medium with verification of genetic cargo via qPCR, and EV size distribution and concertation via Nano sight. A left unilateral incision was made in 15 week old male and female WT mice (N=5-6 per group) with exposure of the L4/5, L5/6, and L6/S1. Groups included: Non-injury control (IVD exposure only), Injury control (puncture with 30G needle), or respective injections of *FOXF1*-EVs or SHAM-EVs. Pain behavioural assessments were performed over 12 weeks including MRI, microCT, histology, and ECM content at 12 weeks.

RESULTS: MRI analysis of relative intensity demonstrated significant restoration of IVD hydration in *FOXF1*-EV treated mice compared to injury. Decreased disc height was observed in injury mice via microCT with increased DHI in *FOXF1* and SHAM treatment groups. (Fig1) Histological sections showed disorganization of AF, and decrease NP height in injury groups with the restoration of structure comparable to healthy controls for *FOXF1* treated groups. This is further evident in increased GAG protein expression. Behavioural assessments demonstrated significant increases in axial discomfort, thermal hyperalgesia, and decreased mobility and grip strength in injury mice with partial restoration in treatment groups with sex-specific differences between male and female mice.

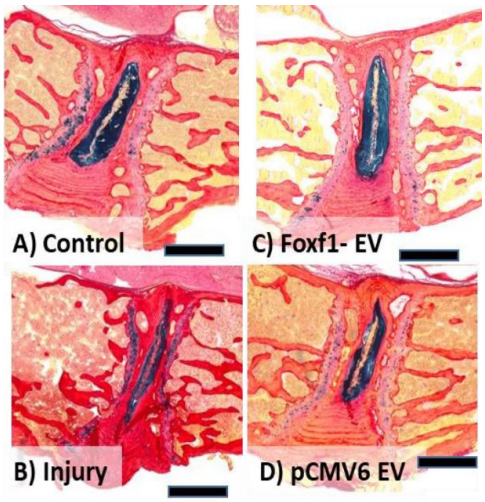

Fig. 1: Alcian Blue/Picrosirius Red stained mouse IVDs of A) non-injury control, B) Injury, C) Foxf1-EV treatment, and D) pCMV6-EV (SHAM). (Scale bar =500µm)

DISCUSSION & CONCLUSIONS: These results demonstrate that *FOXF1* delivered via engineered EVs can repair IVD structure, function and induce pain behavioural changes *in vivo* which highlights its potential as an innovative non-addictive, non-viral, and minimally invasive therapy for discogenic back pain *in situ*.

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Anti-inflammatory and Pro-anabolic Effects of 5-Aminosalicylic Acid on Inflammatory Human Osteoarthritic Chondrocytes *in vitro*

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INTRODUCTION: Low-grade inflammation plays a pivotal role in osteoarthritis (OA) onset and progression. Exploration of effective anti-inflammatory drugs on human chondrocytes could facilitate strategies for OA treatment. 5-aminosalicylic acid (5-ASA), a potent standard drug for inflammatory bowel diseases (IBD) in clinical practice, has not been assessed in OA. In this study, the effects of 5-ASA were explored in inflammatory human osteoarthritic chondrocyte pellet model.

METHODS: Human chondrocyte pellets from 4 donors (male 3, female 1, age range from 65 - 80 years) were first cultured with chondrogenic medium for 1 week, and then stimulated with 1 ng/mL IL-1β and 1 ng/mL TNF-α in the presence (treatment group) or absence (OA group) of 5-ASA at concentrations of 10 mM or 20 mM for 14 days. Pellets were collected after 3-day, 8-day, and 14-day treatment with 5-ASA for transcriptional, biochemical, and histological analysis. In addition, the conditioned media were collected for further analysis. Statistical analysis was performed by one-way analysis of variance (ANOVA).

RESULTS: Gene expression of IL-6, IL-8, and COX-2 were all remarkably downregulated in 5-ASA groups on day 3 in comparison with the OA group (**Fig. 1A)**. Ratio of glycosamino-glycan (GAG) to DNA in pellets, as well as total GAG synthesis (content in pellet and media), were significantly upregulated in 10 mM 5-ASA group after 14-day treatment. Accumulative content of inflammatory stimulators including IL-6, IL-8, and nitric oxide (NO) in media were all markedly mitigated in 5-ASA treated groups. Furthermore, Safranin O/Fast Green staining showed that GAG loss was alleviated in groups treated with 5-ASA both on day 8 (**Fig. 1B**) and day 14.

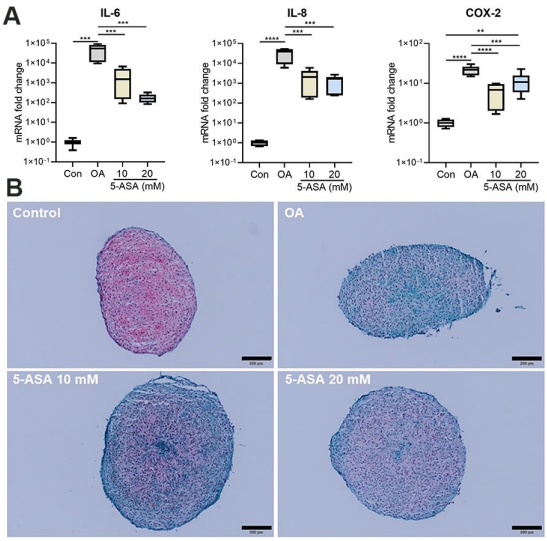

*Fig. 1: (A) Gene expression levels of IL-6, IL-8, and COX-2 in chondrocytes after 3-day 5-ASA treatment compared to non-inflammatory control group (n = 7). ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (B) Safranin O/Fast Green staining of chondrocyte pellets treated with 5-ASA for 8 days. Scale bars = 200 μm.*

DISCUSSION & CONCLUSIONS: The effect of 5-ASA was investigated in an *in vitro* inflammatory OA model, where 3D cultured human chondrocytes were stimulated with inflammatory cytokines. Aforementioned results showed that 5-ASA markedly downregulated OA-related inflammatory markers from both transcriptional and protein levels, and produced more GAG in the pellets, demonstrating its anti-inflammatory and pro-anabolic effects on chondrocytes under an inflammatory OA condition. The translational potential of 5-ASA as a disease-modifying OA drug will be further assessed with a human osteochondral explants OA model.

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Exploring Fully Biodegradable Dendrimers as Nanocarriers for siRNA Delivery to Chondrocytes

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INTRODUCTION: Osteoarthritis and discogenic low back pain are high impact conditions in our society, arising from the degeneration of articular cartilage[1] and intervertebral disc[2], respectively.
The only available treatments in the clinic are not focused on curing these conditions, but on minimizing the symptoms, such as physiotherapy, pain medication and, as a last resource, surgery. Nevertheless, nowadays there are a lot of research efforts being invested in finding regenerative approaches to treat these diseases. Among these, gene therapy has been put forward as a promising approach not only to alleviate the symptoms, but also to cure these conditions by promoting tissue regeneration. One of the open research areas that gene therapy arises is focused on finding effective nucleic acid delivery systems.
Herein we propose to explore a new family of proprietary fully biodegradable PEG-GATGE (Polyethylene Glycol Gallic Acid-Triethylene Glycol Ester) dendrimers, previously reported in our group,[3, 4] to develop a non-toxic fully biodegradable non-viral vector for siRNA delivery to chondrocytes.

METHODS: Fully biodegradable 3rd generation (G3) PEG-GATGE dendritic block copolymers, functionalized with benzylamine groups, were synthesized and characterized by NMR and FTIR. Afterwards, dendriplexes complexing siRNA were prepared at different N/P ratios (moles of dendrimer amines/moles of siRNA phosphate groups) and characterized in terms of their siRNA complexation efficiency using a SYBR™Gold exclusion assay. The dendriplexes size was measured by dynamic light scattering and zeta-potential by laser doppler electrophoresis. Finally, dendrimer and dendriplexes cytotoxicity, as well as their cell internalization, were tested in a chondrocyte cell line expressing the luciferase protein (C28/I2, provided by M.B. Goldring, Hospital for Special Surgery, Weill Medical College of Cornell University, New York, NY, USA).

RESULTS: PEG-GATGE based dendriplexes showed an excellent complexation (>70%) of siRNA at the different N/P ratios tested (5, 10, 20, 40 and 80). Subsequently, dendriplexes at N/P 5, 10 and 20 were tested and proven non-cytotoxic for chondrocytes, while mediating excellent siRNA internalization (>90% as analysed by confocal microscopy and FACS).

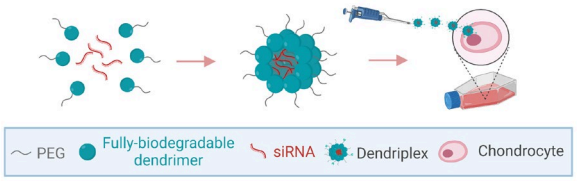

Fig. 1: Schematic representation of dendriplex formation and their application in vitro.

Transfection studies are currently being performed, with luciferase down-regulation being assessed by qPCR.

DISCUSSION & CONCLUSIONS: G3 PEG-GATGE dendritic block copolymers have been successfully synthesized in very good yields and they have shown excellent siRNA complexation efficiencies. Furthermore, they show a very good biocompatibility with chondrocytes and were successfully internalized by these cells, making them promising siRNA vectors for the delivery to the cartilage tissue.

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POLYMERIC NANOPARTICLES ENABLE MRNA DELIVERY INTO CELLS OF HUMAN CARTILAGINOUS TISSUES

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INTRODUCTION: Osteoarthritis (OA) and intervertebral disc degeneration (IVDD) annually affect hundreds of million people worldwide. With pain management and surgical treatment merely alleviating symptoms, currently there are no disease modifying treatment options. Non-viral gene therapy has great potential to address this issue by delivering oligonucleotides that can halt inflammatory and degenerative processes and induce regeneration. In this study we used poly(amido)amine-based polymeric nano-particles (20Medtx) to deliver mRNA to human osteoarthritic chondrocytes.

METHODS: Human OA chondrocytes, human nucleus pulposus (NP) cells or human annulus fibrosus (AF) cells (3 donors in triplicates each) were seeded into 96 well plates (20,000/cm^2). After 24h they were transfected with nanoparticles (uncoated or PEG-coated) loaded with mRNA coding for EGFP at mRNA concentrations of 0.8 to 3.2 µg/ml (weight ratio mRNA:nanoparticles= 1:25). 24h after transfection, SYTOX™ Orange assay was used to assess toxicity. Transfection analysis was done using confocal imaging and calculating the ratio of EGFP positive cells to total cell count (identified by Hoechst stain).

RESULTS: The transfection efficiency reached up to 31% in OA chondrocytes treated with PEG-coated or uncoated nanoparticles (fig.1). NP cells reached a maximum of 58% transfection using PEG-coated or uncoated poly(amido amide) nanoparticles ((SD=3.9 and SD=16.3, respectively; fig. 2). In AF cells the transfection efficiency reached a maximum of 39% (SD=4.8) and 44% (SD=13.0) when transfected with PEG-coated nanoparticles and uncoated nanoparticles, respectively (fig. 3). Dose dependency seemed most clear for the coated nanoparticles. No significant decrease in cell viability (fig 1-3) compared to the untreated control cells was observed in most conditions, except for the 2.4 µg/ml concentration in NP cells, in contrast to lipofectamine, which was toxic for both NP and AF cells.

Figure 1: OA Chondrocytes

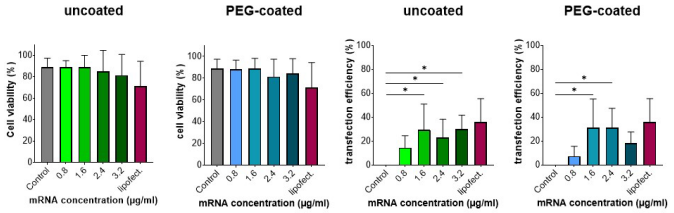


Figure 2: NP cells

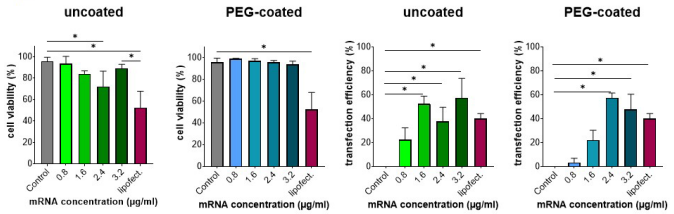
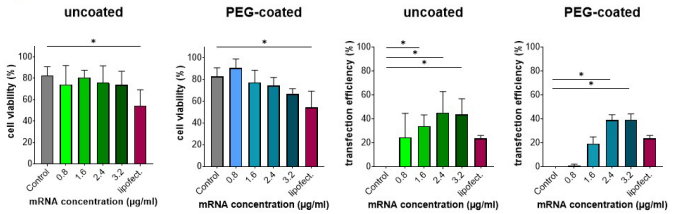


Figure 3: AF cells



DISCUSSION & CONCLUSIONS: - We showed a novel way of transfecting primary human cartilaginous cells with mRNA for future therapy in OA and IVDD. The two different nanoparticle formulations showed similar transfection efficiency and no impairment in cell viability. Next experiments will be focused on the in situ transfection of human cartilaginous cells. Ultimately, the delivery of biologically relevant targets (i.e. mRNA encoding for regenerative factors) will be tested.

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