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Progress report ISSLS Research Grant 2022 sponsored by Taisho Pharmaceutical

Dear ISSLS Research Grant Committee members,

We are grateful to ISSLS and Taisho Pharmaceutical for funding our study entitled "*The effect of notochordal extracellular vesicles on human and bovine nucleus pulposus explants*" in 2022. The Progress Report with detailed information on the results obtained so far and future perspectives is submitted together with this letter.

Please, do not hesitate in contacting us if further questions arise.

Once again, thank you very much for this great support to our research.

Sincerely,

Josette van Maanen, Dr. Frances Bach, Msc, Prof. Keita Ito, Prof. Christine Le Maitre, and Prof. Marianna Tryfonidou

1. Introduction

Notochordal cells (NCs) have attracted increasing interest because of their potential regenerative capacity[1]. NCs initially reside within the embryonic notochord and are only present within the core of the developing IVD[2]. During IVD maturation and ageing, the large, vacuolated NCs are replaced by chondrocyte-like nucleus pulposus cells (NPCs). The replacement of NCs by NPCs precedes the onset of IVD degeneration, implying that NCs may play a role in maintaining IVD health[1]. Affirmatively, the regenerative effect of NCs and their secreted factors has already been demonstrated on NPCs[3–12], MSCs[8,13,14], annulus fibrosus cells[11,15], (endplate) chondrocytes[16,17], nucleus pulposus (NP) explants[18] *in vitro,* and degenerated rat IVDs *in vivo*[19]. The downside of using NCs for regenerative purposes, however, is that they are scarce, and can only be obtained from embryonic/juvenile individuals[20,21]. Thus, identifying strategies to harness the potential regenerative and anti-catabolic capacity of NCs without the requirement to deliver the cells themselves could provide a viable therapeutic approach.

We pioneered the spine field by demonstrating that NCs convey their messages via the secretion of extracellular vesicles (EVs)[7]. EVs are small lipid bilayer-enclosed particles released by cells and serve in intercellular signaling. They carry, protect, and deliver biologically active cargo (*e.g.* RNAs, DNA, proteins, lipids) and have attracted attention for developing regenerative therapies[22,23]. Our group identified vesicle-related proteins in porcine, canine, and human NC-conditioned medium[7]. EVs are abundantly secreted by porcine NCs; these EVs exert anabolic effects on human NPCs from degenerate IVDs[24]. Preliminary work furthermore indicates that porcine NC-derived EVs (pNC-EVs) exert anti-catabolic effects on dog NPCs in a degenerative disc environment (figure 1), evidenced by the reduction in PGE2 release corrected for DNA content in the presence of pro-inflammatory stimulus (i.e. 100 pg/ml IL1β; figure 1A). In line with this finding, in a human chondrosarcoma cell line, reporters of the inflammatory proteins (synthetic response element for transcription factor involved in NF-kB and reporter for IL-6 gene) were decreased when cells were treated with pNC-EVs.

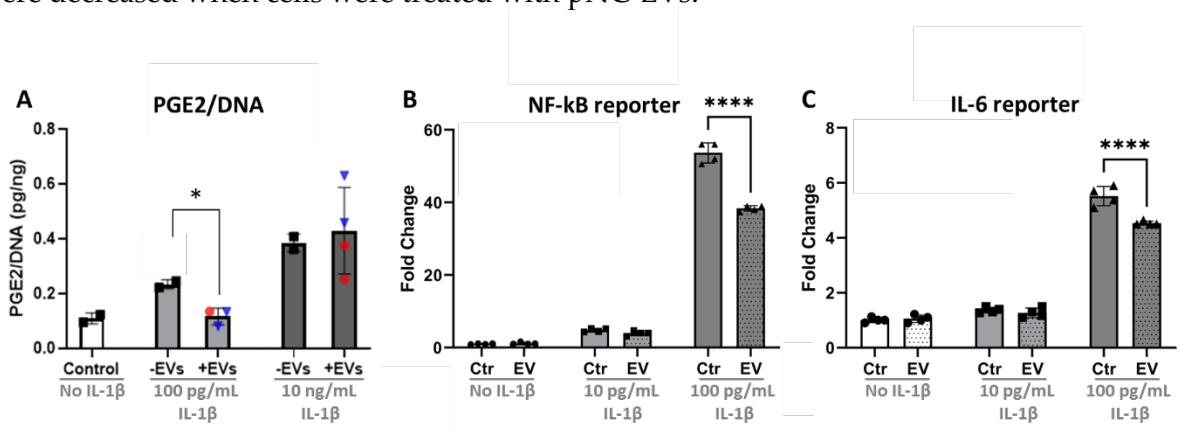


Figure 1. Anti-catabolic effects of porcine NC-derived EVs. Canine NPCs were cultured for 3 days in monolayers with/without IL-1β. Prostaglandin E2 (PGE2) production is decreased in NPCs treated with NC-derived EVs (A). Furthermore, porcine NC-EVs decreased NF-kB and IL-6 signaling activity in a human chondrosarcoma cell line[25] (B and C). *: P<0.05, ***: P<0.001. In collaboration with Prof. Tim Welting and dr. Guus van den Akker, Maastricht University.

Altogether, we and others have demonstrated the anti-catabolic properties of notochordal cells[26] and their secretome[27,28], including notochordal cell-derived EVs, which makes them a promising cell-free therapeutic strategy. To determine whether these NC-derived EVs can be used as a regenerative treatment option for patients with IVD-related LBP, their effects need to be demonstrated in IVD tissue explants. Therefore, the aim of this study was to test whether NC-derived EVs exert anabolic and/or anti-catabolic effects on IVD explants *ex vivo*.

2. Study setup

Porcine NC-derived EVs were isolated, purified, and characterized based on the guidelines of the International Society for Extracellular Vesicles (ISEV)[29,30], by using (ultra)centrifugation, size exclusion chromatography, and DigiWest technology. Thereafter, the presumed anabolic and anti-catabolic effects were studied in two separate models of NP explants. First, a bovine NP explant model was included in the study. The bovine model is considered a suitable biological model for the study of human lumbar discs, due to their similarity in size and cell type, NPCs, present in bovine disc[31]. In addition, bovine tails for the generation of explants are readily available through local abattoirs. NP explants were generated from caudal discs of four bovine donors of 9 to 18 months old. The bovine explants were considered to be non-degenerate[32] and were cultured in a degenerative disc environment. The second model, employed human disc tissue that was obtained from four patients with moderate degenerative disc disease undergoing surgical treatment (micro-discectomy) for nerve root compression (table 1). The human NP specimens act as the proof-of-concept on clinically relevant, degenerated NP tissue.

Donor number	Gender	Age (years)	Spinal level	Histopathological degeneration grade (out of 9)
HD654	Female	61	L5-S1	4
HD655	Female	69	L4-L5	6
HD656	Male	35	L5-S1	6
HD666	Female	27	L4-L5	4

Table 1. Information of the human donors used in the current study. Ethical committee approval to use human tissue taken at the time of spinal surgery for research purposes has been obtained from Sheffield Research Ethics Committee (09/H1308/70). Human discs from patients with moderate disc degeneration, with no prior surgery or other notable spinal deformities were utilized. Only human discs with an intact annulus at time of surgery were utilized to minimize the risk of immune cell contamination. A tissue sample was also collected, formalin-fixed and embedded to wax for histopathological grading. Histopathological scoring of the NP tissue was performed according to Le Maitre et al (2021)[33]

The core of bovine and human NP tissue was placed in a semi-constrained culture system, as previously described[34–36] and cultured in low glucose DMEM, 350 mOsm, 5% O_2 and serum-free conditions to mimic the degenerated disc environment. Explants were maintained under the following conditions: (1) basal degenerated culture media, (2) basal degenerated media with total NC-secretome (notochordal conditioned media (NCCM) - containing EVs and proteins), (3) basal degenerated media with only NC-proteins (EV-depleted NC-conditioned media), and (4) basal

degenerated media with purified pNC-EVs (figure 1). All four conditions were conducted with and without 0.1 ng/mL IL-1β, to further mimic the degenerated disc environment. The concentration of IL-1β was selected based upon previous work to represent physiological IL-1β levels[37]. Following 48 hours of culture, samples were collected to conduct RT-qPCR on anabolic, catabolic, cell cycle, phenotypic and inflammatory-related genes. During the following culture period of 14 days, medium of the explants was refreshed every 48 hours and retained for secretome analysis. After 14 days of culture, samples were collected to determine the effect of the pNC-EVs at histological (H&E, PSR-AB, immunohistochemistry) and biochemical (DNA, glycosaminoglycan, collagen, cytokines) level (figure 2). The conditioned media of human explants was further explored for secreted factors such as cytokines (IL-1α, IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL_17A, TNF, IFNγ, and IL-1 receptor antagonist), chemokines (MCP-1/CCL2, MIP1α/CCL3, MIP1β/CCL4, RANTES/CCL5, and GROA/CXCL1), and growth factors (EGF, TGFα, VEGF, and FGF2) with Luminex technology.

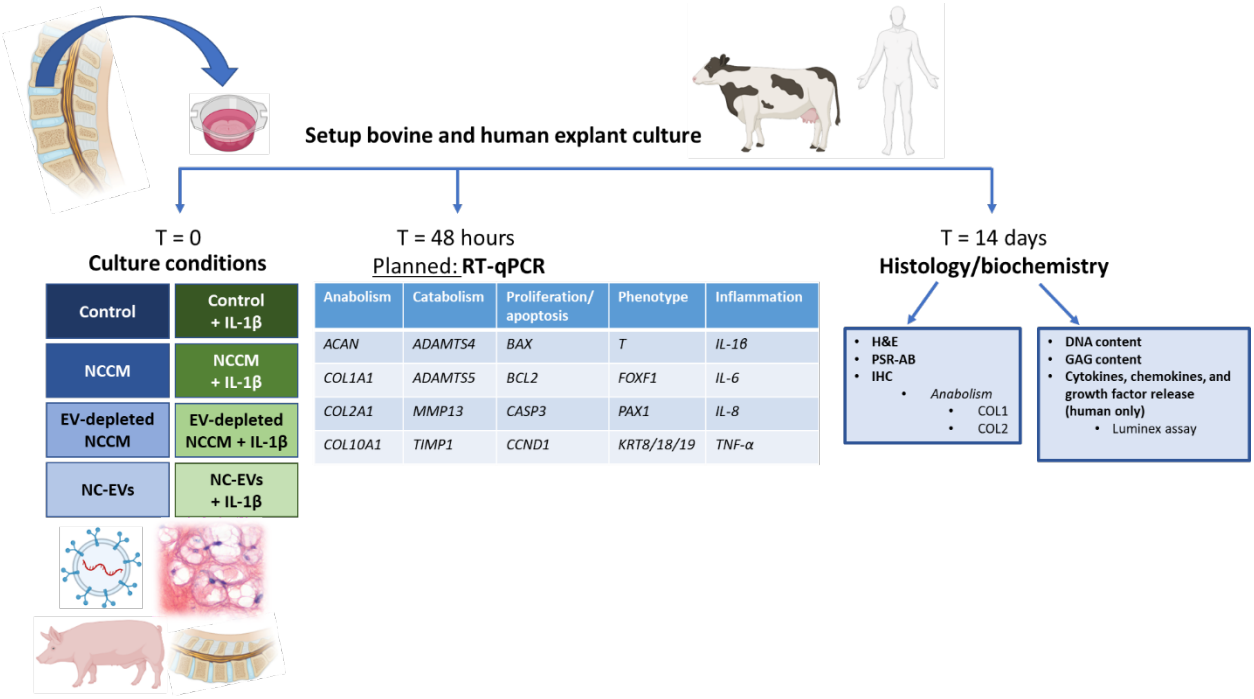


Figure 2. Setup of the bovine and human nucleus pulposus (NP) explant culture study to evaluate the anabolic and/anti-catabolic effect of porcine notochordal cell (NC)-derived extracellular vesicles (EVs). Bovine and on human NP explants were used. After 48 hours of culture, samples for RT-qPCR were collected and a list of targets was selected. After 14 days of culture, the effect of the NC-derived EVs were determined at histological and biochemical level.

Statistical analysis was performed using GraphPad Prism 9.3.1. Data were examined for normal distribution using the D'Agostino & Pearson test. As all data were non-normally distributed, a Friedman test with paired data for each data was performed. Benjamini and Hochberg False Discovery Rate post-hoc tests were performed to correct for multiple comparisons (between conditions). In all tests, a p-value <0.05 was considered significant. In addition, considering the challenges that come with biological samples, tests with a p-value <0.15 were considered to show a trend towards statistical significance. Tests showing a trend were included in the analysis since

the data showed a large inter-donor variation, which may cause a failure to achieve statistical significance in the tests.

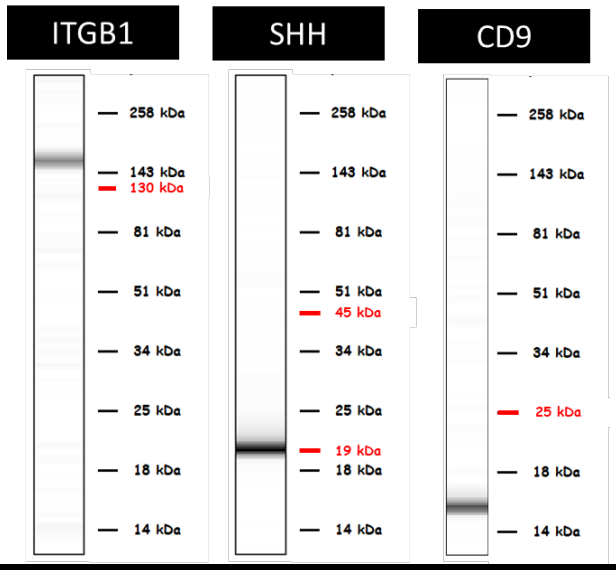
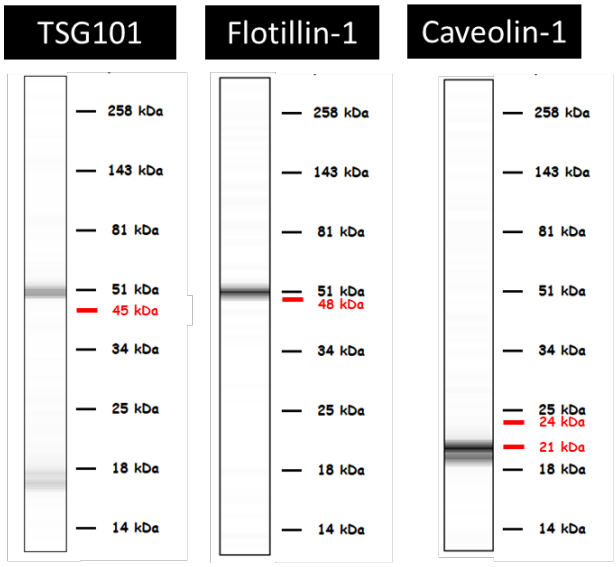
3. Results

3.1 Purification and characterization of porcine NC-derived extracellular vesicles

To demonstrate the EV nature and purity, DigiWest technology was used, a proprietary immunoassay technology which transfers Western Blot to a high-throughput bead-based microarray platform. In collaboration with Dr. Markus Templin (NMI TT, Germany), we have validated a panel of antibodies with this technology for EVs from multiple species, including pig NC-EVs (manuscript under review). The optimized panel mainly entails general EV markers to show the presence of EVs in the sample. Additionally, the panel contains proteins that give an indication on co-isolated proteins.

The DigiWest characterization of the isolated fractions showed the detection of protein markers that demonstrate the lipid-bilayer structure of EVs (i.e. integrin beta-1, sonic hedgehog, and CD9) and proteins that identify luminal protein cargo (i.e. TSG101, flotillin-1, caveolin-1, HSP70/HSC70, HSPA8, Annexin II, and GAPDH) (table 2). Additionally, fibronectin, and enolase-1 were detected in the NC-EVs, which are also considered as general EV-markers. Three possible co-isolated proteins, S6 ribosomal protein - pS235/pS236, keratin 8, and keratin 18, were absent from the EV preparations. Altogether, these results show characterization of our pig NC-EV according to the ISEV guidelines, and confirm that our isolation procedure successfully enriched for NC-EVs.

Category 1. Transmembrane or GPI-anchored proteins associated to plasma membrane/ endosomes			
Target protein	***Detected in EV-sample***	***Antibody specifications***	***Western blot mimics of a subset of marker proteins***
Integrin beta 1 (ITGB1)	✓	Transduction Laboratories 610468	
Sonic Hedgehog (SHH)	✓	C9C5, Cell Signaling 2207	

CD9	✓	EPR2949, Abcam ab92726	
Category 2. Cytosolic proteins recovered in EVs			
TSG101	✓	EPR7130(B), Abcam ab125011	
Flotillin 1	✓	Clone 18, BD transduction laboratories 610820	
Caveolin 1	✓	Cell Signaling, 3238	
HSP70/HSC70	✓	N27F3-4, Enzo ADI-SPA-820	
HSC70 (HSPA8)	✓	D12F2, Cell Signaling 8444	
Annexin II	✓	BD Transduction Laboratories 610068	
GAPDH	✓	D16H11, Cell Signaling 5174	
Category 3. Major components of non-EV co-isolated structures			
S6 ribosomal protein - pS235/pS236	✕	Cell Signaling 2211	
Category 4. Transmembrane, lipid-bound and soluble proteins associated with other intracellular compartments than PM/endosomes			
Keratin 8	✕	Cell Signaling 4548	

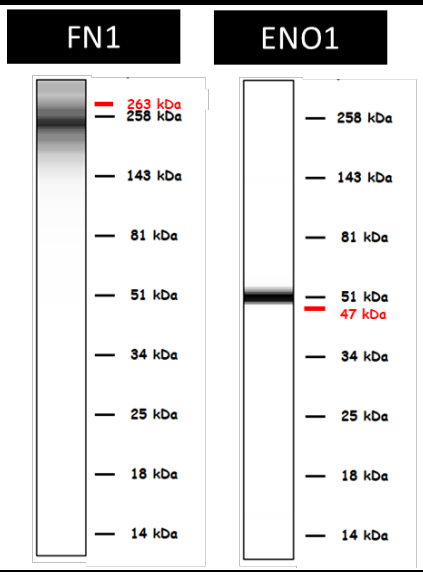
Keratin 18	✕	M20, Santa Cruz sc-52324	
Category 5. Secreted proteins recovered with EVs			
Fibronectin (FN1)	✓	F14, Abcam ab45688	
Category 6. Others of interest			
Enolase-1 (ENO1)	✓	Cell Signaling 3810	

Table 2. List of analyzed target proteins with DigiWest analysis in pig NC-EVs. Protein markers are divided into the categories set out by the International Society for Extracellular Vesicles (ISEV). ✓/✕ indicates if the marker was or was not detected in the EV sample. Clone number, manufacturer and antibody number are included in the table for reference. From the digital signals obtained from the DigiWest analysis, data can be visualized resembling traditional Western blots, so called Western blot mimics. Western blot mimics of a subset of marker proteins are included to visualize the signal obtained for that marker. The red marked molecular weight indicates the predicated molecular weight for that marker, based on the antibody information. n=5 for pig samples.

3.2 Bovine explant culture with porcine NC-derived extracellular vesicles

On histology at 2 weeks follow up cellular morphology was comparable among conditions (figure 3). In all conditions, more elongated spindle-like cells were distributed scarcely through the matrix. The alcian blue – picosirius red staining demonstrated limited blue proteoglycan ECM staining and dominance of collagen staining, which based on immunohistochemistry was mainly collagen type II. Collagen type I showed a faint staining trough the matrix of explants cultured with IL-1β. Based on observations, the histology for all four donors did not reveal a clear trend in the glycosaminoglycan, collagen type II, and collagen type I content in either of the treatment media. Quantification of the histological grade and the (immuno)stains is ongoing.

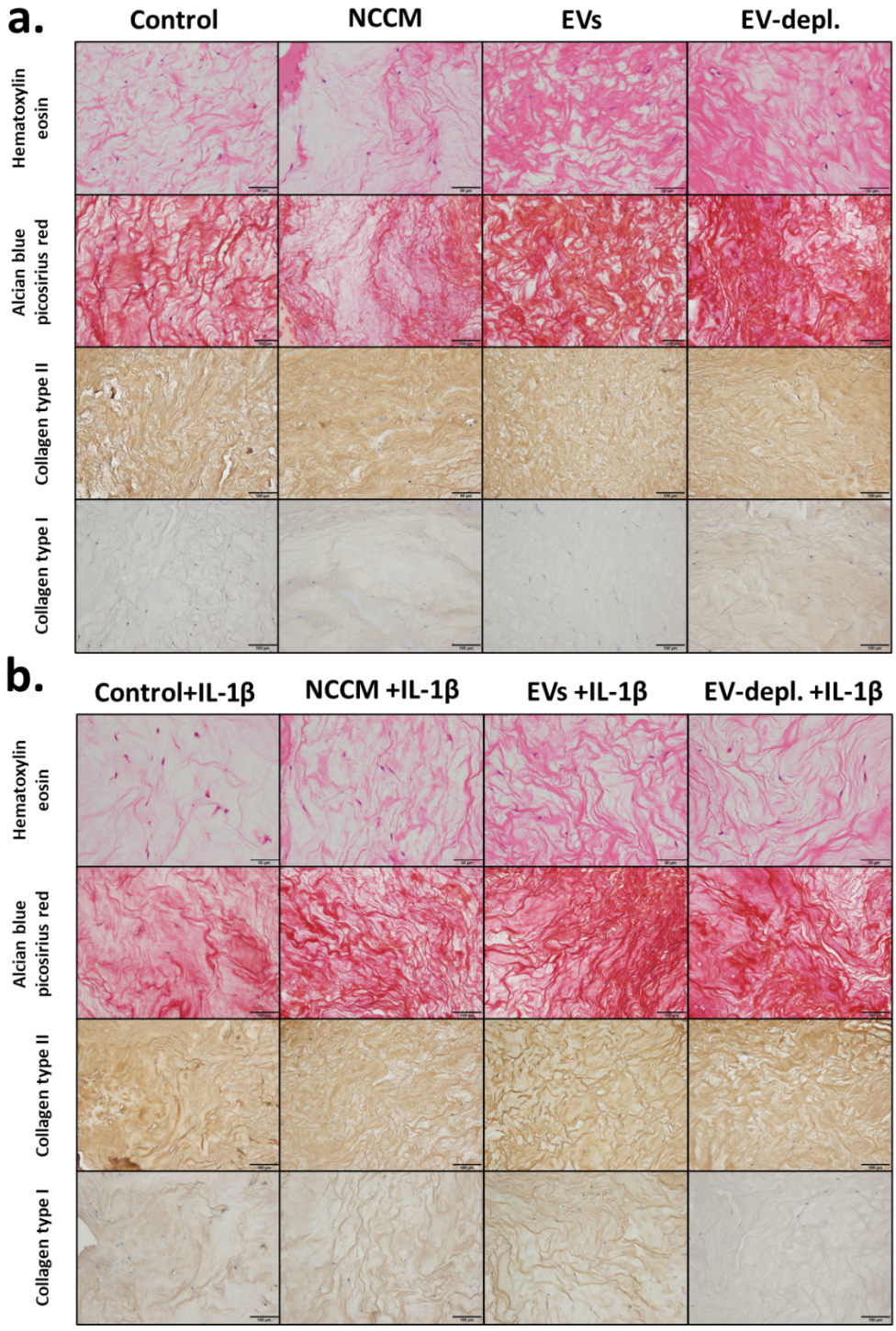


Figure 3. Histology and immunostains of bovine explants from one representative donor (d1) cultured for 2 weeks in treatment media (control, conditioned media (NCCM), NC-EVs, and EV-depleted media) in (a) the absence or (b) presence of 0.1 ng/mL IL-1β. For the alcian blue – picosirius red staining, blue staining indicates the presence of glycosaminoglycans whereas red staining indicates collagens in the matrix. Immunohistochemsitry was performed for collagen type I and type II. The scale bar in the hematoxylin and eosin staining represents 50 μm, in all other picture it represents 100 μm.

DNA and GAG content were analyzed to quantify the biochemical changes NC-EVs might have. The DNA content of bovine explants treated with NC-EVs in the absence of IL-1β tended to be lower than the explants cultured in control medium (p = 0.11) (figure 4a). In contrast, explants treated with NC-EVs + IL-1β displayed a trend towards a higher DNA content compared to control + IL-1β and NC-EVs treatment alone (p = 0.11). To correct for variability in explant size, the data was also corrected for the weight of the explants whereafter the beneficial effect of NC-EVs + IL-1β remained present (p = 0.09) (figure 4b). The GAG content of explants treated with control + IL-1β showed a small decrease compared to control (p=0.09), indicating that GAGs are lost upon IL-1β treatment (figure 4c). Surprisingly, NC-EVs treatment in the absence of IL-1β led to GAG loss (p<0.01). When corrected for the wet weight, this effect remained significant (p<0.05) (figure 4d). No effects on DNA or GAG content were observed in explants cultured with NC-conditioned medium (NCCM) or medium only containing soluble proteins (EV-depleted media). Altogether, these results indicate that the effects on DNA and GAG content observed upon NC-EV treatment are EV-specific and the increase in DNA content seems to be IL-1β-dependent.

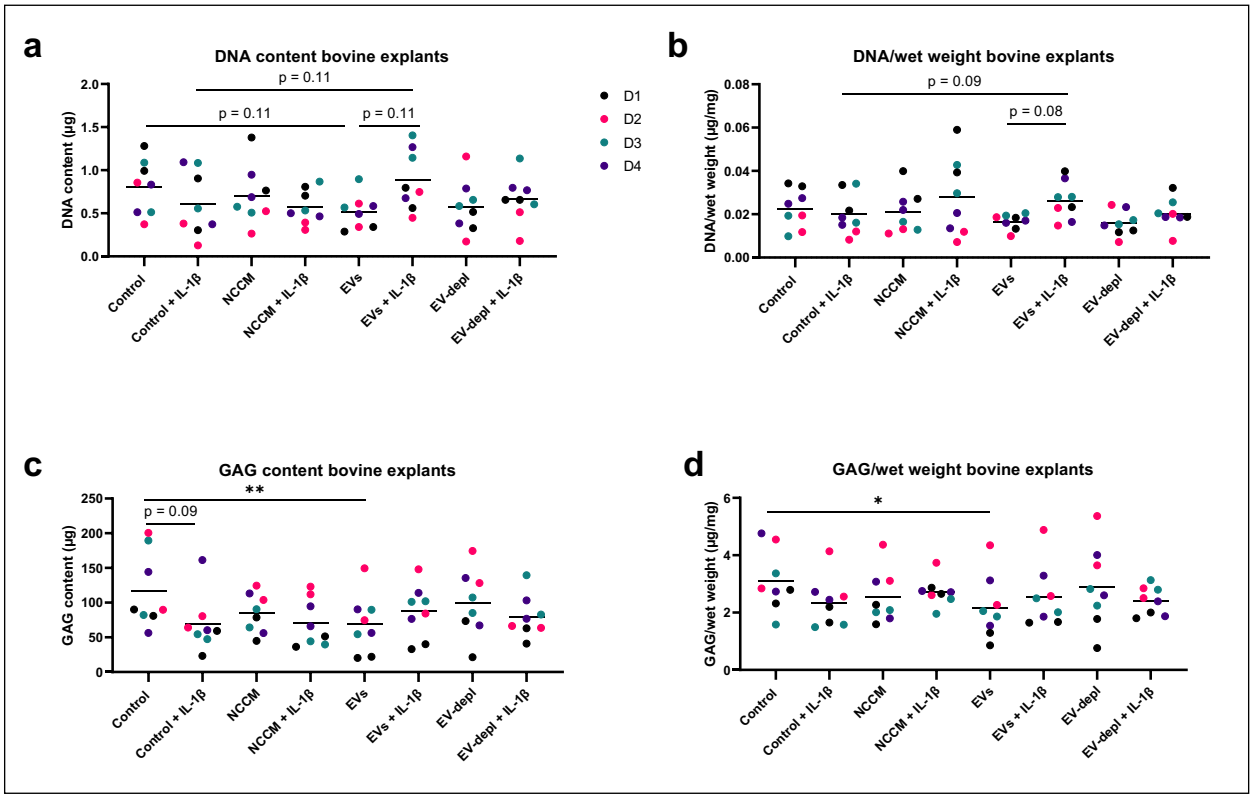


Figure 4. The effect of porcine notochordal conditioned medium (NCCM) derived NC-EVs on matrix production and cell survival in bovine nucleus pulposus disc explants in degenerative culture conditions. N=4 bovine explants donors (D1-D4) were tested with individual porcine NCCM donors (n=4). *,** significantly different from control condition (p<0.05, p<0.01 respectively). Conditions with a statistical trend (p<0.15) are displayed with their individual p-value.

3.3 Human explant culture with porcine NC-derived extracellular vesicles

On histology of the explants, mainly collagen staining was observed in the alcian blue – picosirius red staining, with the exception of blue glycosaminoglycan staining in the pericellular region of the NP cells (figure 5). Follow up stainings will be conducted with Alcian blue alone and aggrecan immunostains to further study the extracellular matrix. Immunohistochemistry revealed the main collagen type in the explants, to be collagen type II. In some of the explants faint matrix staining of collagen type I was observed, although this did not appear to relate to a certain treatment. The histology for all four donors did not reveal a clear trend in the glycosaminoglycan, collagen type II, and collagen type I content upon IL-1β addition or either of the treatment media. Quantification of the histological grade and the (immuno)stains is ongoing.

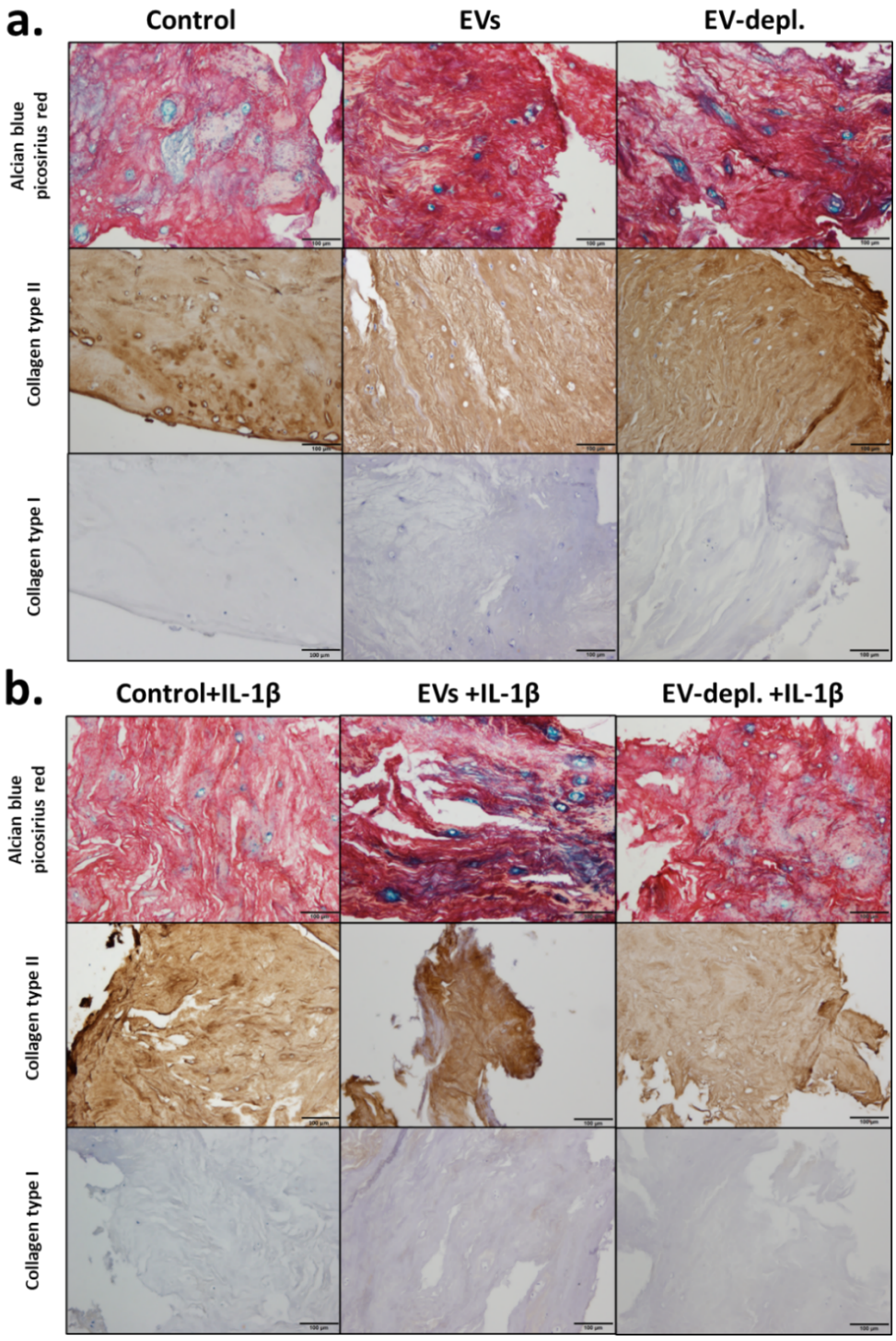


Figure 5. Histology of cultured human explants from one representative donor (HD655) cultured for 2 weeks in treatment media (control, NC-EVs, and EV-depleted media) in (a) the absence or (b) presence of 0.1 ng/mL IL-1β. For the alcian blue – picosirius red staining, blue staining indicates the presence of glycosaminoglycans whereas red staining indicates collagens in the matrix. Immunohistochemsitry was performed for collagen type I and type II. The scale bar represents 100 μm.

Treatment of patient-derived NP explants with NC-EVs in the absence of IL-1β led to a small increase in the DNA content compared to control medium (p=0.07) and to NC-EV treatment in the presence of IL-1β (p=0.08) (figure 6a). This effect was absent in the EV-depleted media. When corrected for the wet weight of the explants the higher DNA content in NC-EV-treated explants did not reach statistical significance (figure 6b). The GAG content of human explants treated with NC-EVs or EV-depleted media was not significantly different from control media with or with IL-1β (figure 6c, 6d).

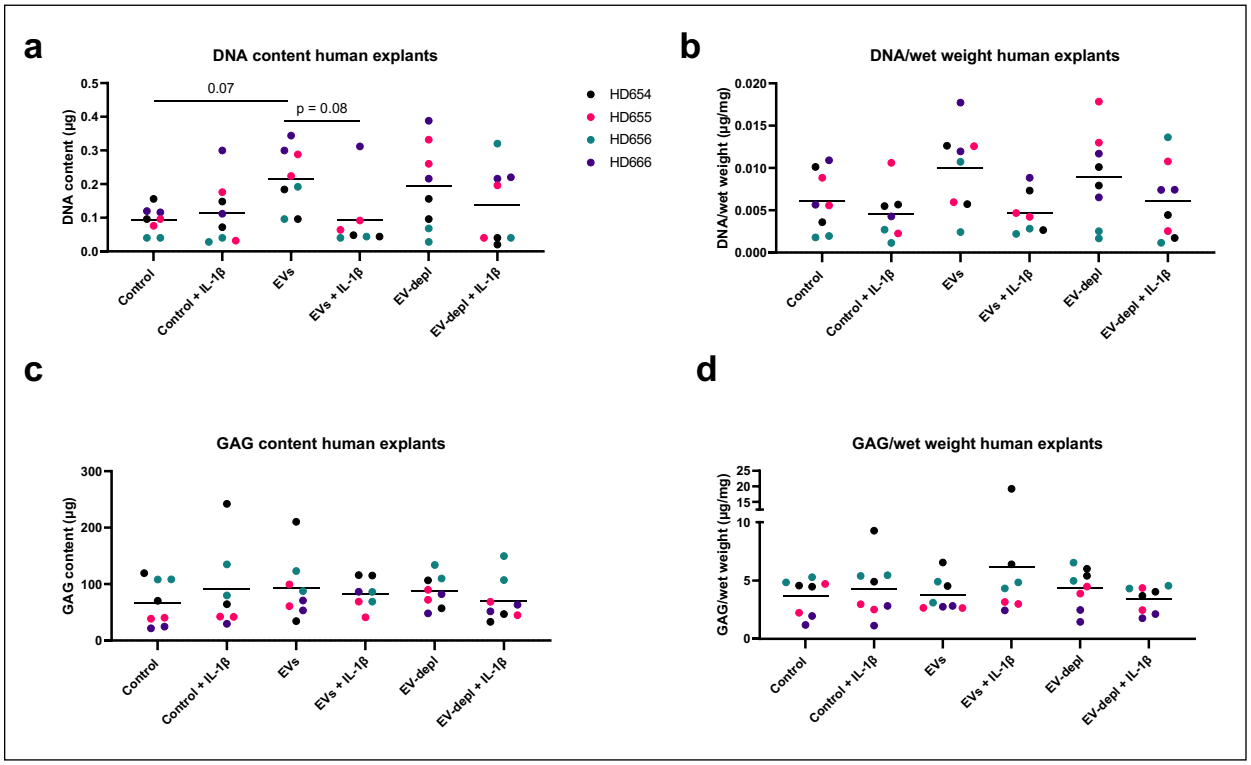


Figure 6. The effect of porcine notochordal conditioned medium (NCCM) derived NC-EVs on matrix production and cell survival in degenerated human nucleus pulposus disc explants. N=4 human explants donors (HD654-HD556, and HD666) were tested with individual porcine NCCM donors (n=4). Conditions with a statistical trend (p<0.15) are displayed with their individual p-value. Data are displayed as the grand mean with the individual donors plotted.

The cytokine, chemokine and growth factor release was measured in the explant media after 2 weeks of culture. The absolute IL-1β levels (i.e. not corrected for wet weight) were consistent with the amount of IL-1β added to the cell culture medium (0.1 ng/mL) (figure 7), which indicates that the assay measured the added IL-1β rather than additional IL-1β released from the explants in the different culture conditions (Figure 8b). The cytokine profiles of human explants showed small changes upon IL-1β stimulation every 48 hours after two weeks, with a non-significant increased release of IL-1α, IL-10, IL-17A, TNF, and IFNγ with IL-1β (figure 8). Cytokines levels in EV- and EV-depleted media alone (without explant culture) were measured to exclude that background level of the cytokine in the treatment media already led to an increase in that cytokine. In control + IL-1β media Il-6 release was decreased, although not significantly (figure

8e). When NC-EVs were added in IL-1β containing medium, the IL-6 release significantly increased. Background levels of IL-6 were absent, indicating that the increased release of IL-6 was produced by the explant itself. In addition, the observed effect seemed to be EV-specific because no effects were observed in explants treated with EV-depleted media. In the other analyzed cytokines, no differences in release were seen upon IL-1β addition or one of the treatments.

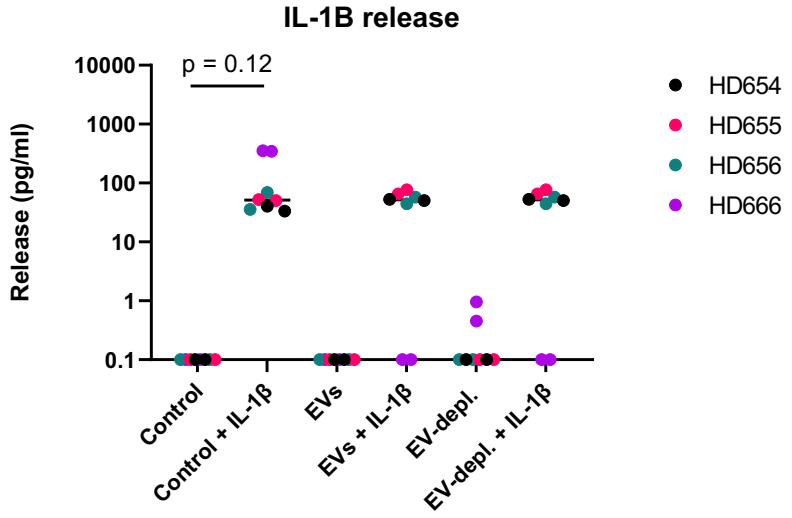


Figure 7. Absolute IL-1β release in the culture media of degenerated human nucleus pulposus disc explants after 2 weeks of culture. N=4 human explants donors (HD654-HD556, and HD666) were tested with EVs isolated from individual porcine NCCM donors (n=4). Conditions with a statistical trend ($p<0.15$) are displayed with their individual p-value. Data are displayed as the grand mean with the individual bovine donors plotted.

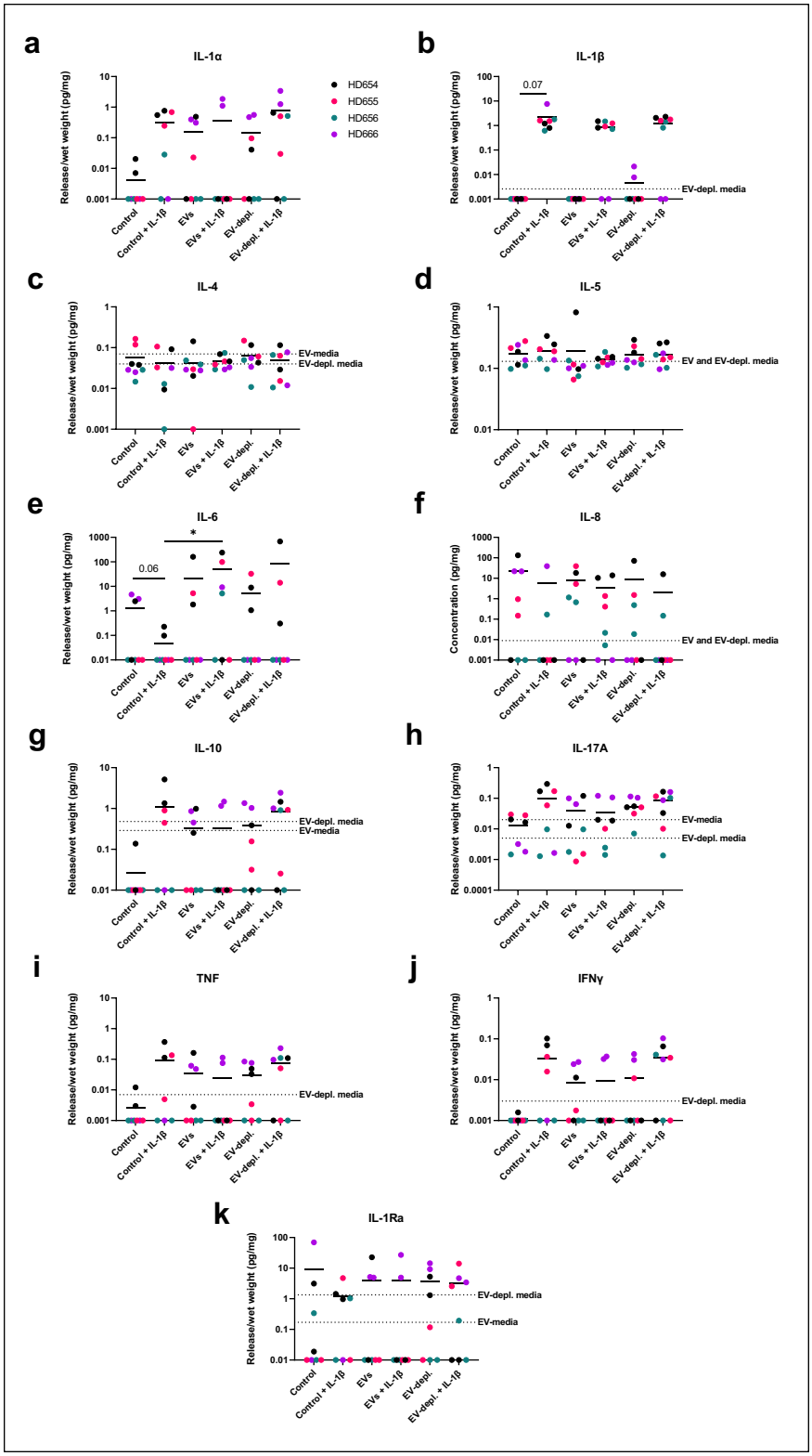


Figure 8. The effect of porcine notochordal conditioned medium (NCCM) derived NC-EVs on cytokine and IL-1 antagonist release in degenerated human nucleus pulposus disc explants after 2 weeks of culture. The data represent the released cytokines in the media during 48 hours of culture. N=4 human explants donors (HD654-HD556, and HD666) were tested with EVs isolated from individual porcine NCCM donors (n=4). * significantly different from

control condition ($p<0.05$). Conditions with a statistical trend ($p<0.15$) are displayed with their individual p-value. Data are displayed as the grand mean with the individual donors plotted. Samples that were not detectable are plotted on the x-axis for visibility. The dotted line in de graphs indicate the concentration of the chemokine in EV- and EV-depleted media alone (without explant culture).

Of the five chemokines analyzed with multiplex ELISA, changes were observed in CCL2 and CXCL1 release upon treatment for 14 days with NC-EVs (figure 9). Upon treatment with NC-EVs, CCL2 release/wet weight was increased (figure 9a). However, if IL-1β was present in the NC-EV culture medium, the CCL2 release was significantly decreased when compared to NC-EVs alone, reaching near the background level for this chemokine in three explant donors. No effects on CCL2 were observed when the explants were cultured with EV-depleted media. Treatment of the explants with control + IL-1β, resulted in an increased release of CXCL1, although not significant (figure 9e). In the presence of IL-1β, the treatment of NC-EVs resulted in a decrease of CXCL1. This effect was absent when EV-depleted media were used, indicating that the effect was NC-EV specific.

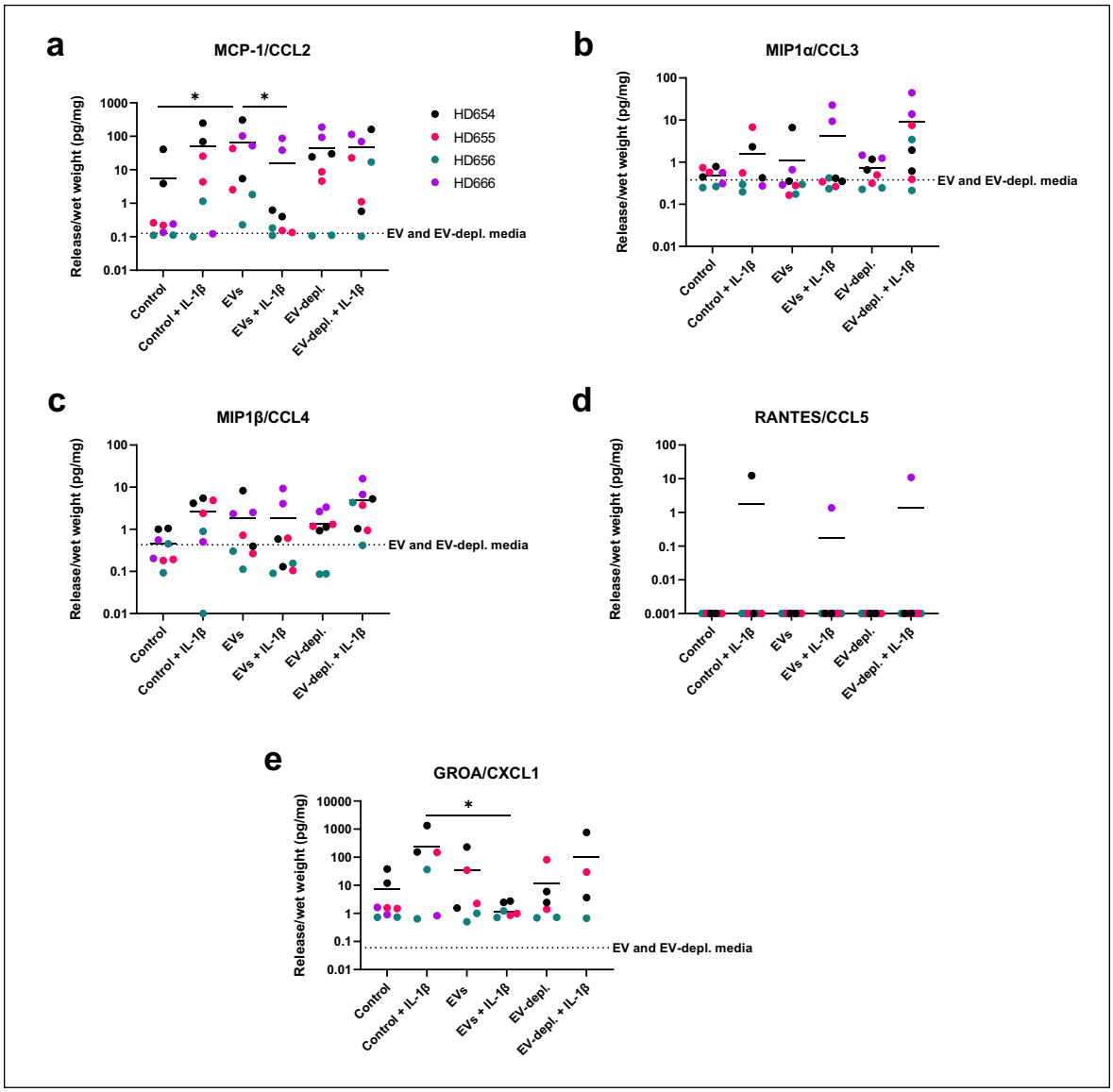


Figure 9. The effect of porcine notochordal conditioned medium (NCCM) derived NC-EVs on chemokine release in degenerated human nucleus pulposus disc explants after 2 weeks of culture. *The* data represent the released cytokines in the culture media during 48 hours of culture. N=4 human explants donors (HD654-HD556, and HD666) were tested with individual porcine NCCM donors (n=4). * significantly different from control condition ($p<0.05$). Data are displayed as the grand mean with the individual donors plotted. Samples that were not detectable are plotted on the x-axis for visibility. The dotted line in de graphs indicate the concentration of the chemokine in EV- and EV-depleted media alone (without explant culture).

The analysis of growth factor release by the explants is of interest in the event the effect of NC-EVs is linked to matrix anabolic effect or changes in vascularization of the degenerating disc. Of the growth factors that were analyzed in the explant media, none were significantly changed upon NC-EV treatment (figure 10). Also, no effects were observed when treating the explants with EV-depleted media. It remains to be determined how growth factors related to pain behave under these conditions, including NGF and substance P.

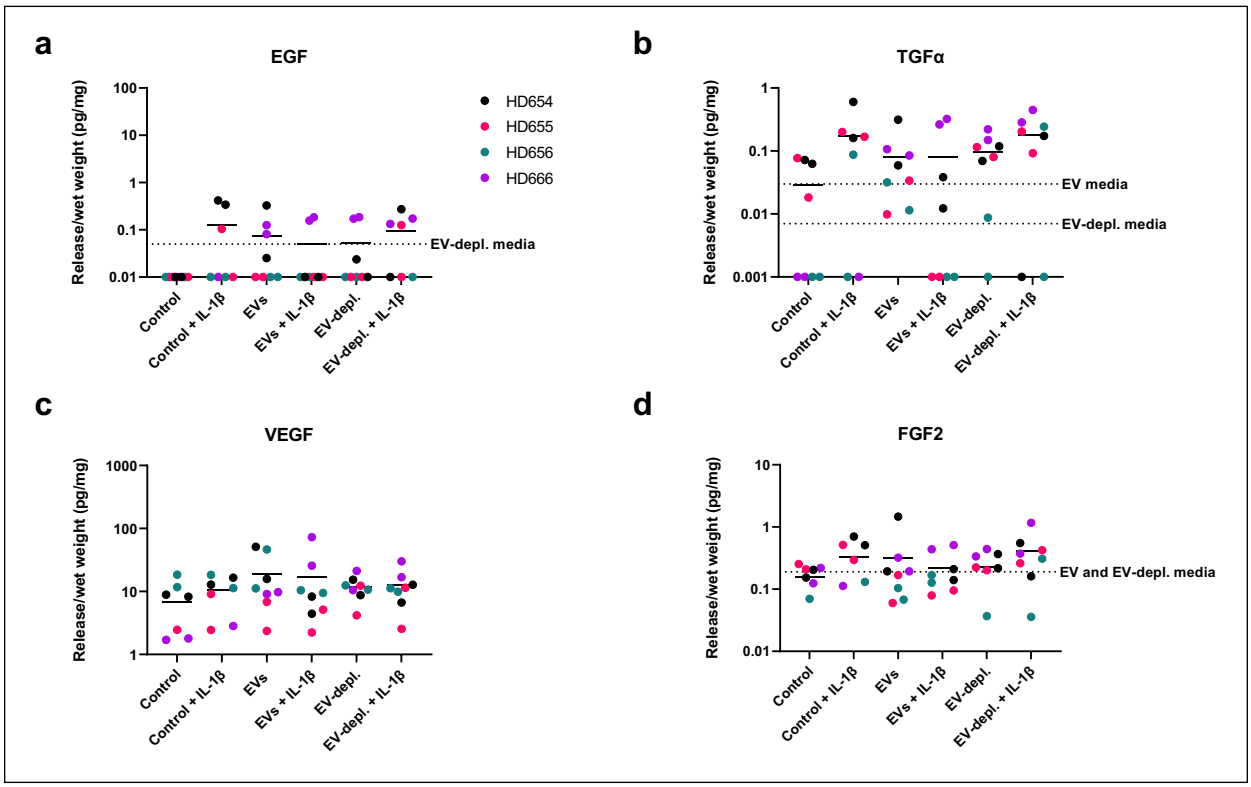


Figure 10. The effect of porcine notochordal conditioned medium (NCCM) derived NC-EVs on cytokine and IL-1 antagonist growth factor release in degenerated human nucleus pulposus disc explants after 2 weeks of culture. The data represent the released cytokines in the culture media during 48 hours of culture. N=4 human explants donors (HD654-HD556, and HD666) were tested with individual porcine NCCM donors (n=4). Data are displayed as the grand mean with the individual donors plotted. Samples that were not detectable are plotted on the x-axis for visibility. The dotted line in de graphs indicate the concentration of the chemokine in EV- and EV-depleted media alone (without explant culture).

4. Discussion

This study is the first to examine the potential anabolic and anti-catabolic effects of NC-EVs in an *ex vivo* semi-constrained culture model of disc degeneration. NP explants from bovine tails were subjected to a pro-inflammatory stimulus with IL-1β to create a model system for human disc degeneration[31] and human surgery samples with moderate disc degeneration were studied in the absence and presence of a pro-inflammatory stimulus. Below we discuss the preliminary results by evaluating the explant models that was used in this study and comparing it to other systems. Thereafter, we discuss the NC-EV specific effects observed within the NP explants from both species.

The model system used to test NC-EVs in this study uses a semi-constrained design which previously has been reported to maintain tissue structure and cellular phenotype in human NP explants[38]. The NP explant model consists of a NP biopsy which is cultured in a silicone ring. This model still allows free swelling of the NP tissue at the top of the explant free swelling, which is anticipated to be much dependent on the initial GAG-content of the tissues, and as such much more pronounced in the bovine NP explants and negligible in moderately degenerate human NP tissue. It is well known that free swelling of NP tissue leads to proliferation of spindle-shaped dedifferentiated NP cells around the edges of the tissue, giving rise to several cell layers of dedifferentiated cells around the explant[39–41]. This characteristic histological change was however, not observed in the histology from this study of the bovine explants, nor in the human explants as shown in this and the previous study using this model[36]. The proliferation of the cells around the edges of the swollen tissue explant leads to a large characteristic increase in DNA[40,41]. The DNA content of bovine explants cultured for 14 days in our semi-constrained model were comparable to studies using a constrained model[8,42], indicating that the semi-constrained design might not have led to cellular alterations in the explant.

Accompanying the cellular change, swelling of NP tissue leads to a drastic loss of GAGs from the matrix due to leakage of the GAGs to the medium[40,41]. The GAG content of bovine explants cultured for 14 days in our semi-constrained model was lower than a study using a bovine constrained NP model[8]. The low abundance of GAGs is our explant model was also visible by the absence of GAG staining on histology. These results might be a results from limited sensitivity of the alcian blue staining in the presence of many collagens, in our study mainly collagen type 2. Previous work has shown that GAG staining of the matrix is maintained in the semi-constrained human NP model[38]. Additional stainings to show the presence of GAGs in study will be safranin-O and an immunohistochemistry staining for aggrecan. However, the low abundance of GAGs might also suggest that in our model there is leakage of GAGs from the matrix. To confirm this, GAG release should be measured in the medium that was collected during the culture period of 14 days. To excluded the possible unwanted effects of the swelling of the explants on the matrix composition, a fully constrained model, such as explant culture in a dyneema jacket[42] or culture in a bioreactor[43], could be considered. A major advantage of culturing the tissue explants in a bioreactor is that loading can be applied to the tissue, increasing its relevance for the *in vivo* situation[43]. The latter come however with limitations related to large culture media volumes

needed which is challenging in light of the cumbersome isolation of EVs from NCCM, and as such would require a considerable upscaling of the method of pNC-EV isolation.

In addition to cellular and matrix changes, it has been shown that free swelling of the NP tissue can result in altered secretory signature of the NP tissue. Free swelling of NP tissue results in an increased release of the pro-inflammatory cytokines prostaglandin E_2 and IL-6[40,41], but not of IL-1β, TNF-α, IL-8, and nitric oxide[40]. In our study, using human explants IL-1β and TNF-α were undetectable in NP tissue cultured in control medium, comparable the data of free swollen bovine NP. To exclude that trapping of the cytokines in the tissue has resulted in a failure to detect them, analysis of the digested tissue could be included, this is however not available due to the study set up. The IL-6 release by the human explants was lower than in a fully constrained bioreactor model of bovine NP explants[44], indicating that the constraining of the model in this study was sufficient. To support this, prostaglandin E_2 can be measured in the collected media from this study and compared to the levels detected in fully constrained bovine and human NP explants while correcting for the differences in tissue weight/media volume ratio among the different culture models.

To mimic the degenerating disc, a more pro-catabolic environment was created with the addition of IL-1β to the culture media. The supplementation of 0.1 ng/mL IL-1β every 48 hours for 2 weeks led to a reduction of GAGs in the bovine explants. This reduction in GAGs was not visible in the alcian blue – picosirius red staining, possibly to a lack of sensitivity. An immunohistochemistry staining for aggrecan will be optimized to visualize these subtle change. In human explants, the decrease GAG content upon IL-1β stimulation was not observed. This difference may be explained by the differences in degeneration grade between bovine and human NP tissue. In human discs an increased effect of IL-1β would be expected on more degenerated disc based on the higher expression of IL-1 receptor in degenerated tissues[45]. It is known that NP cells from degenerated disc respond more pronounced to IL-1β stimulation[46]. To our knowledge, however, it has not be examined if more degenerated tissue responds differently to IL-1β stimulation *in vitro*.

The effect of IL-1β on the explants was further studied, by analyzing the chemokine, cytokine, and growth factor secretome of human explants. After two weeks of culture only very mild changes upon IL-1β stimulation were observed. With the addition of 0.1 ng/mL IL-1β in control degenerative media, multiple chemokines, such as CCL2, CCL4, and CXCL1, and cytokines, IL-1α, IL-10, IL-17A, TNF, and IFNγ, showed visually a higher release with IL-1β in control media, but did not reach statistical relevance. In addition, a small non-significant decrease in IL-6 release was seen in control media + IL-1β. Previous work on the development of a catabolic model for disc degeneration in bovine disc explants showed significant upregulation of cytokines, such as IL-6, IL-8, and PGE_2, and matrix metalloproteinases (MMPs) after treatment with the pro-inflammatory cytokines IL-1β[47] with much higher concentrations (10 and 100 ng/ml) and only at the gene expression level, TNF-α (100 ng/cm^3 disc volume) [44,48] achieving increased NO and IL-8 release in the media or a combination of those two (both employed at 100 ng/ml)[42]. The models between the previous studies and this study, however differ in the levels of the pro-catabolic

stimulus provided (0.1 ng/ml in the present study versus 10-100 ng/ml IL-1β) and in the way they are constrained, with our model being a semi-constrained model versus the aforementioned constrained models. In addition, this study showed differences between donors in the way the respond to IL-1β. The donor variability is further discussed below.

The concentration of IL-1β used in our study (0.1 ng/mL) is considered low when compared to other studies, which use 10-100 ng/mL IL-1β[42,47]. To our knowledge, the exact levels of IL-1β in degenerating discs *in vivo* is unknown. It is believed, however, that a lower concentration of IL-1β better approaches the *in vivo* degenerative niche better. Previous studies on human has shown that lowering the concentration of IL-1β below 1 ng/mL, sufficiently induced catabolic effects[37]. Therefore, the IL-1β concentration in this study was reduced to the minimal concentration of IL-1β in which catabolic effects were observed (*unpublished*). It has been suggested that when using low concentrations of proinflammatory cytokines in explant culture longer time is needed to induce a consistent and significant induction in chemokine and cytokine secretion[44]. In our study, it is evident that there was variability in the responsiveness of the donors to the IL-1β stimulation and it remains to be determined whether this relates to differences in the IL-1β receptor expression which may indirectly also correlate to the degenerative state of the tissue. This may explain the relatively mild overall effects of IL-1β on the disc explants in this study. Immunohistochemistry staining of the cultured explants will be performed to analyze differences in IL-1 receptor expression. Additionally, staining for this receptor could be done on native tissue sample to detect differences between donors.

The anabolic and anti-catabolic effects of NC-EVs were tested in the established NP explant model for both bovine and human species. The anabolic effect of NC-EVs were more pronounced in the bovine explants compared to human NP explant culture. At the cellular level, DNA content increased upon treatment with NC-EVs in the presence of IL-1β in bovine tissues, and only in the absence of IL-1β in the human explants. The increased DNA can be attributed to either increased proliferation of NP cells and/or improved cell survival facilitated by the EVs. Based on histology, there were no indications that there was cell proliferation, i.e. no cell clusters were seen, so it is hypothesized that NC-EVs in these conditions improved cell survival of the NP cells. To evaluate cell proliferation upon NC-EV treatment, immunohistochemistry staining for the proliferation marker Ki-67 can be performed. Furthermore, it may be plausible that the NC-EVs improved cell survival by inhibiting apoptosis, as had been shown for notochordal cell conditioned medium (NCCM) before[3,4]. To confirm the hypothesis that NC-EVs inhibit apoptosis pathways, immunohistochemistry stainings for the apoptosis markers that have been shown to be inhibited by NCCM[3,4], caspase 3 and caspase 9, may be performed. Additionally, for bovine NP explants, samples for qPCR have been collected. The early effects of NC-EVs on the explants in apoptosis can be measured by performing RT-qPCR for the BCl2 family and analyze the BCL2 associated agonist of cell death (BAD) and BCL2 associated X apoptosis regulator (BAX) expression[49].

Contrary to earlier reports conducted on 3D pellet culture of human and dog NP cells, in the present study NC-EVs did not result in a distinct matrix effect in the explant model. In this study, the addition of NC-EVs in media without IL-1β led to a decrease in GAG content in bovine

explants, while no effect of NC-EVs was not observed in human NP explants. A previous study, using a 3D micro-aggregate culture of human NP cells did observe an increase in the GAG content upon NC-EV treatment[50]. Another study, using constrained bovine explant culture, also showed an increase in GAG content when treated with NCCM[8]. This anabolic effect of NCCM was not observed in our study, suggesting that the semi-constrained model may not have been enough to preserve the GAGs in the matrix and to show an increase in GAG content. Additional measuring of GAG release in the media would give an indication as to the cumulative amount of GAGs in the condition. The interpretation of the cumulative release are however limited due to a high GAG concentration in the uncultured NCCM and NC-EV media themselves[24]. Further analysis of the anabolic effects of NC-EVs will be performed by determining gene expression of matrix proteins (aggrecan, collagen 2, collagen 1) on bovine explant samples that have been collected during the study and combined with aforementioned (immune)histochemical quantitative analysis of the respective matrix components.

The effect of NC-EVs on the secretory signature of human disc explants cultured for 2 weeks was limited to CCL2, CXCL1, and IL-6. The NC-EV effect seemed to be dependent on the presence of IL-1β, as CCL2 increased, but decreased again in the presence of IL-1β upon treatment with NC-EVs. Similarly, CXCL1 secretion reduced with the addition of NC-EVs and IL-1β. Both CCL2 and CXCL1 are considered pro-inflammatory cytokines and function as a key chemokines for leukocyte migration[5152]. CCL2 levels directly corelate with progression of disc degeneration[53] and CXCL1 expression is significantly higher in infiltrated disc samples[53]. Infiltration of immune cells can further amplify the release of pro-inflammatory cytokines and thereby stimulate the degeneration of the disc[54]. Interestingly, treatment of the explants with NC-EVs in the presence IL-1β alone resulted in an increase in IL-6. IL-6 is increased during disc degeneration[54] and upregulated in protruded tissue[55]. Increased release upon NC-EV treatment would thus suggest the opposite effect of NC-EVs. However, current literature has mainly focused on the catabolic response of IL-6 in the disc, whereas in other tissues it also has been suggested to have a role in tissue regeneration. The regenerative effect of IL-6 is suggested to act via three pathways, STAT3, AKT, and ERK1/2 signalling[56]. In rat NP cells, activation of AKT signaling increased matrix synthesis[57] which could argue that stimulation of this pathway by IL-6 may promote an anabolic response in the disc. Supporting data for this hypothesis can be generated by gene expression analysis for these three pathways. Altogether these data show that NC-EVs may inhibit immune cell infiltration in the degenerating disc which could facilitate an pro-anabolic response in the degenerating disc. While the role CCL2 signaling in extracellular matrix turnover has been studied within the context of its effects on macrophages it remains to be determined whether this also may hold true for the direct effects of CCL2 signaling on the resident NP cells.

Overall, it can be noted that the effect of NC-EVs on the chemokine and cytokine profile showed quite some donor variation, with some donors showing no response in cytokine and chemokines release upon IL-1β or NC-EV treatment. Differences between the donors might relate to the age of the donor or the degeneration grade of the disc material. Surprisingly, the two oldest donors, HD654 and HD655, aged 61 and 69 respectively, showed the clearest improvement in the chemokine profile upon treatment. Degeneration grade based on histology did not seem to

influence this, since the degeneration grades between the older and younger donors were similar. For other EV types it is suggested that their effects on target cells may be context dependent[58,59]. The different ages of the patients may give rise to a different context on cellular levels, influencing the effect of NC-EVs on the explants.

A possible limitation of the study might be the administration of the NC-EVs to the explants. In the current study, the treatments were supplemented to the media, requiring penetration of the NC-EVs into the explant. With increased degeneration, the matrix of the disc becomes more fibrotic and therefore more stiff[60]. The fibrotic tissue could limit the penetration of the EVs into the core of the explant. Penetration of EVs into stiff tissues, like cartilage tissue, is still challenging[61]. One study showed the tissue penetration of mesenchymal stromal cell-derived EVs in a cartilage explant to be 30-40 μm after a five hour time-lapse[62]. Affirmatively, EVs derived from neutrophils did not reach a tissue penetration of more than 100 μm in a rat cartilage explant model[63]. These data would suggest that penetration of NC-EVs in the denser matrix of degenerated disc explants is limited. However, EVs from other cell types have been shown to contain matrix degrading enzymes, facilitating their penetration through the matrix[64]. It is however unknown if NC-EVs carry such enzymes. To demonstrate tissue penetration in our explant model, localization of the NC-EVs within the explant can be considered using labelled EVs[62,63]. To circumvent the need for tissue penetration by the EVs and to simulate better the clinical application of EVs, injection of the NC-EVs in the explant could be applied.

5. Future perspectives

Altogether, this study is the first to explore the effect of NC-EVs on two clinically relevant *ex vivo* models for disc degeneration. Initial results indicate that NC-EVs might have anti-apoptotic and anti-inflammatory effects in the disc explant model of two species. The decrease in chemokine production upon treatment with NC-EVs suggest that these might inhibit immune cell infiltration in the degenerating disc, which may allow them to break the vicious catabolic circle in the pathophysiology of disc degeneration[54]. Furthermore, it remains to be determined if these chemokines are also directly involved in the extracellular matrix turnover by the resident NP cells. Analysis of the samples collected in this study is still ongoing and will focus on further establishing the anti-catabolic properties of NC-EVs. After conformation of their potential regenerative effects in the current model, the next step would be to evaluate NC-EVs in an *ex vivo* disc model, including physiological loading of the disc[48,65]. As an addition to the biological data obtained in these studies, it is of interest to unravel the mechanism of action of NC-EVs, which is currently still unknown. In a parallel study, we are currently comprehensively analyzing the protein content of pig NC-EVs using mass spectrometry. Data from that study will be combined with the data from this study to obtain a better biological view on the mechanism of action of NC-EVs and their potential to be implemented as a treatment for future disc degeneration patients. Thereafter, the first steps towards animal models can be made, *e.g.* by intradiscally injecting NC-derived EVs in (slow-release facilitating) scaffolds/hydrogels.

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