

## DEGENERATED HUMAN INTERVERTEBRAL DISCS CONTAIN AUTOANTIBODIES AGAINST EXTRACELLULAR MATRIX PROTEINS

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### Abstract

Degeneration of intervertebral discs (IVDs) is associated with back pain and elevated levels of inflammatory cells. It has been hypothesised that discogenic pain is a direct result of vascular and neural ingrowth along annulus fissures, which may expose the avascular nucleus pulposus (NP) to the systemic circulation and induce an autoimmune reaction. In this study, we confirmed our previous observation of antibodies in human degenerated and post-traumatic IVDs cultured *in vitro*. We hypothesised that the presence of antibodies was due to an autoimmune reaction against specific proteins of the disc. Furthermore we identified antigens which possibly trigger an autoimmune response in degenerative disc diseases. We demonstrated that degenerated and post-traumatic IVDs contain IgG antibodies against typical extracellular proteins of the disc, particularly proteins of the NP. We identified IgGs against collagen type II and aggrecan, confirming an autoimmune reaction against the normally immune privileged NP. We also found specific IgGs against collagens types I and V, but not against collagen type III. In conclusion, this study confirmed the association between disc degeneration and autoimmunity, and may open the avenue for future studies on developing prognostic, diagnostic and therapy-monitoring markers for degenerative disc diseases.

**Keywords:** Intervertebral disc, nucleus pulposus, annulus fibrosus, extracellular matrix.

### Introduction

The intervertebral disc (IVD) is the largest avascular structure in the human body. It is composed of an outer annulus fibrosus (AF), made up of concentric lamellae rich in collagen type I, and a central gelatinous nucleus pulposus (NP), rich in proteoglycans and collagen type II (Raj, 2008). Collagens type I and type II give tensile strength and anchor the disc tissue to the bone. On the other hand, tissue hydration of the disc is sustained by the major proteoglycan, aggrecan, which with the help of its chondroitin and keratin sulphate chains, maintains the osmotic pressure of the disc (Urban and Roberts, 2003).

With increasing age and degeneration, the disc becomes disorganised: annular lamellae become irregular (Raj, 2008) and aggrecan degradation results in a loss of hydration, which leads to progressive loss of space between vertebrae (Urban and Roberts, 2003). The fall in concentration of aggrecan allows penetration of large molecules into the disc, such as growth factors and cytokines (Raj, 2008). Molecular mediators of inflammation are present in pathologic disc tissues. Interleukin-1 beta (IL-1 $\beta$ ) and tumour necrosis factor alpha (TNF- $\alpha$ ) (Le Maitre *et al.*, 2007), IL-6 and IL-8 (Burke *et al.*, 2002), IL-17 and IFN $\gamma$  (Shamji *et al.*, 2010) may correlate with the grade of degeneration and have been linked to back pain. Additionally, aggrecan in the intervertebral disc inhibits nerve growth and repels sensory nerves, suggesting that loss of aggrecan can cause vascularisation and innervation of the disc (Johnson *et al.*, 2002). Structural changes and increased vascular and neural ingrowth in degenerated discs have been closely associated with chronic back pain (Stefanakis *et al.*, 2012). Low back pain is a very common multifactor condition in the general population; it becomes chronic in 30 % of cases and causes great economic impact, due to loss of productivity and increased health care costs (Strong *et al.*, 2013). Back pain is related to ageing, mechanical stresses (Adams, 2004) and genetic predisposition (Patel *et al.*, 2011), and it is attributed to disc degeneration in around 40 % of patients (Rodrigues-Pinto *et al.*, 2013). Pathogenesis of chronic low back pain has been also hypothesised to have an autoimmune component (Bobechko and Hirsch, 1965; Kim *et al.*, 1981). Healthy IVDs are avascular and NP is contained within the AF, hidden from the immune system. Vascularisation along the annular fissures exposes the degenerated disc to contact with cells of the immune system, and epitopes or degradation products of disc proteins may trigger an autoimmune reaction (Geiss *et al.*, 2007). The ability of NP to induce an inflammatory response and autoimmune

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reaction has been shown in dogs (McCarron *et al.*, 1987), pigs (Geiss *et al.*, 2007) rabbits (Bobechko and Hirsch, 1965), and the presence of IgGs has been identified in canine NP by ELISA assay (Pennington *et al.*, 1988). Murai *et al.* (2010) have also demonstrated in rats, that non-degenerated NP cells *in vitro* and transplanted NP tissues *in vivo*, can be recognised by macrophages and natural killer cells (NK), which may have an immunological function in the early phase of disc herniation. Patients with prolapsed IVD and sciatica had an increased disc to serum antibody ratio, measured by nephelometry (Spiliopoulou *et al.*, 1994), while immunohistochemistry studies have detected antigen-antibody complexes in the pericellular space of human herniated discs (Satoh *et al.*, 1999). Deposits of immunoglobulin G and M in human disc herniated tissue suggests a correlation between inflammation and autoimmune response (Habtemariam *et al.*, 1996). Furthermore, we have recently shown that significant amounts of IgG antibodies remain in human degenerated and post-traumatic IVDs, despite thorough washing after discectomy (Bertolo *et al.*, 2011). Until now however, there are no studies in humans, which show the specificity of the antibodies present in the disc.

In this article we want to pose the question what kind of antibodies are produced during the degeneration of the disc. We focused on the hypothesis that antibodies present in degenerated IVDs are against major proteins of the disc, such as collagen type I and type II or minor components, such as collagen type III and type V. Considering that disc degeneration is related to a decrease in proteoglycan content, we included aggrecan and native proteoglycan

protein extracts as other putative targets of the autoimmune response. To test the hypothesis, by immunohistochemistry and immunoblotting techniques, we analysed human degenerated and post-traumatic IVD fragments, as well as media collected from cultured *in vitro* discs, for the presence of disc specific IgGs and used bovine caudal discs as healthy negative controls.

## Materials and Methods

### Intervertebral disc fragment harvest

Human degenerated and post-traumatic IVDs (AF and NP separately) were obtained from patients ( $n = 20$ ) undergoing surgical intervention, after informed consent and approval by ethics committee of canton Luzern (collected samples are listed in Table 1). IVD samples were immediately harvested in *Hank's* Balanced Salt Solution (Gibco – LuBioScience GmbH, Lucerne, Switzerland) and the solution was collected after 1-3 h (disc medium day 0). Samples were washed 3-4 times in phosphate-buffered saline (PBS) to eliminate residual blood, and cultured in DMEM/F12 + Gluta-MAX, supplemented with 10 % FBS, (100 units/mL) penicillin / (100 µg/mL) streptomycin (all Gibco) and 2.5 µg/mL amphotericin B (Sigma – Fluka Chemie GmbH, Buchs, Switzerland) at 37 °C and 5 % CO<sub>2</sub>. After three days, disc medium of thoroughly washed disc fragments cultured *in vitro* was collected (disc medium day 3). Remaining fragments were washed three times in PBS and frozen at -80 °C for storage or embedded in Tissue-

**Table 1.** Demographic details of annulus fibrosus and nucleus pulposus donors.

Sample	Sex	Donor's age (Years)	Thompson Grading Scale	Type of operation
1	F	71	III	DDD. Discectomy L4/5
2	M	41	III	DDD. Discectomy L5/S1
3	M	65	III	DDD. Discectomy L3/4–L4/5
4	M	65	IVa	Trauma. Discectomy Th11/12/L1
5	M	49	III	DDD. Discectomy L5/S1
6	M	57	III	Trauma. Discectomy C5/C6
7	F	40	IV	DDD. Discectomy L5/S1
8	F	59	III	DDD. Discectomy L4/5
9	M	44	II	Trauma. Discectomy L3/4/5
10	M	67	III	DDD. Discectomy L4/5
11	M	58	III	DDD. Discectomy L5/S1
12	F	76	IV	DDD. Discectomy L4/5–L3/4
13	M	31	IV	DDD. Discectomy L4–L5–S1
14	F	59	III	DDD. Discectomy L4/5
15	F	70	III	DDD. Discectomy L4/5
16	F	51	IV	DDD. Discectomy L2-5
17	F	73	IV	DDD. Discectomy C4/5/6/7
18	M	36	III	DDD. Discectomy L5/S1
19	M	41	III	DDD. Discectomy L4/5
20	M	41	III	DDD. Discectomy L3/4

Average age = 55 ± 14 years old; DDD = Degenerative Disc Disease.

Tek (Sakura – Sysmex Suisse AG, Horgen, Switzerland) for immunohistochemical analysis.

As healthy controls, we isolated discs from bovine tail, obtained from a local slaughterhouse. Bovine discs were dissected and nucleus pulposus (bNP) was separated from inner and outer annulus fibrosus (bAF).

### RNA extraction and gene expression of IVD fragments

IVD fragments ( $n = 10$ ), frozen at  $-80\text{ }^{\circ}\text{C}$ , were pulverised on dry ice in a stainless steel mortar and total RNA was extracted by Aurum Total Mini Kit (Bio Rad, Reinach, Switzerland), following the manufacturer's instructions. One  $\mu\text{g}$  of total RNA was used for synthesis of cDNA (SuperScript VILO cDNA Synthesis Kit, Invitrogen – LuBioScience GmbH, Lucerne, Switzerland), followed by dilution (1:5) with ultrapure water. Template cDNA ( $5\text{ }\mu\text{L}$ ) was mixed with PCR reaction solution (IQ SYBR Green Supermix, BioRad) containing  $0.25\text{ }\mu\text{M}$  specific primers, listed in Table 2. Final volume of  $25\text{ }\mu\text{L}$  was placed in 96-well plates (BioRad) and amplified according to the following settings: denaturation at  $95\text{ }^{\circ}\text{C}$  for 3 min (1 cycle);  $95\text{ }^{\circ}\text{C}$  for 15 s,  $64\text{ }^{\circ}\text{C}$  for 20 s and  $72\text{ }^{\circ}\text{C}$  for 20 s (40 amplification cycles), followed by a melting curve analysis. Specific products (GAPDH, IL-17A, IL-6, and IL-8) were amplified by a quantitative PCR (CFX96™ Real Time System, BioRad) in triplicates. The results were normalised to the expression of GAPDH. The correct products of PCR were validated on 2.5 % agarose gel with EZ Load 100 bp Molecular Ruler (BioRad) and detected with Gene Genius Bio Imaging System (Syngene - Biolabo Scientific Instruments SA, Chatel St Denis, Switzerland).

### Protein extraction from IVD fragments

IVD fragments ( $n = 10$ ), frozen at  $-80\text{ }^{\circ}\text{C}$ , were pulverised on dry ice in a stainless steel mortar, and lysed with  $500\text{ }\mu\text{L}$  of CellLytic MT Mammalian Tissue Lysis Extraction Reagent (Sigma). To prevent degradation, Protease Inhibitor Cocktail (Sigma) was added and lysates were homogenised using a Dispomix device (Axonlab, Baden, Switzerland). After 15 min incubation at room temperature, lysates were centrifuged at  $17000\text{ g}$  for 15 min at  $4\text{ }^{\circ}\text{C}$  to remove debris. Protein concentrations of supernatants were measured by Bradford assay (Axonlab).

### Measure of total IgG antibodies concentration by ELISA

The concentration of IgGs, in culture media ( $n = 20$ ) collected at day 0 and day 3, and in protein extracts ( $n = 10$ ) from degenerated IVDs, was quantified using a standard indirect ELISA procedure. Briefly, a 96-well plate (Nunc MaxiSorp, Fluka Chemie GmbH, Buchs, Switzerland) was coated with  $50\text{ }\mu\text{L}/\text{well}$  of  $1\text{ ng}/\mu\text{L}$  Protein A (LuBioScience GmbH, Lucerne, Switzerland) in PBS. After incubation overnight at  $4\text{ }^{\circ}\text{C}$ , wells were washed with PBS by plate washer (Beckman-Coulter, Nyon, Switzerland), and non-specific binding sites were blocked for 3-4 h with  $100\text{ }\mu\text{L}/\text{well}$  of blocking solution containing 5 % TopBlock (LuBioScience) and  $1.5\text{ mg}/\text{mL}$  casein (Axonlab) in Tris-buffered saline (TBS). The coated plate was incubated overnight at  $4\text{ }^{\circ}\text{C}$  with  $50\text{ }\mu\text{L}/$

**Table 2.** Human marker genes used in quantitative RT-PCR.

Gene	Primer nucleotide sequence (5'-3')	Product size (bp)
GAPDH	F—TGGACTCCACGACGTA CTCA R—GGAAGCTTGT CATCAATGGAA	102
IL-17A	F— TGGGAAGACCTCATTGGTGT R—GGATTTCGTGGGATTGTGAT	84
IL-6	F—AGCCAGAGCTGTGCAGATG R—CTGCAGCCACTGGTTCTGT	145
IL-8	F—AGACAGCAGAGCACACAAGC R—AGGAAGGCTGCCAAGAGAG	119

F, forward; R, reverse.

well of disc medium diluted 1:100, 1:1000 and 1:5000, or 1 h at RT with  $10\text{ ng}/\mu\text{L}$  of disc protein extract. Dilutions of purified human IgG (Bethyl – LuBioScience GmbH, Lucerne, Switzerland) were used for a standard curve. After washing with PBS, anti-human-IgG HRP-conjugated secondary antibody (A80-119P Bethyl), diluted 1:5000 in blocking mix ( $50\text{ }\mu\text{L}/\text{well}$ ), was added for 1 h at room temperature. Following washing with PBS,  $100\text{ }\mu\text{L}$  of o-phenylenediamine substrate (OPD, dissolved at  $15.3\text{ mg}/\text{mL}$  in citrate buffer, pH 5.0, AppliChem – Axonlab, Baden, Switzerland) were added and left shortly to develop. The reaction was stopped with 10 % sulphuric acid. Absorbance was measured at  $450\text{ nm}$  by DTX 880 Multimode Detector (Beckman-Coulter) and concentration of IgGs ( $\text{ng}/\text{mL}$ ) was determined from the standard curve.

### Detection of total and disc specific IgGs by immunohistochemistry analysis

Bovine and human IVD fragments embedded in Tissue-Tek (Sakura) were sectioned at  $10\text{ }\mu\text{m}$  using a cryostat (Microm HM560, HistoCom AG, Zug, Switzerland). Endogenous peroxidase was quenched by 3 %  $\text{H}_2\text{O}_2$  in PBS at room temperature for 10 min, and then washed with PBS. Before incubation with primary antibodies, sections were pre-treated with  $2500\text{ U}/\text{mL}$  hyaluronidase (Sigma) in PBS for 1 h at  $37\text{ }^{\circ}\text{C}$ . Non-specific background was blocked with 10 % normal goat serum (LuBioScience) for 30 min. To demonstrate the presence of IgG antibodies, human degenerated disc sections were incubated with anti-human HRP-conjugated secondary antibody diluted 1:1000 (A80-119P, Bethyl) for 1 h at room temperature. Control healthy bovine disc sections were incubated with anti-bovine HRP-conjugated secondary antibody diluted 1:1000 (A10-130P, Bethyl). As a positive control, anti-collagen type II Mab (II-II6B3, Development Studies Hybridoma Bank (DSHB), University of Iowa, Iowa City, IA, USA); diluted 1:40 in PBS) followed by anti-mouse HRP-conjugated secondary antibody diluted 1:1000 (A90-216P, Bethyl) was used.

To analyse the presence of IgGs against proteins of the disc, bovine healthy disc sections were incubated overnight at  $4\text{ }^{\circ}\text{C}$  with disc media collected after 3 d from human degenerated IVDs cultured *in vitro*. After washing with PBS, sections were incubated with anti-human HRP-conjugated secondary antibody diluted 1:1000 (A80-119P, Bethyl) for 1 h at room temperature.

Sections were developed with TMB SP HRP substrate (SPPM-1000-01, BioFX - LuBioScience GmbH, Lucerne,

Switzerland), mounted with VectaMount AQ (H-5501, Vector Lab. – Reactolab, Servion, Switzerland) and analysed by light microscopy (Olympus IX71, Olympus Schweiz AG, Volketswil, Switzerland).

#### Detection of IgGs against disc ECM proteins by immunoblotting

Purified ECM proteins: Human Collagen type I (288, Yo Proteins – LuBioScience GmbH, Lucerne, Switzerland), Human Collagen type II (30410424, ProtealImmune – Anawa, Wangen, Switzerland), Human Collagen type III (889 Yo Proteins), Human Collagen type V (436 Yo Proteins), bovine aggrecan (A1960 Sigma) and proteins extracted from bovine healthy discs (oAF – iAF – NP) were diluted to 100 ng/μL in PBS. In dot blot assay 100 ng (1 μL) of each protein were spotted onto nitrocellulose membrane (Whatman – GE Healthcare, Glattbrugg, Switzerland) and dried for 1 h at room temperature. After blocking of non-specific sites with 5 % fat-free milk (Rapilait, Migros, Switzerland) in PBS, the membrane was incubated with disc media collected after 3 d from human degenerated IVDs cultured *in vitro* (1 μg of total IgGs, as determined by ELISA) or monoclonal primary antibodies, diluted in 5 % milk-PBS, overnight at 4 °C. Monoclonal primary antibodies were used to control spotted proteins: monoclonal anti-Collagen type I (M38-s, DSHB; diluted 1:10); anti-Collagen type II (II-II6B3, DSHB; diluted 1:500); anti-Collagen type III clone MC3-HA (380, Yo Proteins; diluted 1:500); anti-aggrecan (AHP0022, Biosource – LuBioScience GmbH, Lucerne, Switzerland; diluted 1:500). After washing with PBS, the membrane was incubated 1 h at room temperature with anti-IgG secondary antibody conjugated with HRP (anti-mouse A90-216P; anti-human A80-119P Bethyl) diluted 1:5000 in 5 % milk-PBS, followed by washing with PBS and incubation with chemiluminescence substrate (EZ ECL, Biological Industries – LucernaChem, Lucerne, Switzerland). Acquisition was performed with a digital SLR camera (Nikon D600, Nikon, Zürich, Switzerland) (Khoury *et al.*, 2010). Dot blots of 10 patients with degeneration of IVD were analysed and quantified by Image J version 1.46r for Windows (ImageJ Inc.) measuring the integrated density of each dot.

In Western blot assays, proteins were separated by SDS-PAGE using Tris-Glycine 5-15 % gels (BioRad) with a Protein Standard 10-250 kD (Precision Plus Protein Standard, BioRad) and transferred onto a nitrocellulose membrane using the semi-dry Trans-Blot Turbo system (BioRad). After blocking of non-specific sites with 5 % milk (Rapilait) in PBS, the membrane was incubated for 4 h at room temperature with disc media collected after 3 d from human degenerated IVDs cultured *in vitro* (1.75 μg of total IgGs, as determined by ELISA) diluted in 5 % milk-PBS. After washing with PBS, the membrane was incubated 90 min at room temperature with anti-human IgG secondary antibody conjugated with HRP (A80-119P Bethyl) diluted 1:10000 in 5 % milk-PBS, and after 3 washes with PBS, incubated with chemiluminescence substrate (LumiGlo Reserve KPL – Bio Concept,

Allschwil, Switzerland). Acquisition and analyses were performed as described above.

#### Isolation and culture of human and bovine disc cells

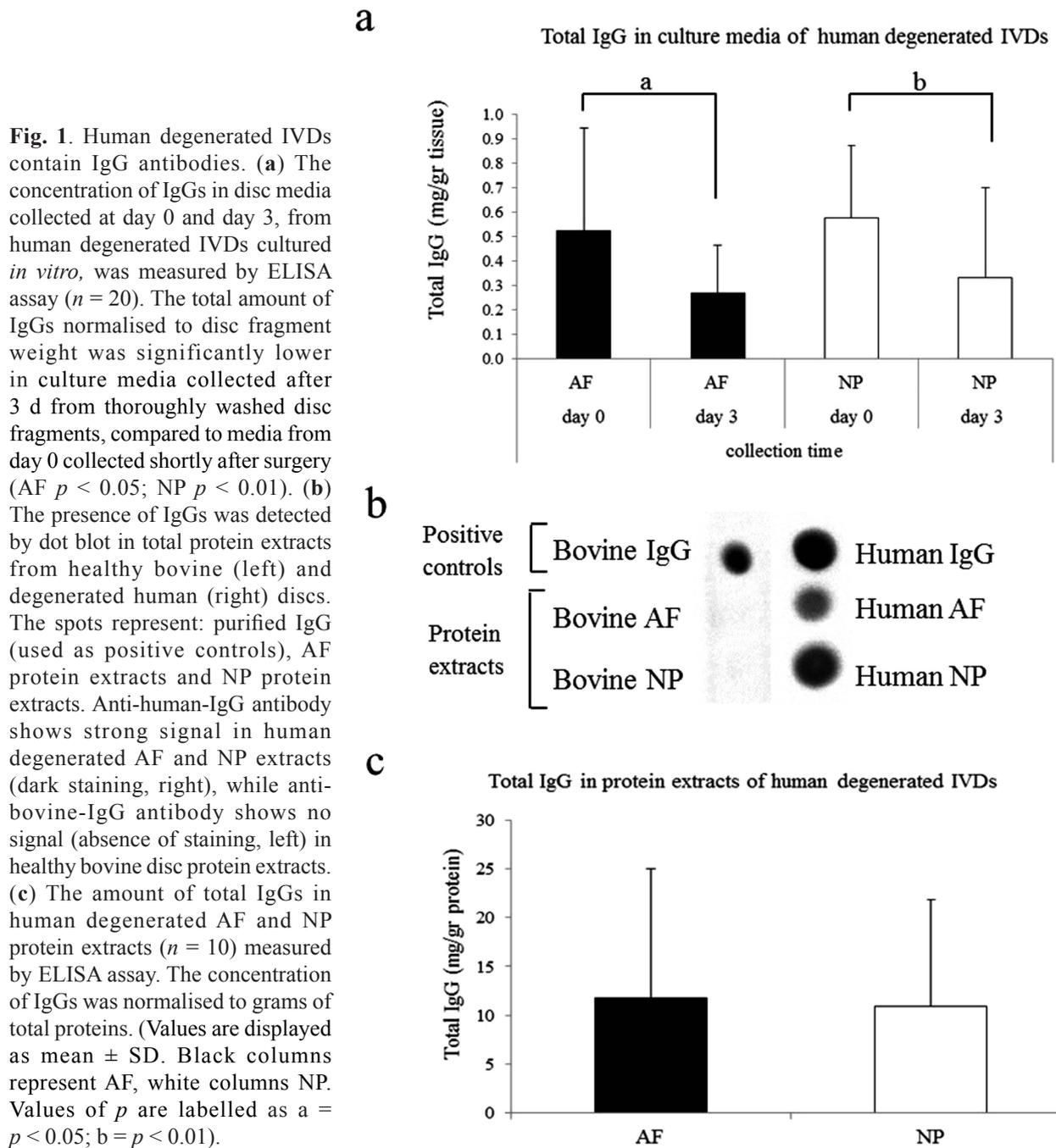
Human IVD fragments (AF and NP separately) obtained from patients undergoing surgical intervention, were digested with 0.05 % collagenase (Worthington – Bio Concept, Allschwil, Switzerland), 5 % FBS, penicillin (100 units/mL) /streptomycin (100 μg/mL) in DMEM/F12 + GlutaMAX (all Gibco) for 6 h at 37 °C. Healthy bovine disc fragments, obtained from tails of adult animals, were dissected and digested (1) with 0.3 % pronase (Sigma-Aldrich), 5 % foetal bovine serum (FBS), penicillin (100 units/mL) /streptomycin (100 μg/mL) in DMEM/F12 + GlutaMAX for 1 h at 37 °C; (2) with 0.05 % collagenase (Worthington), 5 % FBS, penicillin (100 units/mL) /streptomycin (100 μg/mL) in DMEM/F12 + GlutaMAX, overnight at 37 °C. Disc cells were expanded in monolayer culture for 15 d in DMEM/F12 + GlutaMAX, supplemented with 10 % FBS, penicillin (100 units/mL) / streptomycin (100 μg/mL), 2.5 μg/mL Amphotericin B (Sigma) and 5 ng/mL recombinant bFGF (Peprotech – LuBioScience GmbH, Lucerne, Switzerland) at 37 °C in a humid atmosphere containing 5 % CO<sub>2</sub>.

At passage 1, cells were then grown in three dimensional cubes with 3 mm side length, made of gelatine, a partially hydrolysed collagen (Spongostan, Ferrosan AS, Soeborg, Denmark). Cell suspension was carefully pipetted on the construct (72,000 cells for cube, at density of 4,000,000 cells/mL) allowing cells to be completely absorbed. Constructs were kept at RT in a 6-well plate for 30 min to allow cells to anchor to the matrix, before adding media. To induce chondrogenesis, disc cell constructs were cultured for up to 5 weeks in chondrogenic medium: DMEM/F12 + GlutaMAX supplemented with 2.5 % FBS, 40 ng/mL dexamethasone (Applichem), 50 μg/mL ascorbate-2-phosphate (Sigma), 50 μg/mL L-proline (Sigma), 100 U/mL penicillin/100 μg/mL streptomycin, 2.5 μg/mL amphotericin B, 1X insulin (10 μg/mL), transferrin (5.5 μg/mL), selenium (0.67 ng/mL), X supplement (ITS, Gibco), and 10 ng/mL transforming growth factor-β1 (TGF-β1) (Peprotech). Controls were maintained in control medium without TGF-β1.

To extract proteins, frozen constructs were hammer and lysed with 300 μL of CellLytic M Cell Lysis Reagent (Sigma). To prevent degradation, Protease Inhibitor Cocktail (Sigma) was added. Extracted disc cell proteins were used to perform Western blot assay with disc media collected after 3 d from human degenerated IVDs fragments cultures *in vitro*.

#### Statistical analysis

For statistical analysis we used the non-parametric Mann-Whitney-Wilcoxon U test for dependent variables. Data were expressed as the mean ± SD. Values of *p* were labelled as significant when *p* < 0.05 (a); *p* < 0.01 (b); *p* < 0.001 (c). Data analysis was performed with SPSS version 14.0 for Windows (SPSS Inc.).

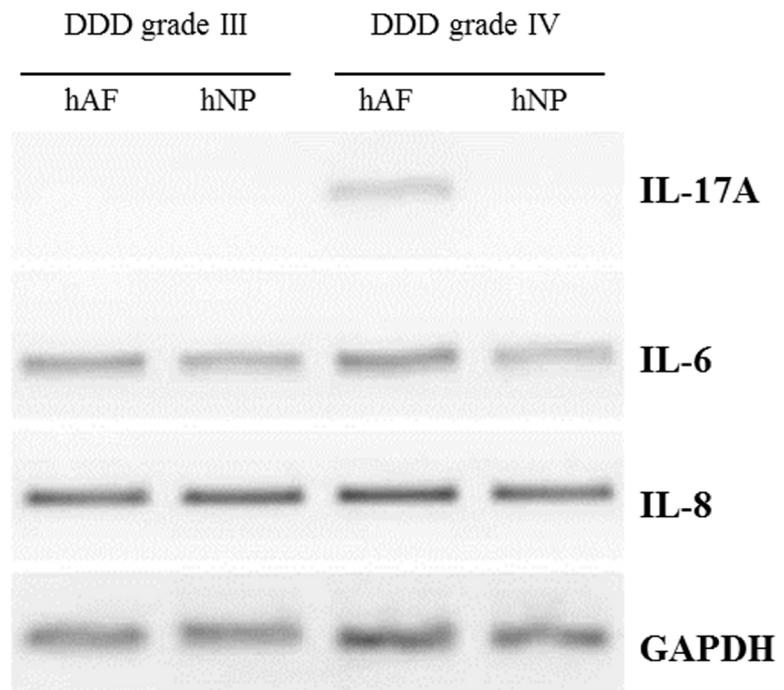


## Results

### Human degenerated IVDs contain IgG antibodies

Degenerated and post-traumatic human AF and NP fragments, obtained from patients undergoing discectomy (Table 1), were washed to remove blood residues from operative field and cultured *in vitro* for a period of three days. We collected disc media in contact with disc fragments shortly after surgery (disc medium day 0), and after 3 d of thoroughly washed disc fragment *in vitro* cultures (disc medium day 3). Using ELISA, we demonstrated that IgG antibodies were still present in washed degenerated discs fragments and measured that the concentration of IgGs in disc culture media collected after 3 d, was significantly lower (AF  $p < 0.05$ ; NP  $p < 0.01$ )

compared to media collected at day 0 (Fig. 1a). There were no differences between AF and NP fragments at the same collection time. As a healthy control, we cultured *in vitro* bovine caudal discs, but we could not detect bovine antibodies in disc media (data not shown). By dot blot assay, we demonstrated the presence of IgGs in total protein extracts of human degenerated IVD fragments, further washed after 3 d of culture *in vitro*. Immunoblot revealed robust signal in human degenerated protein extracts incubated with anti-human IgG antibody, while no signal was detected in healthy bovine controls after incubation with anti-bovine-IgG antibody. Purified human and bovine IgGs were used as positive controls (Fig. 1b). The concentration of IgGs in total protein extracts from human degenerated IVDs was determined by ELISA



**Fig. 2.** Human degenerated IVDs express molecular mediators of inflammation. Representative image of agarose electrophoresis shows gene expression of interleukins 17A (IL-17A), 6 (IL-6), 8 (IL-8) and GAPDH (house-keeping gene), by RT-PCR, in human degenerated discs (hAF and hNP) with degeneration grade III and IV.

assay (Fig. 1c). No significant differences between protein extracts from AF and NP fragments and no correlation with degeneration grade could be observed.

#### Human degenerated IVDs express molecular mediators of inflammation

Gene expression analysis of human degenerated IVDs revealed the presence of molecular mediators of inflammation mRNAs. Interleukin-6 (IL-6) and Interleukin-8 (IL-8) were present in all tissue samples and there were no differences between AF and NP or degeneration grade. Interleukin 17A (IL-17A) was present at very low levels and only in AF samples with high degeneration grade (Fig. 2).

#### Detection of IgGs in human degenerated IVDs

Immunohistochemistry (IHC) analysis showed the presence of IgG antibodies in human degenerated IVD sections (Thompson grade III). AF and NP tissues displayed a strong signal when incubated with anti-human-IgG antibody, while the negative control anti-bovine-IgG antibody showed no signal (Fig. 3a). The absence of infiltrated IgGs in healthy disc samples was demonstrated by lack of signal in IHC analysis of healthy bovine AF and NP sections incubated with anti-bovine and anti-human-IgG antibodies (Fig. 3b). In both human and bovine discs, IHC staining with monoclonal anti-Collagen type II antibody was used as positive control.

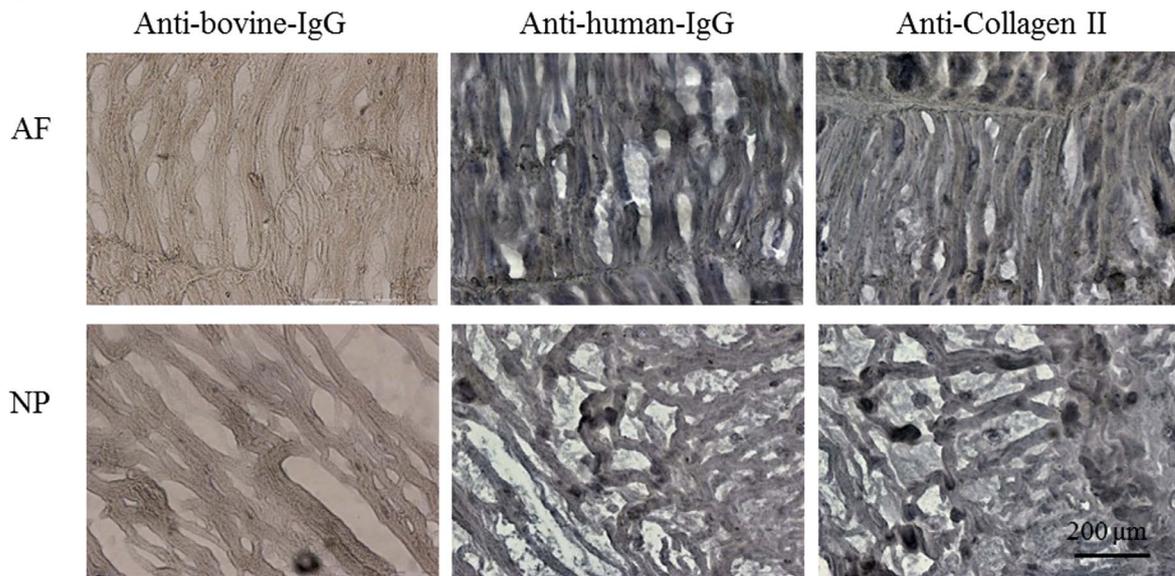
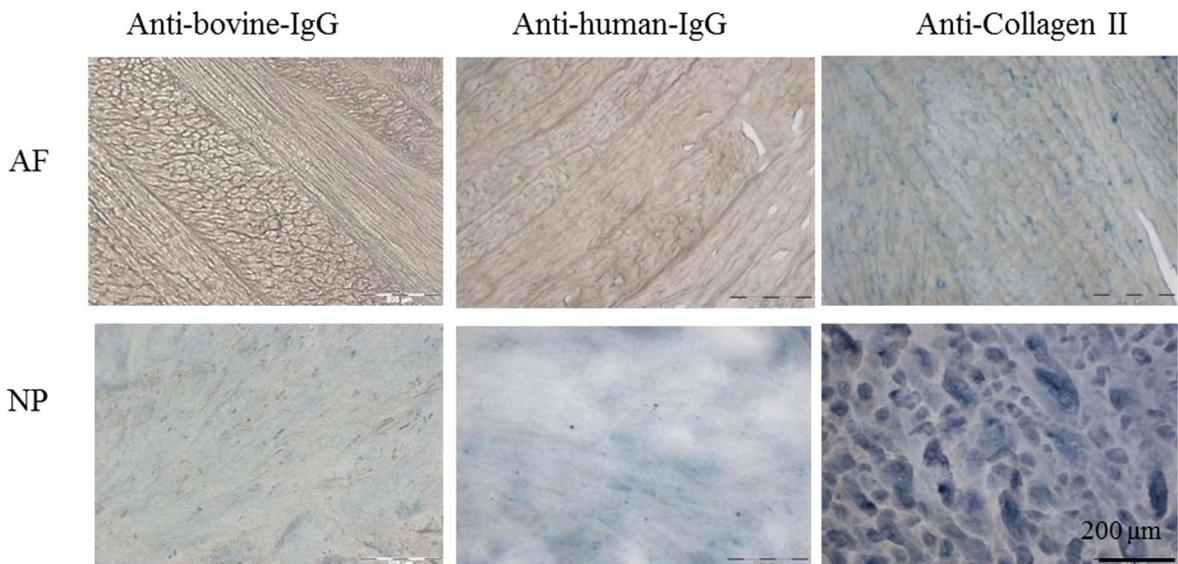
#### Culture media of human degenerated IVDs contain IgG antibodies against proteins of healthy disc

We detected the release of IgGs into *in vitro* culture media, collected at day 3 from human degenerated IVDs (Thompson grade III), by using these media as primary antibodies against healthy bovine AF and NP sections (Fig.

4a). The IHC analysis showed disc specific IgGs (dark blue staining), mainly against NP sections ( $p < 0.05$ ) (Fig. 4b). Similar trends were observed in disc media collected at day 0 (data not shown). As shown in the previous paragraph, we could not use human disc sections in IHC analysis for the presence of antibodies inside (see Fig. 3a). Therefore we used healthy bovine discs, which did not give signal when incubated with anti-human IgG secondary antibody (see Fig. 3b).

#### IgGs present in human degenerated IVDs target native ECM proteins

To identify if antibodies found in human degenerated IVDs, recognise proteins of the disc, we spotted on nitrocellulose membranes, proteins extracts from healthy bovine discs (bovine AF outer, AF inner and NP), along with purified proteins of extracellular matrix (ECM), such as human Collagens type I, type II, type III, type V and bovine aggrecan. We detected IgGs against bovine disc protein extracts in human disc media collected after 3 d from AF and NP cultured *in vitro*, and we also found specific IgGs against ECM purified proteins (Fig. 5a). None of the samples showed IgGs against purified human Collagen type III. Anti-human IgG antibody, used as negative control, did not non-specifically stain any of the proteins, whereas monoclonal primary antibodies against Collagen type I, type II, type III and aggrecan, used as positive controls, showed reactivity with their respective proteins. The quantitative analysis of dot blots (Fig. 5b) revealed more IgGs against NP extracts compared to AF ( $p < 0.01$ ). In particular, more anti-collagen type II ( $p < 0.01$ ) and anti-aggrecan ( $p < 0.05$  for NP culture disc media) IgGs were detected, compared to anti-collagen type I antibodies. The analysis also showed a strong amount of antibodies against Collagen type V.

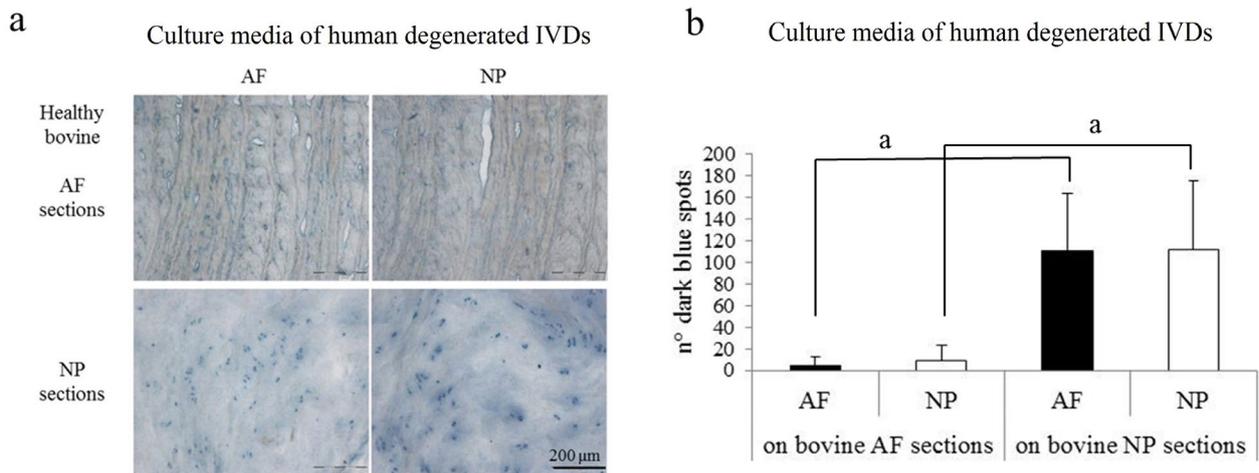
**a** Degenerated human disc sections:**b** Healthy bovine disc sections:

**Fig. 3.** Detection of IgGs in human degenerated IVDs. **(a)** Immunohistochemistry shows the presence of human IgGs (dark blue staining) in degenerated IVD sections (AF and NP). In negative controls human disc tissues were incubated with anti-bovine-IgG antibody. **(b)** No IgGs were detected in healthy bovine disc sections incubated with anti-human and anti-bovine-IgG antibodies. Anti-Collagen II monoclonal antibody was used as a positive control for both human and bovine disc sections (Scale bar = 200 µm).

### Western blot detection of ECM proteins by IgGs released from human degenerated IVDs

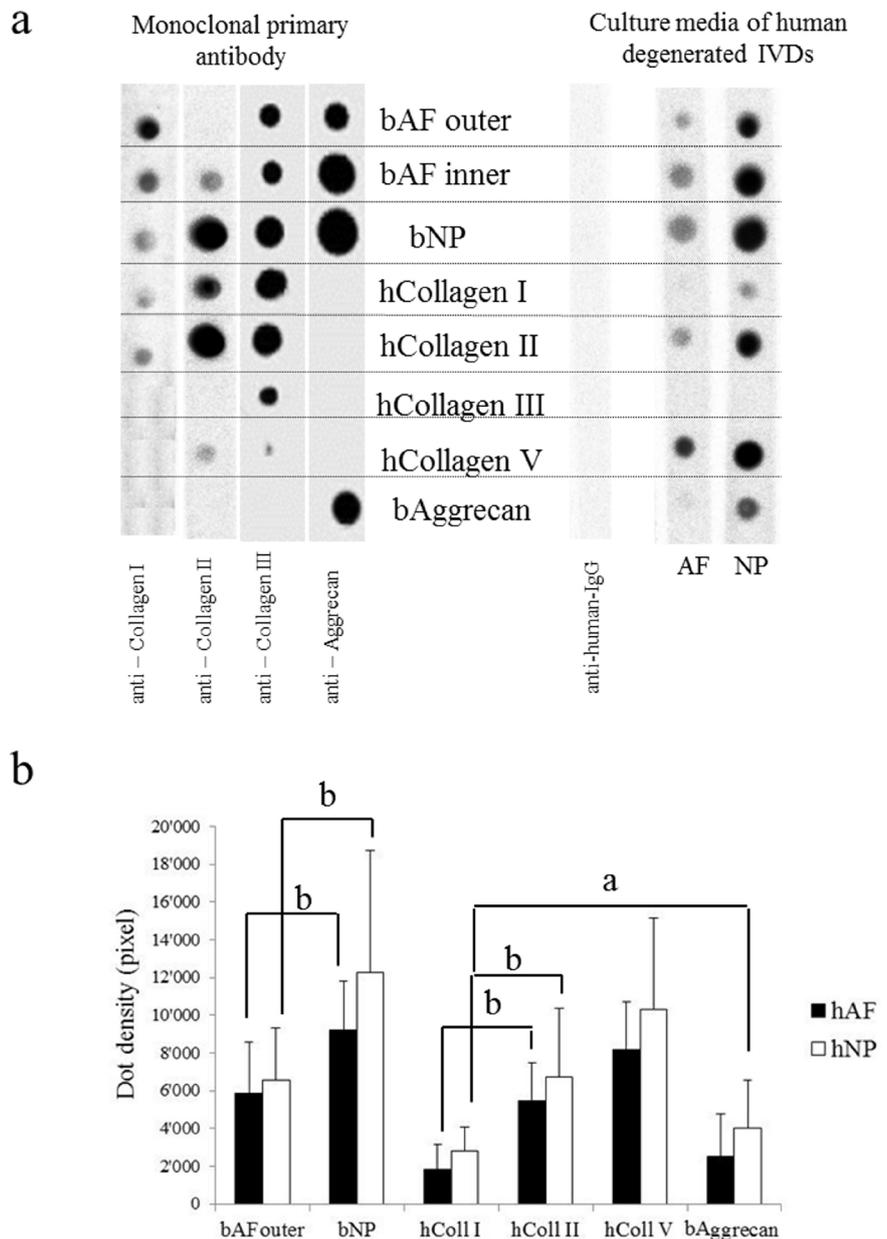
Western blot of healthy bovine disc proteins (bAF and bNP) and purified ECM proteins (human Collagen type I, type II, type V and bovine aggrecan) separated by SDS PAGE, was probed with disc media, collected at day 3 from human degenerated IVDs cultures (Fig. 6a). The assay revealed a major band with a molecular mass about of 130 kDa, matching the monomeric form of collagen. The higher bands in Collagen type I and type II lanes correspond to molecular weights of dimeric (~270 kDa)

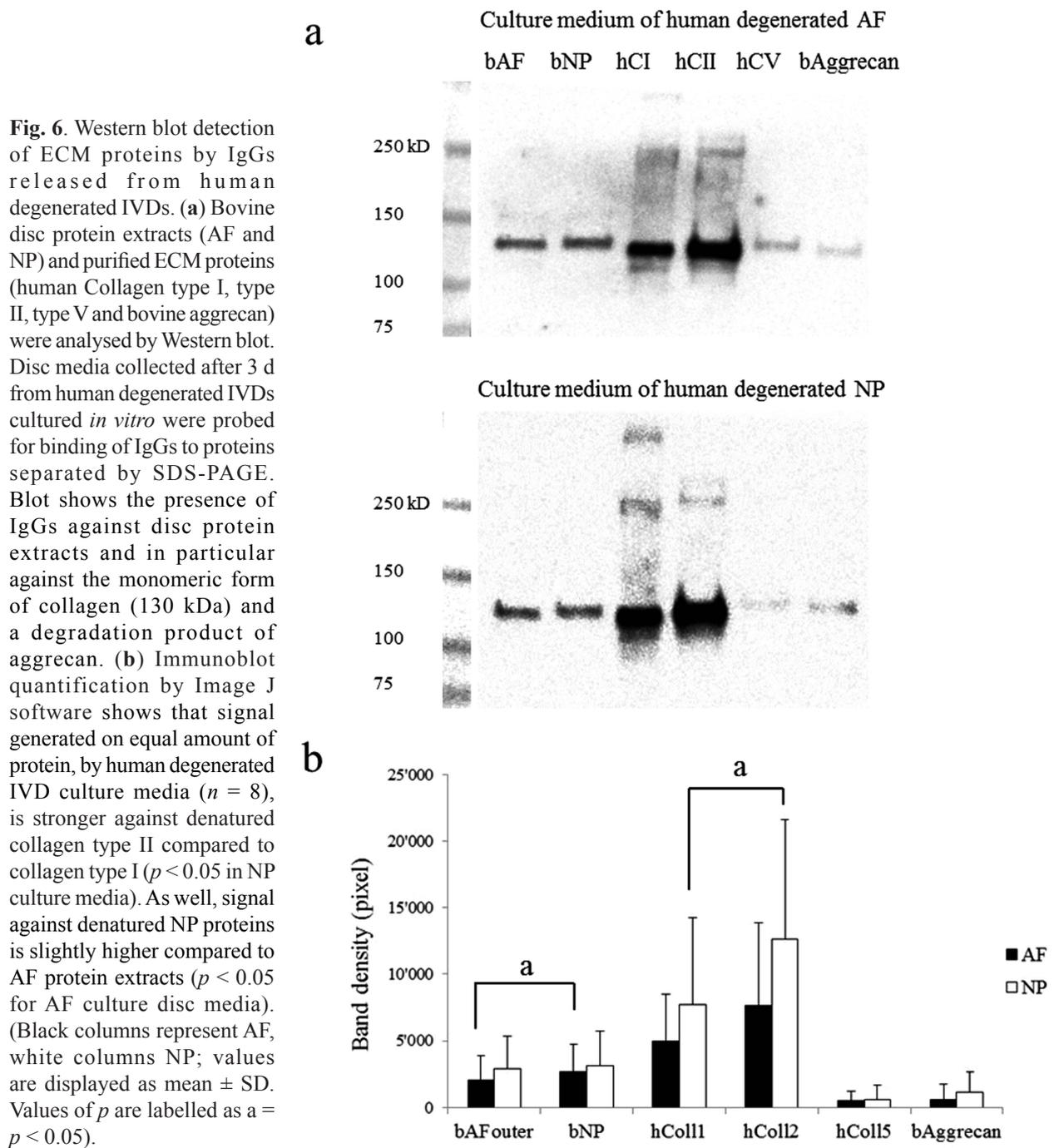
and trimeric (~400 kDa) collagen forms. The assay also showed a weak signal against a degradation product of aggrecan (~130 kDa). Quantification of densitometry (Fig. 6b) revealed that signal generated by equal amounts of denatured proteins, was significantly stronger against collagen type II, compared to collagen type I ( $p < 0.05$  in NP culture media; not significant in AF culture media). Furthermore, more reactivity was measured against healthy bovine NP compared to AF protein extract ( $p < 0.05$  in AF culture media; not significant in NP culture media).



**Fig. 4.** Culture media of human degenerated IVDs contain IgG antibodies against proteins of healthy disc. **(a)** Immunohistochemistry on bovine healthy disc sections (AF and NP) shows the presence of disc specific antibodies (dark blue staining) in media collected at day 3 from human degenerated IVD cultured *in vitro* (Thompson grade III) (Scale bar = 200 μm). **(b)** The quantification of dark blue staining revealed disc specific IgGs mainly against NP ( $p < 0.05$ ). (Values are displayed as mean ± SD. Black columns represent AF, white columns NP. Values of  $p$  are labelled as a =  $p < 0.05$ ).

**Fig. 5.** IgGs present in human degenerated IVDs target native ECM proteins. **(a)** Dot blot on healthy bovine disc protein extracts (bovine AF outer, AF inner and NP) and purified ECM proteins (human Collagen type I, type II, type III, type V and bovine aggrecan) shows the presence of specific IgGs for all detected proteins except Collagen type III, in culture media collected at day 3 from human degenerated IVDs. Monoclonal primary antibodies (anti-Collagen type I, type II, type III and anti-aggrecan) were used as positive controls, while anti-human-IgG antibody was used as a negative control. Rows represent spotted proteins, while columns represent monoclonal antibodies and disc culture media used as primary antibodies. **(b)** Culture media collected from human degenerated IVDs ( $n = 10$ ) showed more IgGs against NP than AF bovine protein extracts ( $p < 0.01$ ); in particular more IgGs against collagen type II ( $p < 0.01$ ) and aggrecan ( $p < 0.05$  for NP culture media) compared to collagen type I. (Dot blot quantification by Image J software – black columns represent IgGs in AF, white columns in NP culture media; values are displayed as mean ± SD. Values of  $p$  are labelled as a =  $p < 0.05$ ; b =  $p < 0.01$ ).





### IgGs present in human degenerated IVDs recognise disc cell proteins

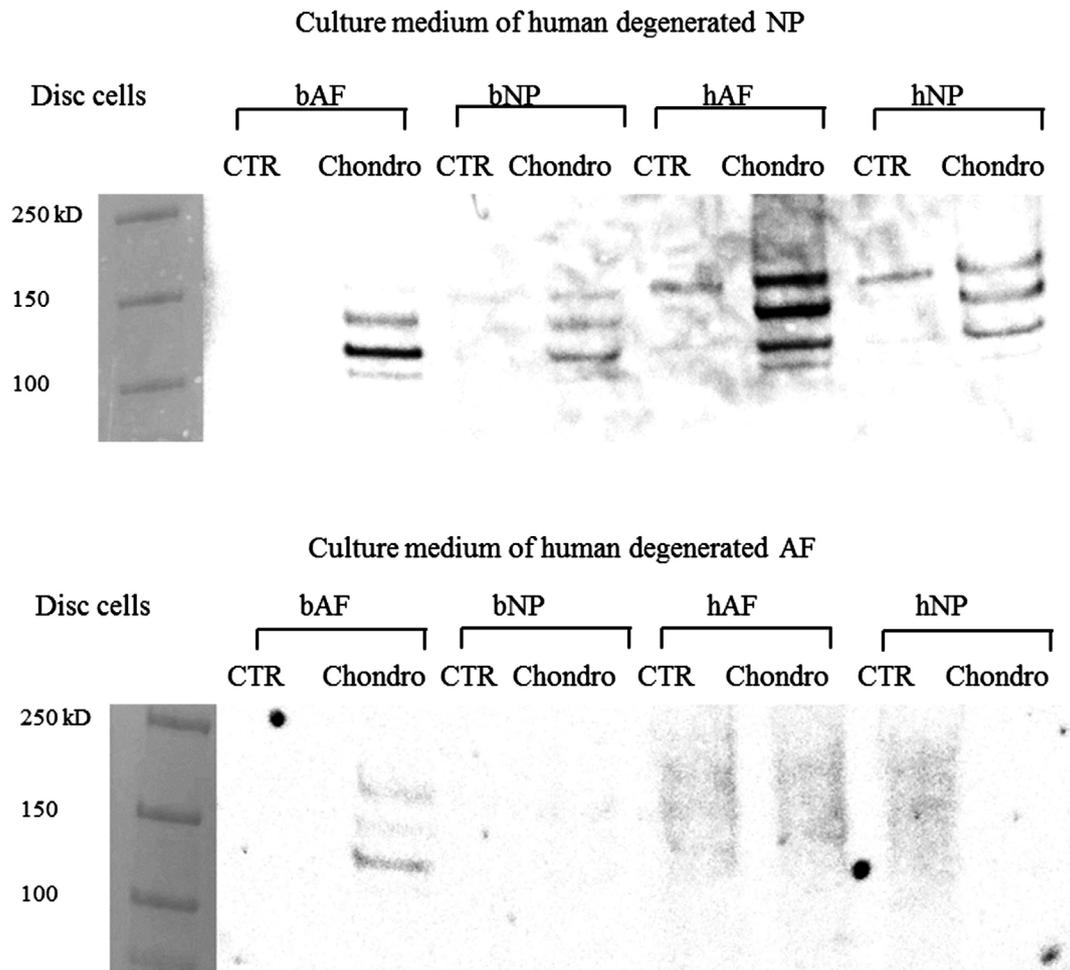
We stimulated cells extracted from human degenerated AF and NP fragments to produce specific disc proteins, in chondrogenic and non-chondrogenic (control) three-dimensional (3D) cultures. The chondrogenic differentiation was confirmed by gene expression of specific markers by RT-PCR analysis (data not shown). Protein extracts from stimulated human and bovine disc cells and respective controls were probed with culture media collected after 3 d from human degenerated discs. Western blot analysis revealed characteristic bands with molecular weights between 100 and 150 kDa, in human degenerated NP culture medium (Fig. 7, top). Disc medium from human

degenerated AF gave similar but weaker signal, detectable only against bovine stimulated disc cells (Fig. 7, bottom).

### Discussion

In this study we confirmed the presence of IgG antibodies, as a sign of lymphocyte infiltration, in degenerated and post-traumatic IVDs, and we found that these antibodies are directed against typical extracellular proteins of the disc, mostly against proteins of the NP.

We quantified IgGs released by disc fragments in media collected shortly after surgery (disc medium day 0), and after 3 d from thoroughly washed disc fragments cultured



**Fig. 7.** IgGs present in human degenerated IVDs recognise disc cell proteins. Proteins extracted from bovine and human disc cells, cultured in a three-dimensional (3D) scaffold and stimulated to produce proteins specific of disc (chondrogenic differentiation), were separated by SDS-PAGE. Controls (CTR) correspond to non-stimulated disc cells. Western blot with disc medium collected after 3 d from human degenerated NP cultured *in vitro*, reveals bands with molecular weight between 100 and 150 kDa, against both human and bovine stimulated disc cells (top). Disc medium from human degenerated AF gives similar but weaker signal, detectable only against bovine stimulated disc cells (bottom).

*in vitro* (disc medium day 3). We demonstrated that specific IgG antibodies were still present in media collected from washed degenerated disc fragments, but in significantly less amounts compared to media collected at day 0, which could contain additional non-specific antibodies from surgery blood residues. To confirm that antibodies found in disc culture media are released by the degenerated disc, and not from operative bleeding, we measured the concentration of IgGs in protein extracts from further washed degenerated AF and NP fragments. The average of IgGs concentration appeared similar between AF and NP extracts, probably because advanced degeneration and surgical routine make it difficult to separate well the AF from the NP in a degenerated disc. However, if disc tissues were not severely degenerated and thus easily separated by the surgeon, we found a higher concentration of antibodies in the NP compared to the AF protein extracts. As no healthy human samples were obtainable, we used healthy bovine caudal discs as controls and demonstrated that protein extracts from healthy discs contain no antibodies.

Additionally, no antibodies were found in healthy bovine disc tissue sections probed with anti-bovine IgG antibody.

We also found the presence of some molecular mediators of inflammation, such as IL-6, IL-8 and traces of IL-17, in analysed degenerated samples. Previous studies reveal the presence of cytokines (Le Maitre *et al.*, 2007) and inflammatory mediators (Burke *et al.*, 2002) in herniated and degenerated discs, and suggest the involvement of lymphocytes in disc degeneration (Shamji *et al.*, 2010). Inflammation and back pain are correlated with increasing innervation and vascularisation of the disc (Stefanakis *et al.*, 2012) and it is hypothesised that NP, in contact with the systemic circulation, can trigger lymphocyte activation and an autoimmune response (Habtemariam *et al.*, 1996). Undefined antigen-antibody complexes have been found in herniated disc tissues (Satoh *et al.*, 1999), but the authors did not show that the antigens are collagens or proteoglycans.

In an earlier study we have detected IgG antibodies in human degenerated and post-traumatic disc fragments

cultured *in vitro* (Bertolo *et al.*, 2011), but we did not identify their targets. Based on the above mentioned data and studies, we put to test the hypothesis that if the integrity of vertebral endplates is compromised by trauma or degeneration, antibodies against disc proteins would be present locally. After confirming the presence of human IgGs, we showed that they correlated to the amount of collagen II in degenerated AF and NP sections, further supporting the autoimmune hypothesis. To investigate if the antibodies found in degenerated IVDs are produced against proteins of the disc, we used healthy – antibody free – bovine AF and NP disc sections as targets in immunohistochemistry assays. Human IgGs from degenerated IVDs probed strongly NP specific proteins in bovine disc sections. Immunoblot assays confirmed independently this strong autoimmune response against NP proteins and to lower extent against AF proteins. To test the specificity of the immune response, we used highly purified extracellular matrix proteins as antigens. Greater amounts of antibodies against a major constituent of the NP – collagen type II – were extracted from human IVDs, compared to IgGs against the major constituent of AF – collagen type I. Animal model studies also have demonstrated that autologous NP can induce inflammation and an autoantibody response in rabbits (Bobechko and Hirsch, 1965), dogs (McCarron *et al.*, 1987), pigs (Geiss *et al.*, 2007) and rats (Murai *et al.*, 2010).

Different antibodies can bind different epitopes of the same protein, or even distinguish between native and denatured proteins. We separated purified human proteins and bovine disc extracts by SDS-PAGE and performed a Western blot using culture media of human degenerated discs as the primary polyclonal antibody probe. The assay confirmed the presence of IgGs principally against the monomeric form of collagen type II and similar, but weaker signal, against collagen type I. Involvement of collagen type II in the autoimmune reaction is highly probable; IVD degeneration has been correlated with increased degradation of collagen II (Antonioni *et al.*, 1996), yet collagen II might trigger articular cartilage diseases (Burkhardt *et al.*, 2002) and antibodies to denatured collagens have been found in patients with rheumatoid arthritis (RA) (Morgan *et al.*, 1987). We also found antibodies against the minor fibrillar human collagen type V, which plays a role in the organisation of heterotypic type I/V collagen fibrils (Birk, 2001). Interestingly strong signal against native collagen type V was observed only with dot blot assay, but not with SDS electrophoresis and Western blot, probably because under denaturing conditions collagen V epitopes are no longer properly recognised by specific antibodies. Identifying antibodies against all these proteins raises the question if autoimmune reactivity in the disc is not generic to the whole collagen family? Probably not, as we could not find IgGs against collagen type III, a collagen present in outer AF of degenerated IVDs and correlated with altered metabolism of disc tissue (Adam and Deyl, 1984).

Additionally, we investigated if the dominant component of the NP – the proteoglycan aggrecan – can also be a target of autoimmune response in the process of IVD degeneration. Indeed, with dot blot assay, we detected

antibodies against purified bovine aggrecan in culture media of human degenerated discs. However, upon further analysis of IgGs against denatured protein by western blot, we observed a low intensity band, which does not correspond to the molecular weight of the native aggrecan core protein (210-250 kDa), but to a degradation product of the protein (approx. 130 kDa). This observation makes biological sense, as cleavage events at metalloproteinase and aggrecanase sites are normal in aggrecan catabolism (Sztrolovics *et al.*, 1997). Slow release of aggrecan degradation products into the blood seems to be related to progression of IVD degeneration (Roughley *et al.*, 2006). Furthermore, an approx. 140 kDa aggrecan degradation product generated by calpain cleavage, and correlating with the stage of degeneration, has been found in degenerated disc tissue (Fukuta *et al.*, 2011).

Proteins in the extracellular matrix often have quaternary structures and modification, and also are partially degraded, compared to proteins freshly synthesised from the cell. It was a fundamental question to understand if the autoimmune response is targeting the extracellular matrix alone or also cells which synthesise fresh proteins. Indeed, we demonstrated that autoantibodies from degenerated IVDs recognise freshly synthesised disc cell proteins. To mimic the *in vivo* environment and induce chondrogenic differentiation (Bertolo *et al.*, 2012), we grew cells from human degenerated discs and bovine healthy discs in 3D scaffolds, extracted their total protein content and observed that antibodies from human degenerated discs recognise proteins from the extracts.

Disc degeneration, as a multifactorial disease, correlates with different variables, such as genetic predisposition, altered metabolism, structural changes and neurovascular ingrowth (Adams and Roughley, 2006). In this study we looked at autoimmune response as an additional source of degenerative changes. Although it may seem to be secondary and to be triggered by an already present degeneration, these findings may have implications for diagnostics beyond self-reported back pain. Yet the diagnostic value of our findings is unclear, as patients suffering of degenerative disc disease (DDD) could also have osteoarthritis (OA), making it difficult to separate between these two conditions based only on the immunological profile. Nevertheless, epitope specificity could differ between diseases, as well as specific epitopes found to contribute to development of arthritis (Nandakumar *et al.*, 2008), so hope remains that further studies will find unique epitopes, which will bring the needed diagnostic specificity and sensitivity.

Looking at the underlying biology of the process, we positively proved the association between autoimmunity and disc degeneration, previously shown as presence of autoantibodies in sera of patients with degenerated and herniated IVDs (Mihn and Kim, 2011). Autoantibodies present in degenerated discs may contribute to a vicious circle of “frustrated healing”, where structural damage and consequential stress within the disc, lead to progressive degeneration (Adams and Dolan, 2012). Future discovery of epitopes specific for the immune response in the disc can lead to the development of diagnostic tests for disc degeneration in early stages and possibly to treatments,

which can limit the autoimmune damage and initiate tissue repair. From a diagnostic perspective it is more interesting to detect circulating plasma antibodies in patients with degenerative disc disease. This might require using new, more sensitive methods – a previous study identified only 4 out of 60 sera of patients with back pain which reacted to proteins extracted from herniated NP (Kim *et al.*, 1981). Early detection can change the way disc degeneration is treated – either by pharmaceutical interventions or by advanced cell therapies. Presumably, cell-based therapies applied in very early stages of disc degeneration (Paesold *et al.*, 2007) may have better outcomes than when the degenerative changes are advanced.

### Conclusions

We demonstrated that degenerated and post-traumatic IVDs can activate an autoimmune reaction, producing antibodies against specific proteins of the disc and more specifically the NP. Discrete antigens, mainly collagen type II and aggrecan degradation products are among the targets of an autoimmune response in the degenerated IVDs. We believe that our findings will draw new attention to the possible development of monitoring markers for prognosis, diagnosis and therapy related to disc degenerative diseases.

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## Discussion with Reviewers

**Reviewer I:** Did these patients have any symptoms of an autoimmune disease or autoantibodies in the sera?

**Authors:** We did not have data for autoimmune diseases in the patients group. There are neither unique patient numbers nor central clinical data repositories in Switzerland, hence any option to check retrospectively.

**Reviewer I:** The presence of autoantibodies against matrix proteins has been demonstrated in arthritis and now in the disc. What are the implications in biological repair of these tissues?

**Authors:** As patients suffering from degenerative disc disease (DDD) could also have osteoarthritis (OA), further studies are needed to find unique and specific epitopes, which will bring diagnostic specificity and sensitivity. Beyond the question of diagnostics, the underlying implication is that patients having one type of degenerative problem, such as osteoarthritis, may be more prone to accelerated aging and degeneration of disc tissues after initial annulus fibrosus trauma.

**Reviewer II:** From a diagnostic viewpoint, the authors view the approach as providing a new approach to early diagnosis of disc disease. However, many patients who suffer from DDD also have OA, where there are raised levels of IgG collagen type II, MMP's and MMP degradation peptides. Thus, how can this approach be used diagnostically?

**Authors:** Clearly there are a number of questions to be answered before using these findings in diagnostics. It is possible that the auto-antibodies are the same across different cartilage/disc disease, and then no differential diagnosis would be possible. In the best case scenario, the discovery of epitopes specific for the immune response in the disc will be the important step towards the development of diagnostic tests for early stage disc degeneration. Early detection can permit one to treat conservatively the degeneration in the early stage, limit the autoimmune damage, and help tissue repair. Cell-based therapies or pharmaceutical interventions applied in very early stages of disc degeneration may have better outcomes, than when the degenerative changes are advanced.