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THE TISSUE-RENIN-ANGIOTENSIN SYSTEM OF THE HUMAN INTERVERTEBRAL DISC

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Abstract

Symptomatic intervertebral disc (IVD) degeneration accounts for significant socioeconomic burden. Recently, the expression of the tissue renin-angiotensin system (tRAS) in rat and bovine IVD was demonstrated. The major effector of tRAS is angiotensin II (AngII), which participates in proinflammatory pathways. The present study investigated the expression of tRAS in human IVDs, and the correlation between tRAS, inflammation and IVD degeneration.

Human IVD tissue was collected during spine surgery and distributed according to principal diagnosis. Gene expression of tRAS components, proinflammatory and catabolic markers in the IVD tissue was assessed. Hydroxyproline (OHP) and glycosaminoglycan (GAG) content in the IVD tissue were determined. Tissue distribution of tRAS components was investigated by immunohistochemistry.

Gene expression of tRAS components such as angiotensin-converting enzyme (ACE), Ang II receptor type 2 (AGTR2), angiotensinogen (AGT) and cathepsin D (CTSD) was confirmed in human IVDs. IVD samples that expressed tRAS components (n = 21) revealed significantly higher expression levels of interleukin 6 (IL-6), tumour necrosis factor α (TNF-α), a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) 4 and 5 compared to tRAS-negative samples (n = 37). Within tRAS-positive samples, AGT, matrix-metalloproteinases 13 and 3, IL-1, IL-6 and IL-8 were more highly expressed in traumatic compared to degenerated IVDs. Total GAG/DNA content of non-tRAS expressing IVD tissue was significantly higher compared to tRAS positive tissue. Immunohistochemistry confirmed the presence of AngII in the human IVD.

The present study identified the existence of tRAS in the human IVD and suggested a correlation between tRAS expression, inflammation and ultimately IVD degeneration.

Keywords: Intervertebral disc, renin angiotensin system, degeneration, regeneration, spine, inflammation.

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Introduction

Low back pain (LBP) is one of the most prevalent musculoskeletal conditions and a leading cause of disability worldwide, resulting in an enormous socioeconomic burden (Vos *et al.*, 2017). Degeneration of the intervertebral disc (IVD) is a major contributor to LBP. IVD degeneration features extracellular matrix (ECM) degradation, accelerated cartilage and bone remodelling, the release of proinflammatory cytokines, altered spine biomechanics, angiogenesis and neoinnervation; altogether causing chronic LBP and disability (Adams and Roughley, 2006; Lang *et al.*, 2018; Risbud and Shapiro, 2014). Disc degeneration has a multifactorial pathogenesis that can be triggered and aggravated by mechanical stress, infection and trauma as well as genetic predisposition (Lang *et al.*, 2018). There is evidence that these events can be followed by an inflammatory response, changing the biomolecular conditions within the IVD (Risbud and Shapiro, 2014). Proinflammatory cytokines contribute to disc degeneration, inflammation, discogenic pain and also regulate the expression of major catabolic enzymes for ECM deterioration, such as a disintegrin

and metalloprotease with thrombospondin motifs (ADAMTS) and matrix metalloproteinases (MMP) (Risbud and Shapiro, 2014). Tumour necrosis factor-alpha (TNF-α) and interleukin (IL)-6 have been shown to be highly expressed in degenerated and symptomatic human IVD cells (Risbud and Shapiro, 2014; Takahashi *et al.*, 1996).

Currently, there are no treatment options addressing the underlying pathological changes leading to disc degeneration. Many symptomatic patients in early stages of IVD degeneration do not benefit from conservative treatments and, at the same time, do not qualify for surgery. Many surgical techniques such as spinal fusion or total disc replacement have been established and implemented to treat symptomatic patients, although the long-term benefit compared to conservative therapies remains elusive (Airaksinen *et al.*, 2006; Kaiser *et al.*, 2014). In lack of a sufficient self-repair capacity of the IVD and satisfactory treatment options, new biomolecular therapies targeting the inflammatory and catabolic pathways have been considered recently. These strategies aim at trying to reduce the inflammatory microenvironment of the IVD in order to slow down the progressive degenerative cascade. As a promising alternative to established therapies, the biomolecular approach could preserve the IVD's mechanical and biological functions and also relieve pain at early to moderate stages of degeneration. However, before therapeutic strategies can be developed, how, where and when inflammation contributes to IVD degeneration needs to be elucidated.

The renin-angiotensin system (RAS) plays a fundamental role in regulating the physiology of the cardiovascular system. The major effector of this system is angiotensin II (AngII), a peptide hormone that increases blood pressure (Timmermans *et al.*, 1992). The precursor protein angiotensinogen (ATG) is cleaved by the protease renin to form biologically inactive AngI, which is converted to active AngII by angiotensin-converting enzyme (ACE) (Liao *et al.*, 2017). Recently, AngII was also demonstrated to have haemodynamic-independent effects, such as contribution to inflammation, hypersensitivity and fibrosis in many organs, including liver, kidney and skin (Kranzhofer *et al.*, 1999; Paul *et al.*, 2006; Ruiz-Ortega *et al.*, 2001). Previous studies have also shown that inflammatory cells expressed RAS components and AngII contributed to axon sprouting and regeneration (Chakrabarty *et al.*, 2008; Hoch *et al.*, 2009). Therefore, the definition of "*local*" or "*tissue*" renin-angiotensin system (tRAS) was introduced (Paul *et al.*, 2006). RAS inhibitors belong to the group of most commonly prescribed drugs globally (Stagnitti, 2001). Recent research reveals evidence that ACE inhibitors diminish TNF-α production *in vivo* and *in vitro* (Fukuzawa *et al.*, 1997). Price *et al*. demonstrated, in a rodent model of rheumatoid arthritis (RA), that TNF-α release and subsequent knee joint swelling were significantly inhibited by the application of ACE inhibitors (Price *et al.*, 2007). Other groups confirmed these findings revealing beneficial anti-arthritic effects of ACE inhibitors as well as angiotensin II receptor blockers (ARBs) by reducing inflammation, neutrophil recruitment, hypernociception, disease activity, oxidative stress levels, and ultimately joint destruction (Agha and Mansour, 2000; Dalbeth *et al.*, 2005; Fahmy Wahba *et al.*, 2015; Flammer *et al.*, 2008; Guerra *et al.*, 2016; Liu and Wang, 2014; Perry *et al.*, 2008; Queiroz-Junior *et al.*, 2015; Refaat *et al.*, 2013; Sagawa *et al.*, 2005; Shi *et al.*, 2012; Silveira *et al.*, 2013; Tang *et al.*, 2015; Wang *et al.*, 2013). Renal injury models revealed that inhibition or reduction of AngII actions by ACE inhibitors or angiotensin receptor antagonists reduced proteinuria, recruitment of proinflammatory cells, fibrosis and catabolic gene expression (Mezzano *et al.*, 2001; Ruiz-Ortega *et al.*, 2001; Wolf and Neilson, 1993).

Systemic inflammatory actions of the RAS have been studied extensively during the last 20 years. However, the expression of local RAS in the IVD is largely unknown, except for a recent study by Morimoto *et al*. who described the existence of tRAS in the rat IVD (Morimoto *et al.*, 2013). In human IVD cells, the expression of local RAS components might contribute to disc degeneration, inflammation and discogenic pain. The objectives of the present study were to determine if tRAS is expressed in the human IVD tissue and if the expression of tRAS is associated with IVD inflammation. An additionally aim was to identify distinct molecules within the tRAS system as new anti-inflammatory therapeutic targets for early intervention of IVD degeneration.

Materials and Methods

Ethical considerations

The study was approved by the local institutional review board (protocol number 188/15) at the Medical Centre, Faculty of Medicine, Albert Ludwig University of Freiburg, Germany. Informed consent was obtained from all patients before surgery. All studies were performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Study population

A prospective experimental, single-centre study was conducted of patients with symptomatic single or multilevel cervical, thoracic and/or lumbosacral spinal pathologies who underwent spinal fusion procedures (transforaminal lumbar interbody fusion (TLIF), extreme lateral interbody fusion (ELIF), anterior cervical discectomy and fusion (ACDF), lumbar/thoracic corpectomy and fusion between 2015 and 2016 (Table 1). Patients had been referred for spinal fusion for various indications including degenerative disc disease (DDD), low-grade spondylolisthesis, and foraminal stenosis (Table 1). The diagnosis of DDD at the institution was based

on a synopsis of diagnostic findings, assessed within a local standardised diagnostic algorithm. Patients most commonly described chronicity of symptoms after ≥ 6 months, indicating failed conservative therapy. For patients presented with a combination of certain clinical (*e.g.* nonradicular chronic LBP) and other correlating diagnostic characteristics (*e.g.* "black disc", significant disc height loss or Modic changes), the diagnosis DDD was concluded if alternative potential sources of LBP were excluded. Human IVD tissue (nucleus pulposus and annulus fibrosus, but without cartilage endplate) was harvested during discectomy surgeries and transferred into a tube filled with 0.9 % NaCl solution. As minimally invasive techniques were mainly being used for spinal fusion surgery there was only a small corridor for the introduction of instruments. Hence, the discectomies for the present study were comprised of an initial squarish incision of the IVD followed by a small piece-by-piece resection of the disc tissue, utilising pituitary rongeurs, starting at the outer AF and progressing towards NP and contralateral AF. In young traumatic or scoliosis patients, the discs were less degenerated so that anatomic regions of the disc could be assessed macroscopically. However, in severely degenerated and osteochondrotic IVDs, the discs were very dry, thin and fibrotic so that a sufficient differentiation of IVD tissue regions was not always possible.

Assessment of clinical, radiographic, and laboratory scores

In order to assess clinical, radiographic, and laboratory scores of patients undergoing spinal fusion, visual analogue scale (VAS) for pain of back, buttock and leg, leucocytes, C-reactive protein (CRP) and radiographic imaging were documented preoperatively (Modic *et al.*, 1988; Pfirrmann *et al.*,

Table 1. Demographic characteristics of the study population. *n*: absolute number of patients, %: relative number of patients in percentage, [1]Mean ± SD, BMI: Bone mineral density, Spondylolisthesis (I-II): Spondylolisthesis grade I and II according to the Meyerding classification; ASA-score: American Society of Anaesthesiologists classification of physical status.

Parameter		*n* = 58	%
Age in years		58.19 ± 18.23[1] (14-96)	
Sex	Male	34	58.6 %
	Female	24	41.4 %
BMI in kg/m^2		26.42 ± 4.23[1] (17-42)	
Intervertebral disc level	C4-C7	11	18.9 %
	T5-T12	6	10.2 %
	L1-L3	8	13.8 %
	L3/L4	9	15.5 %
	L4/L5	18	31.0 %
	L5/S1	6	10.3 %
Nicotine abuse		18	31.0 %
ASA-score	2.22 ± 0.65[1] (1-3)		
	1	7	12.1 %
	2	31	53.4 %
	3	20	34.5 %
Diabetes mellitus Type II		8	13.8 %
Hypertension		28	48.3 %
Rheumatoid arthritis		1	1.7 %
Osteoporosis		4	6.9 %
Neoplasm		5	8.6 %
Degenerative disc disease		33	56.9 %
Scoliosis	Degenerative	7	12 %
	Idiopathic	3	5.1 %
Spondylolisthesis (I-II)		10	17.2 %
Spinal stenosis		43	74.1 %
Vertebral fracture		7	12.1 %
Postdiscectomy syndrome		7	12.1 %
Motoric deficit		8	13.8 %
Sensible deficit		21	36.2 %

2001). The clinical, radiographic (Modic changes, Pfirrmann grade) and laboratory scores were evaluated and correlated to each other by blinded investigators retrospectively.

Gene expression analysis

Approximately 150 mg of IVD tissue from each patient was used for RNA extraction. Tissue samples were preserved in RNAlater™ (Sigma-Aldrich, St. Louis, MO, USA) after resection and stored at – 80 °C. After thawing and before RNA isolation, the tissue was washed with red blood cell lysis buffer to avoid sample contamination with blood cells. For analysis, the preserved tissue was pulverised in liquid nitrogen and then homogenised within TRI Reagent (Molecular Research Centre, Cincinnati, OH, USA) using a Tissue Lyser (Qiagen, Venlo, Netherlands) (Caprez *et al.*, 2018). Total RNA was extracted with bromochloropropane phase separation followed by Rneasy MINI kit (Qiagen). Reverse transcription was performed with SuperScript VILO cDNA Synthesis Kit (Life Technologies, Carlsbad, CA). Quantitative real-time PCR was performed using the Step-One-Plus instrument (Life Technologies). IVD cells were assessed for anabolic and catabolic gene expression, including aggrecan (*ACAN*), collagen type 2 (*COL2*), *MMP3*, *MMP13*, *ADAMTS4* and *5*, transforming growth factor-beta (*TGFB1*), and insulin-like growth factor 1 (*IGF1*). Additionally, the inflammatory phenotype of disc cells was assessed by analysing gene expression levels of interleukin *(IL)-1*, *IL-6*, *IL-8* and *TNF-α*. Gene expression of tRAS components for angiotensin II receptor type 1a (*AGTR1A*), angiotensin II receptor type 2 (*AGTR2*), Cathepsin D (*CTSD*), Angiotensinogen (*AGT*), *ACE* and renin was also measured. The sequence of custom designed primers-probes (Microsynth, Balgach, Switzerland) or catalogue number of Assay on Demand primers-probes (Thermo Fisher, Waltham, MA, USA) are listed in Table 2. Relative quantification of target mRNA was performed using the comparative Ct method with 18S ribosomal RNA as an endogenous control. Only significant differences in gene expression are shown in the figures.

Table 2. Sequence of custom-designed primer-probes and catalogue number of assay-on-demand primer-probes.

Gene acronym	Gene full name	Primer-probe sequence or catalogue number	Reporter/quencher
h18S	Human 18S ribosomal RNA	Hs99999901_s1	FAM/NFQ-MGB
hACAN	Human aggrecan	Forward primer seq.: 5′-AGT CCT CAA GCC TCC TGT ACT CA-3′ Reverse primer seq.: 5′-CGG GAA GTG GCG GTA ACA-3′ Probe seq.: 5′-CCG GAA TGG AAA CGT GAA TCA GAA TCA ACT-3′	FAM/TAMRA
hACE	Human angiotensin-converting-enzyme	Hs00174179_m1	FAM/NFQ-MGB
hADAMTS4	Human a disintegrin like and metallopeptidase with thrombospondin type 1 motif 4	Hs00192708_m1	FAM/NFQ-MGB
hADAMTS5	Human a disintegrin like and metallopeptidase with thrombospondin type 1 motif 5	Hs01095518_m1	FAM/NFQ-MGB
hAGT	Human angiotensinogen	Hs01586213_m1	FAM/NFQ-MGB
hAGTR2	Human angiotensin-II receptor type 2	Hs02621316_s1	FAM/NFQ-MGB
hCTSD	Human cathepsin D	Hs00157205_m1	FAM/NFQ-MGB
hCOL2	Human collagen type 2	Forward primer seq.: 5′-GGC AAT AGC AGG TTC ACG TAC A-3′ Reverse primer seq.: 5′-GAT AAC AGT CTT GCC CCA CTT ACC-3′ Probe seq.: 5′-CCT GAA GGA TGG CTG CAC GAA ACA TAC-3′	FAM/TAMRA
hIGF1	Human insulin-growth-factor 1	Hs01547656_m1	FAM/NFQ-MGB
hIL1β	Human interleukin 1β	Hs00174097_m1	FAM/NFQ-MGB
hIL6	Human interleukin 6	Hs00985639_m1	FAM/NFQ-MGB
hIL8	Human interleukin 8	Hs00174103_m1	FAM/NFQ-MGB
hMMP13	Human matrix-metalloproteinase 13	Forward primer seq.: 5′-CGG CCA CTC CTT AGG TCT TG-3′ Reverse primer seq.: 5′-TTT TGC CGG TGT AGG TGT AGA TAG-3′ Probe seq.: 5′-CTC CAA GGA CCC TGG AGC ACT CAT GT-3′	FAM/TAMRA
hMMP3	Human matrix-metalloproteinase 3	Hs00968305_m1	FAM/NFQ-MGB
hTGFβ1	Human transforming growth factor β1	Hs00171257_m1	FAM/NFQ-MGB
hTNFα	Human tumor necrosis factor α	Hs01113624_g1	FAM/NFQ-MGB
hRenin	Human renin	Hs00982555_m1	FAM/NFQ-MGB
hAGTR1	Human angiotensin-II receptor type 1	Hs00258938_m1	FAM/NFQ-MGB

Biochemical analysis

Biochemical analysis was performed as described previously (Lang *et al.*, 2018). For glycosaminoglycan (GAG), collagen and DNA content measurement 50 mg of IVD tissue from each patient was digested within 0.5 mg/mL proteinase K at 56 °C overnight. The GAG content was determined using the 1,9-dimethylmethylene blue dye (DMMB) method (Lang *et al.*, 2018). Quantification of the total amount of collagen was performed using the hydroxyproline (OHP) assay with 4-dimethylaminobenzaldehyde (DABA) as described previously (Lang *et al.*, 2018). DNA content was measured spectrofluorometrically using Hoechst (33258) dye.

Immunohistochemistry

Tissue samples were fixed in 4 % paraformaldehyde (customised). The samples were then embedded in paraffin wax and serial 5 µm sections were used for immunohistochemical analysis. After blocking endogenous peroxidase activity, sections were heated for 45 min at 95 °C in citrate buffer (0.01 mol/L, pH 6.0) and incubated overnight at 4 °C with anti-AngII antibody (cat. no.: T-4007, Peninsula Laboratories LLC, San Carlos, CA, USA) diluted 1 : 200 in LowCrossBuffer® (CANDOR Bioscience, Wangen, Germany). A secondary HRP-Polymer-Reagent (Zytomed Systems, Berlin, Germany) followed by 3-Amino-9-Ethylcarbazol (AEC) (Vector Laboratories, Peterborough, UK) were applied to the sections and formed a visible precipitate. Sections were counterstained with haematoxylin (Sigma-Aldrich, St. Louis, MO, USA). Stained sections were imaged using an Olympus BX51 microscope (Olympus, Tokyo, Japan). Tissue from human hepatocellular carcinoma served as control.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0e software (GraphPad Software, Inc., La Jolla, CA, USA) for experimental research data and SPSS v23 (IBM Corp., New York, USA) for clinical parameters. Continuous variables were reported as mean ± standard deviation, and discrete variables were reported as frequency (%). For normally distributed data, the differences were assessed using the independent *t*-test or ANOVA, as appropriate. Mann-Whitney U test was used to determine differences between groups when data were not normally distributed as well as χ^2-test to compare variants between two groups. All p values were two-sided with statistical significance evaluated at $\alpha = 0.05$. Undetermined gene expression values were included in statistical analysis using a rank-based test.

Results

Baseline characteristics

A total of 58 patients were included in the study (mean age: 58 ± 18 years; range: 14-96 years; Table 1). Patients were distributed into 4 groups according to principal diagnosis: degeneration (75.9 %, $n = 44$), trauma (10.3 %, $n = 6$), scoliosis (10.3 %, $n = 6$) and

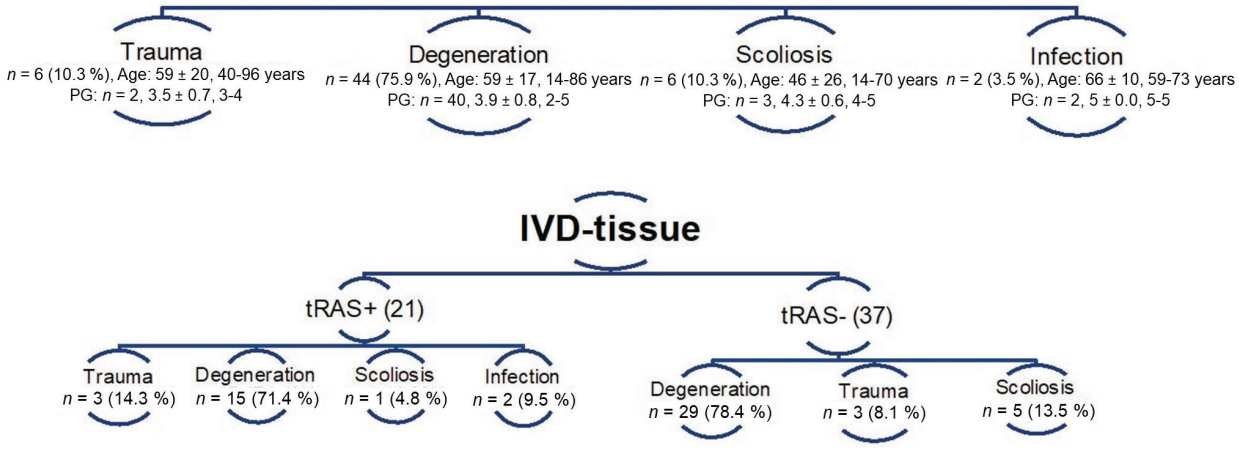

Fig. 1. Distribution of tRAS positive samples according to principal diagnosis. The top part shows the sample number (n), age (mean ± SD, age range) and Pfirrmann grade (n = number of samples with PG available, mean ± SD, range) of patients from 4 groups according to principal diagnosis. tRAS: tissue-renin-angiotensin system, PG: Pfirrmann grade.

Table 3. Number of undetected samples for tRAS factor gene expression in the different groups (Fig. 1).

Parameter	Degeneration	Trauma	Scoliosis	Infection
n	44	6	6	2
ACE	29 (66 %)	3 (50 %)	5 (83 %)	0 (0 %)
AGT	10 (23 %)	2 (33 %)	1 (17 %)	0 (0 %)
AGTR2	1 (2 %)	0 (0 %)	0 (0 %)	0 (0 %)
CTSD	0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)

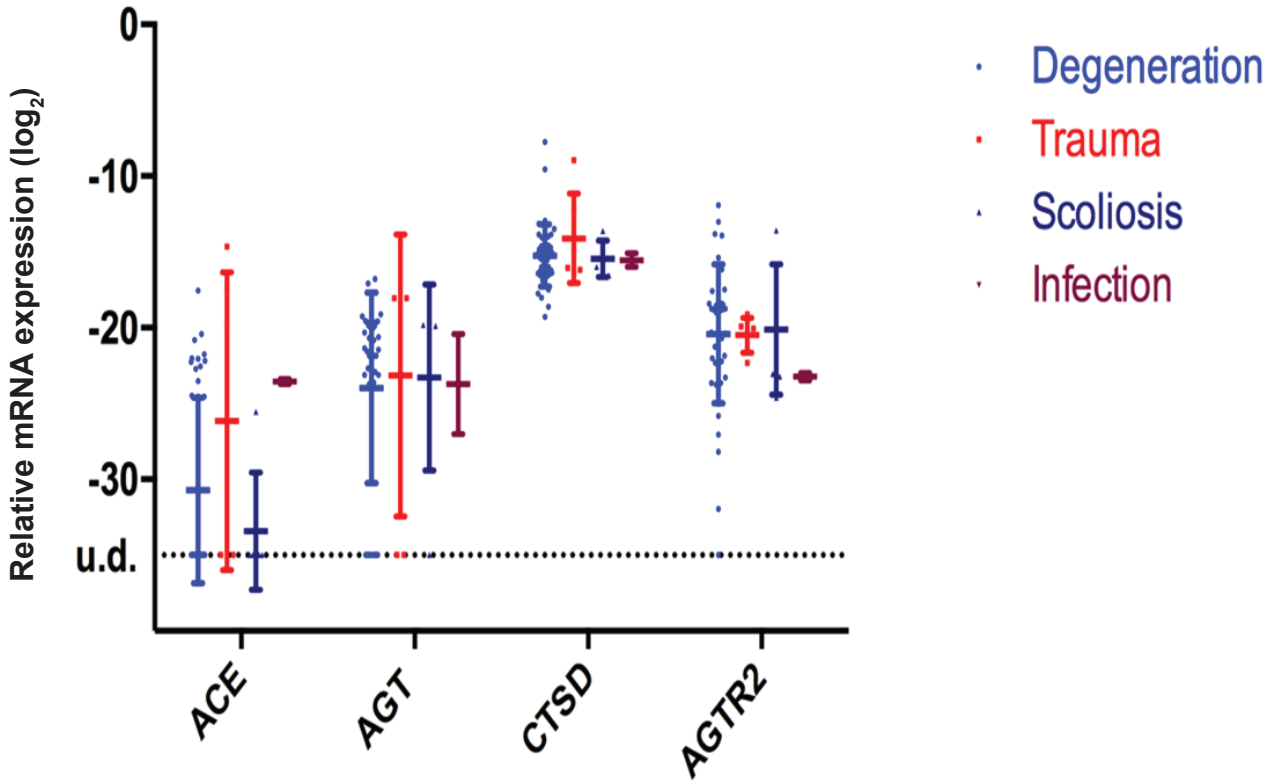

Fig. 2. Relative gene expression levels of tRAS factors classified by diagnosis. No significant differences were observed between the Degeneration ($n = 44$), Trauma ($n = 6$), Scoliosis ($n = 6$) or Infection ($n = 2$) groups. Expression levels normalised to the expression of 18S rRNA. u.d. = samples with undetected values. ACE: angiotensin-converting-enzyme, AGT: angiotensinogen, CTSD: cathepsin D, AGTR2: Ang II receptor type 2.

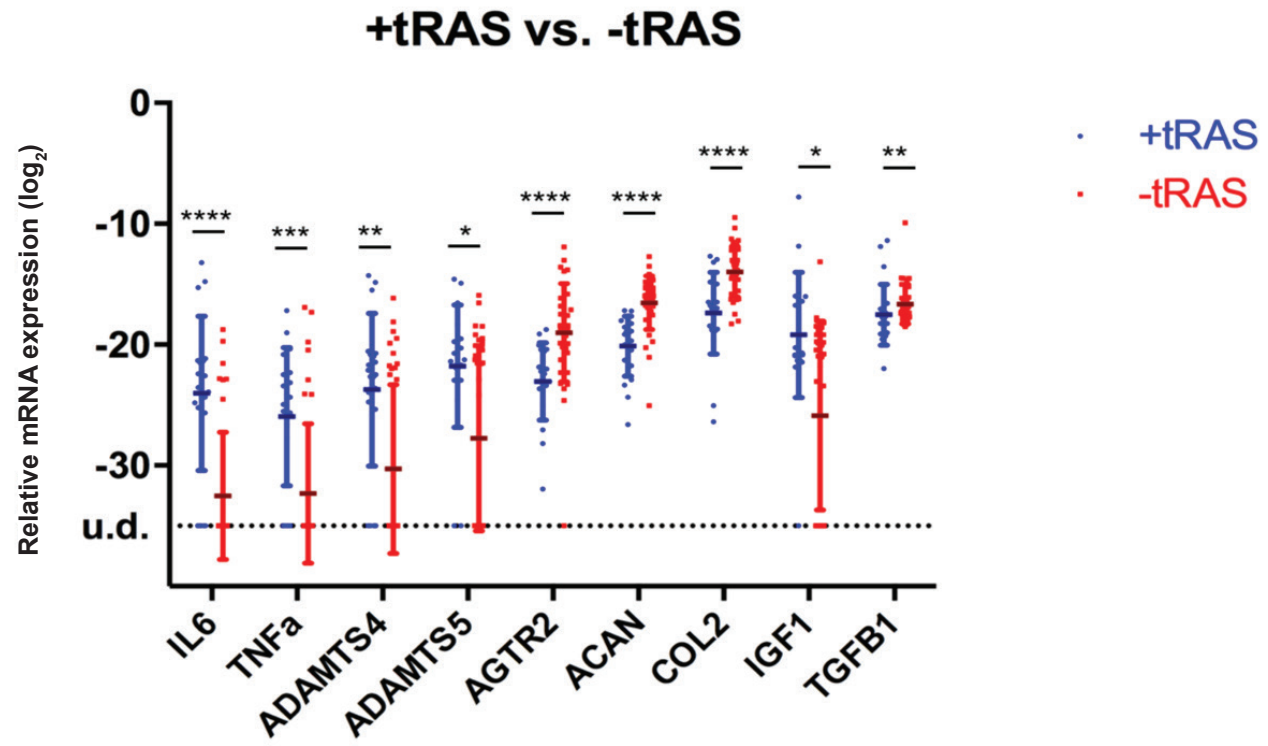

Fig. 3. Significant relative gene expression levels of catabolic, anabolic, and proinflammatory genes in human IVDs as classified by tRAS expression. *IL-6*: interleukin 6, *TNF-α*: tumour necrosis factor α, *ADAMTS*: a disintegrin and metalloproteinase with thrombospondin motifs, *AGTR2*: Ang II receptor type 2, *ACAN*: aggrecan, *COL2*: collagen type 2, *IGF1*: insulin-like growth factor 1, *TGFB1*: transforming growth factor-beta, +: tRAS-positive samples ($n = 21$), –: tR)AS-negative samples ($n = 37$). u.d = samples with undetected values. Expression levels normalised to the expression of 18S rRNA. Mann-Whitney U test was used for statistical analysis, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

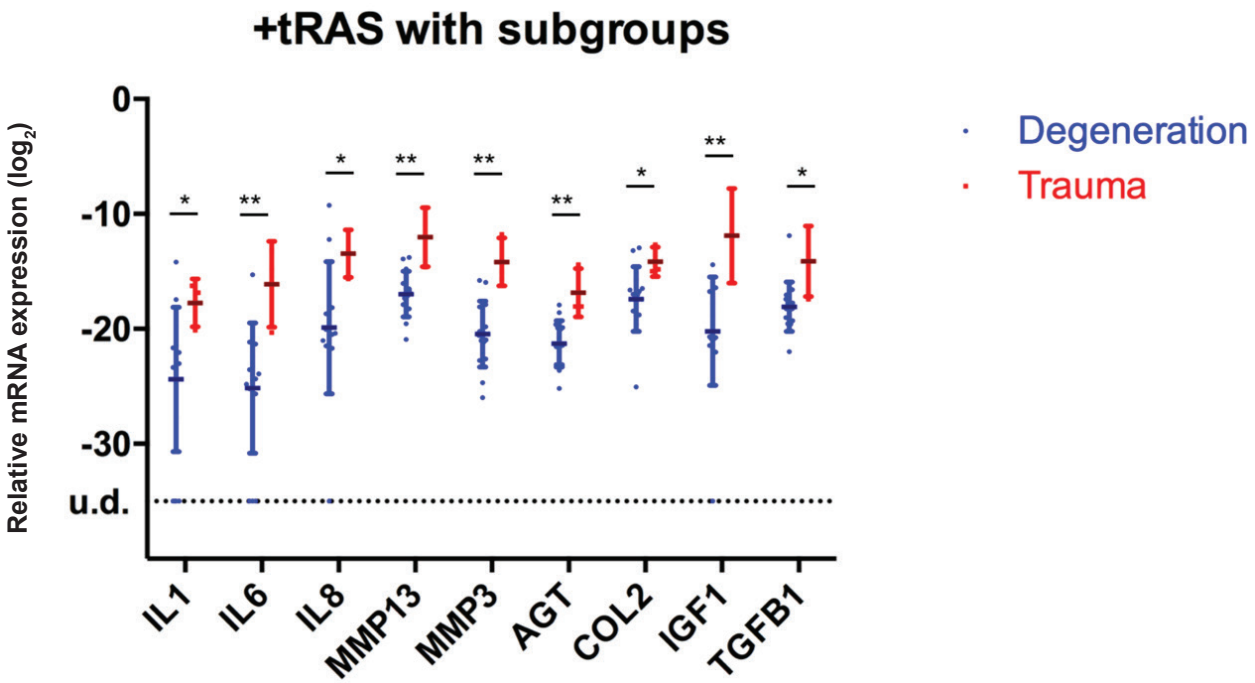

Fig. 4. Significant relative gene expression levels of trauma *vs.* degenerative human IVDs in +tRAS samples. *IL*: interleukin, *MMP*: matrix-metalloproteinases, *AGT*: angiotensinogen, *COL2*: collagen type 2, *IGF1*: insulin-like growth factor 1, *TGFB1*: transforming growth factor-beta, +tRAS: IVD samples which express all the four markers *ACE*, *AGTR2*, *AGT* and *CTSD*. Trauma (n = 3), Degeneration (n = 15). u.d. = samples with undetected values. Expression levels normalised to the expression of 18S rRNA. Mann-Whitney U test was used for statistical analysis, *$p < 0.05$, **$p < 0.01$.

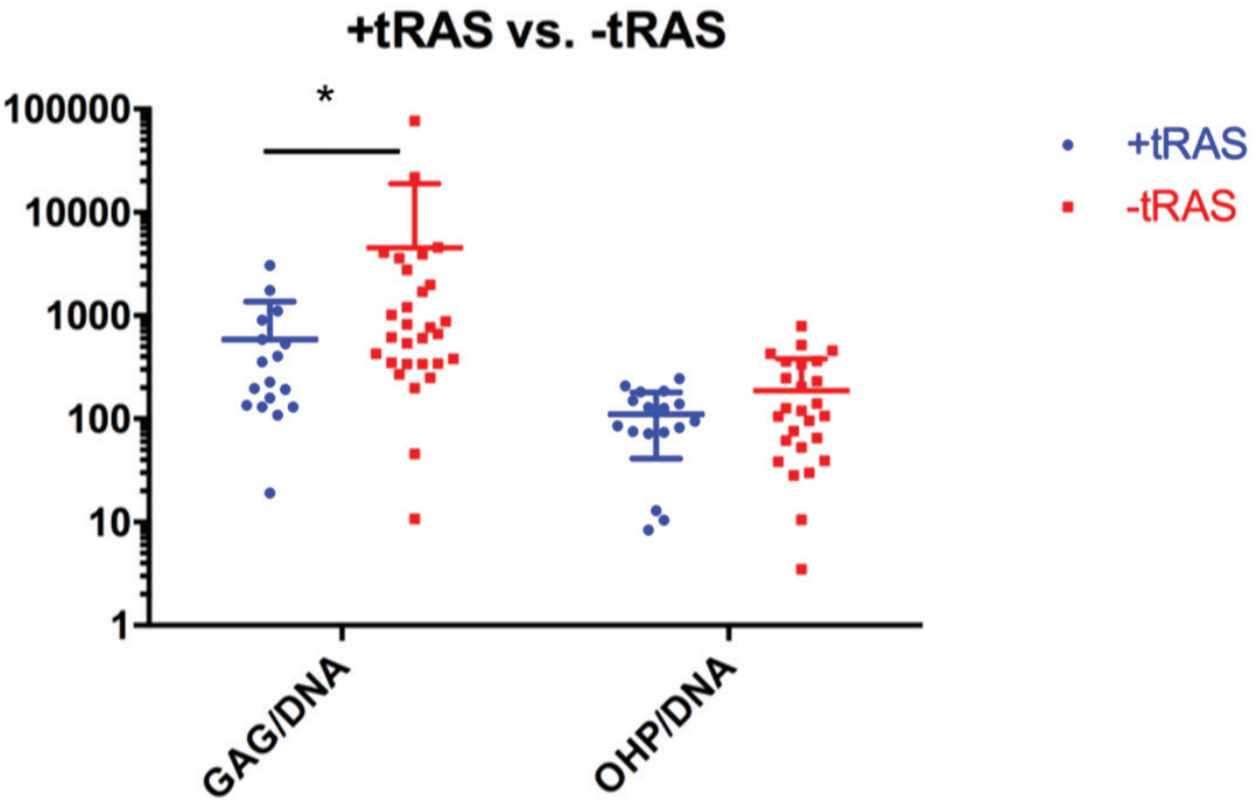

Fig. 5. Biochemical analysis of human IVD tissue stratified by tRAS expression. Glycosaminoglycan (GAG)/DNA and Hydroxyproline (OHP)/DNA content in +tRAS and -tRAS groups. Mann-Whitney U test was used for statistical analysis, *$p < 0.05$.

Table 4. IVD tissue samples used for immunohistochemistry staining of Ang II.

Sample number	35	49	70	104	130	94	99
tRAS Gene expression	+	+	+	+	+	-	-
Principal diagnosis	Trauma	Degeneration	Trauma	Scoliosis	Degeneration	Scoliosis	Trauma
IHC of AngII	+	+	+	+	+	+	+

infection (3.5 %, n = 2) as illustrated in Fig. 1. For these principal diagnoses, the percentage of several disease phenotypes in the whole study population are summarised in Table 1, including spinal stenosis (74 %), spondylolisthesis (17 %), postdiscectomy-syndrome (12 %) and fractures (12 %). Most commonly, IVDs were acquired from spinal levels L4/5 (31 %), L3/4 (16 %), L5/S1 (10 %), whereas cervical (19 %) or thoracic (10 %) motion segments were less frequently involved (Table 1).

Gene expression

Gene expression of tRAS components such as *ACE*, *AGTR2*, *AGT*, and *CTSD* was largely detectable in human IVDs (Fig. 2). The number of samples in which the genes were undetected in each group is shown in Table 3. The scoliosis group had the highest percentage in undetermined ACE expression comparing with the degeneration, trauma, and infection groups. *AGTR1A* and renin were not detected in any tested samples. IVD samples expressing *ACE* always simultaneously expressed *AGTR2*, *AGT* and *CTSD*. IVD samples were classified expressing all of the four markers (*ACE*, *AGTR2*, *AGT* and *CTSD*) as tRAS-positive IVDs (n = 21) (Fig. 1). tRAS-negative IVDs (n = 37) did not express *ACE*. For all the genes illustrated in Fig. 2, no significant differences were observed among the 4 groups at the expression level.

The levels of gene expression were analysed to investigate the hypothesis that tRAS, being associated with catabolic and proinflammatory processes, participates in symptomatic disc degeneration. Samples that expressed tRAS revealed significantly higher gene expression levels of proinflammatory genes such as *IL-6*, *TNF-α*, and the catabolic markers *ADAMTS4* and *5*, as well as *IGF1*, compared to tRAS-negative samples ($p < 0.05$, Fig. 3). Gene expression of ECM components such as *ACAN* and *COL2* ($p < 0.0001$, Fig. 3) as well as *AGTR2* and *TGFβ1* was lower in tRAS-positive discs compared to tRAS-negative tissue. Other analysed genes revealed no significant differences. Furthermore, within tRAS-positive samples, *AGT*, the catabolic and proinflammatory markers *MMP13*, *MMP3*, *IL-1*, *IL-6* and *IL-8* were significantly higher expressed in trauma compared to the degeneration group (Fig. 4). Other genes, especially factors of tRAS (*CTSD*, *AGTR2* and *ACE*) showed no significant differences within the tRAS-positive group.

Biochemical Analysis

Biochemical analysis of IVD tissues revealed significantly higher GAG/DNA values of non-tRAS expressing IVD tissue (median: 665) compared to tRAS positive tissue (median: 228, $p < 0.05$; Fig. 5). There was no difference in the collagen/DNA amount between the two tissue groups.

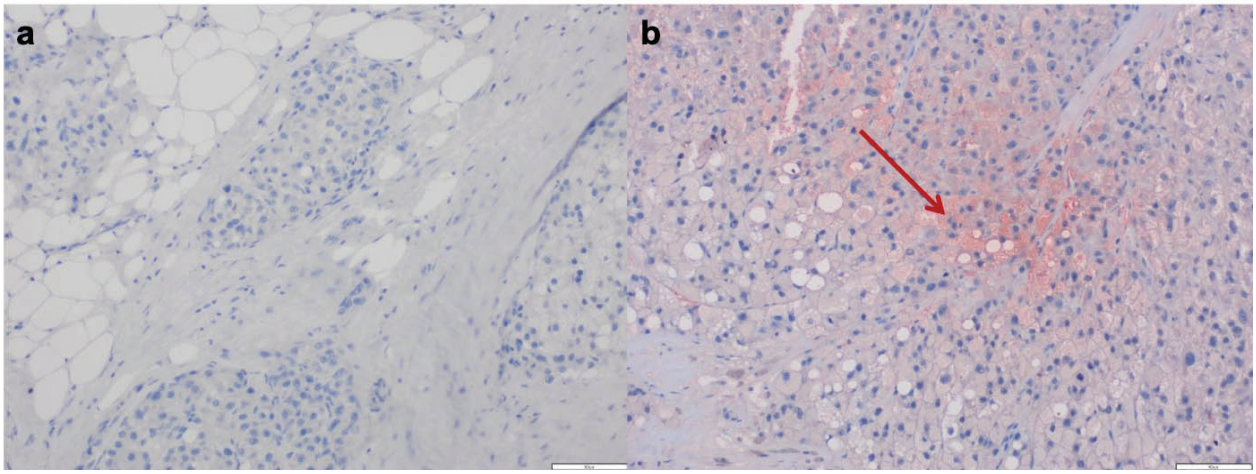

Fig. 6. Immunohistochemical staining of AngII in human hepatocellular carcinoma (HCC) tissue serving as control. (**a**) Negative control, no red staining. (**b**) positive control, AngII is stained red, especially in the stroma. Blue: cell nucleus; red: AngII proteins. Scale bar: 100 μm.

Immunohistochemistry

Seven IVD tissue samples (Table 4) were utilised to perform immunohistochemistry staining against anti-AngII antibody to reveal the qualitative expression of tRAS in the human IVD at protein level. Three samples were from trauma patients (2× tRAS+ and 1× tRAS- on gene-expression level). Two IVD samples were from the subgroup degeneration (2× tRAS+ on gene-expression level) and scoliosis (1× tRAS+ and 1× tRAS- on gene-expression level), respectively. Immunoreactivity to AngII in human hepatocellular carcinoma (HCC) tissue served as the positive control (Fig. 6) (Xu *et al.*, 2017). As illustrated in Fig. 7, intense red staining of AngII was observed in the cytoplasm of human IVD cells which were positive for tRAS gene expression, confirming the existence of tRAS within human IVDs. The qualitative analysis confirmed the presence of AngII in all the analysed samples.

Correlation of tRAS expression and clinical, laboratory and radiographic parameters

tRAS-negative patients were significantly older (62.4 ± 16.9 *vs.* 50.9 ± 18.6, p = 0.02) and yielded

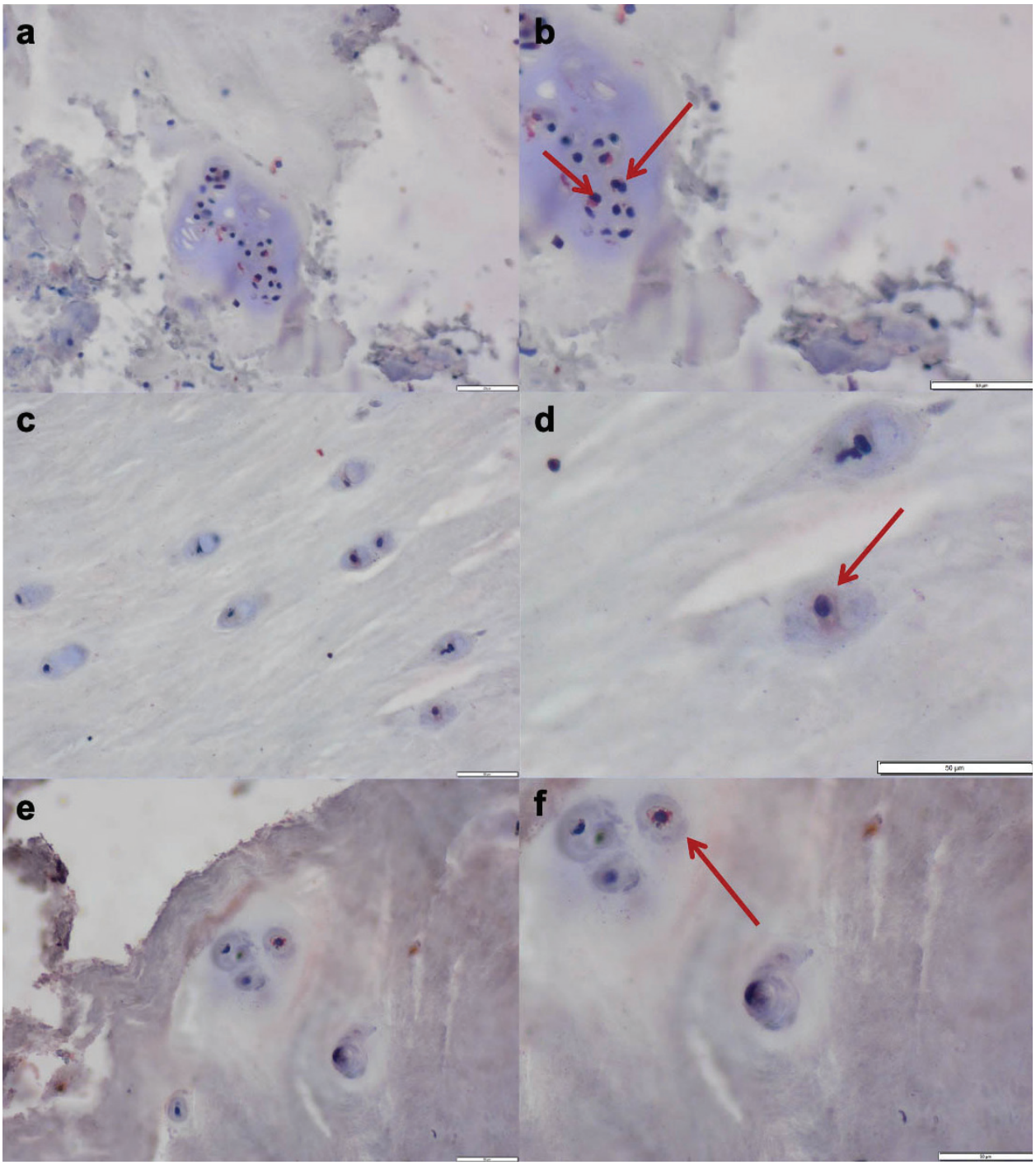

Fig. 7. Immunohistochemical staining of AngII in human IVD tissue. (**a**) and (**b**) IVD tissue from scoliosis patient. (**c**) and (**d**) IVD tissue from degeneration patient. (**e**) and (**f**) IVD tissue from trauma patient. AngII is positively expressed in human disc cells. Blue: cell nucleus, red: AngII proteins. Scale bar: 50 μm.

higher American Society of Anaesthesiologists' (ASA) classification of physical-health scores (2.38 ± 0.55 *vs.* 1.95 ± 0.74, $p = 0.02$) at time of surgery compared to patients expressing tRAS (Table 5). tRAS expressing patients were more frequently (33.3 % *vs.* 2.7 %) treated with steroid medication than tRAS negative patients ($p = 0.002$). No effects on tRAS expression were observed in patients taking ACE inhibitors or AT1-receptor antagonists. A correlation test was performed on the samples which has a Modic score, Pfirrmann grade and pain level, to investigate if any of these degeneration scores are correlated with the tRAS gene expression level. No significant differences were observed between tRAS expressing and non-expressing patients on the Modic score, Pfirrmann grade or pain level.

Discussion

The present study sought to investigate whether tRAS was expressed in the human IVD. The study population consisted of a representative patient cohort (slight majority of male sex) and comprised a broad spectrum of indications (infection, degeneration, trauma and scoliosis) and localisations (cervical, thoracic and lumbar spine) for discectomy and spinal fusion and electronic medical records.

The results also provided evidence for a correlation of tRAS with inflammatory and degenerative changes in IVD tissue. tRAS-positive IVDs revealed significantly higher gene expression levels of proinflammatory (*IL-6* and *TNF-α*) and catabolic markers (*ADAMTS 4* and *5*) compared with tRAS-negative samples (Fig. 3). Furthermore, gene expression of the NP phenotype markers such as *ACAN* and *COL2* was significantly reduced in tRAS-positive compared with tRAS-negative tissue ($p < 0.0001$; Fig. 3). tRAS-positive IVD samples revealed lower GAG/DNA ratios compared with tRAS negative samples, implying an accelerated state of catabolism.

The renin-angiotensin-system (RAS) plays an important role in the induction and progression of tissue injuries in the cardiovascular system (Namsolleck *et al.*, 2014). During previous years, a

Table 5. Sub-analysis of tRAS positive and negative tissue samples according to patient demographics.
n: absolute number of patients, %: relative number of patients in percentage, [1]Mean ± SD, ASA-score: American Society of Anaesthesiologists classification of physical status, SSSI: Spine surgical invasiveness index, ACE: Angiotensin-converting enzyme, AT1: AngiotensinII-receptor type-1. For statistical analysis, χ^2-test or Fisher's Exact test was used, depending on the number of samples.

Parameter	tRAS	*n* = 58	Range	%	Significance +tRAS *vs.* -tRAS
Age in years	+	50.86 ± 18.56[1] (14-80)			***0.02**
	-	62.35 ± 16.90[1] (17-96)			
Sex	+	Male	16	76.2 %	
	-	Male	18	48.6 %	
BMI in kg/m²	+	26.31 kg/m²	21-42	± 4.86	0.31
	-	26.48 kg/m²	17-34	± 3.90	
Nicotine abuse	+		6	28.6 %	0.76
	-		12	32.4 %	
ASA-score	+	1.95 ± 0.74[1] (1-3)			***0.02**
	-	2.38 ± 0.55[1] (1-3)			
Diabetes mellitus Type II	+		2	9.5 %	0.70
	-		6	16.2 %	
Hypertension	+		9	42.9 %	0.53
	-		19	51.4 %	
Degenerative disc disease	+		12	57.1 %	0.98
	-		21	56.8 %	
Motoric deficit	+		1	4.8 %	0.24
	-		7	18.9 %	
Sensible deficit	+		4	19.0 %	***0.04**
	-		17	45.9 %	
Steroids	+		7	33.3 %	***0.002**
	-		1	2.7 %	
ACE inhibitors	+		4	19.0 %	1.0
	-		6	16.2 %	
AT1-receptor antagonists	+		1	4.8 %	0.40
	-		5	13.5 %	
SSSI	+	5.48 ± 3.56[1] (1-17)			0.24
	-	6.62 ± 4.07[1] (1-17)			

diversity of locally acting renin-angiotensin systems had been identified in multiple tissues, such as skin, kidney, liver, vulva, gastrointestinal tract, nervous system, lymphatic tissue and bone – revealing participation in inflammatory and nociceptive processes (Patil *et al.*, 2010; Paul *et al.*, 2006). While AngII is well accepted as a classical cardiovascular hormone inducing vasoconstriction, latest findings also demonstrate that inflammatory cells express RAS component AngII, which induces axon sprouting and nerve growth (Chakrabarty *et al.*, 2008; Cote *et al.*, 1999; Gendron *et al.*, 2002; Hoch *et al.*, 2009; Jurewicz *et al.*, 2007).

Recent research supports the hypothesis that the RAS, and especially AngII, may be considered as a locally-acting modulator of tissue homeostasis, regeneration and repair. AngII is markedly increased after wounding and induces the production of proinflammatory cytokines, such as IL-6 and TNF-α, and adhesion molecules. It also functions as a proinflammatory cytokine that regulates inflammation, growth and fibrosis (Ekholm *et al.*, 2015; Ekholm *et al.*, 2009; Han *et al.*, 1999; Moriyama *et al.*, 1995; Ruiz-Ortega *et al.*, 2002).

Morimoto and Price *et al*. were the first to describe local renin-angiotensin systems and their contribution to inflammation in the musculoskeletal system of rats (Morimoto *et al.*, 2013; Price *et al.*, 2007); mRNA was detected, as well as protein of several tRAS factors including ACE, AGT, CTSD, AGTR1 and AGTR2; renin remained undetermined. After stimulation with Ang II, cells in culture elevated cell proliferation and expression of genes such as *COL2*, *ACAN* and *ADAMTS5*. In the present work, expression of *AngII* in degenerated and symptomatic discs was confirmed indirectly by gene expression analysis of genes participating in Ang II synthesis and directly by immunohistochemistry. *CTSD* expression was identified in every IVD sample while *ACE* expression was undetected in 37 samples. This observation could be explained by different specificities of these two enzymes. CTSD was identified as a key enzyme in alternative AngII synthesis pathways in the human uterus as well as in rat ganglia and IVD (Hackenthal *et al.*, 1978; Morimoto *et al.*, 2013; Patil *et al.*, 2010). In the current study, AGT, CTSD, ACE and AGTR2 mRNAs were identified in the IVD, while renin expression remained below the detection limit. These findings suggest a possible signalling pathway of tRAS in human IVD tissue: the reaction from AGT to AngI could be catalysed by CTSD, AngI could be converted into AngII by ACE.

For tRAS-positive discs, analyses in Table 5 show hypertension and diabetes as co-morbidities. Patients having both conditions benefit from the use of ACE inhibitors for the management of high blood pressure. While these patients did not show less disc inflammation due to lesser concentration of converted AngII. These results indicate that other molecules, such as cathepsin D, may serve as an alternate mechanism for generating AngII and manifesting increased inflammation. Renin-like enzymes, such as CTSD, are proteinases that degrade collagens and proteoglycans and are known to catalyse the formation of Ang I (Genest *et al.*, 1983; Morimoto *et al.*, 2013). In other organs such as the liver, CTSD levels can help to predict paediatric hepatic inflammation (Walenbergh *et al.*, 2015). The catabolic role of CTSDs has also been investigated in damaged articular cartilage affected by osteoarthritis and rheumatoid arthritis (RA) (Keyszer *et al.*, 1995). Additionally, previous work by Ariga *et al*. provides evidence for the involvement of CTSDs in the degeneration of the human intervertebral disc. Interestingly, CTSD is typically found at the site of degeneration within the IVD (Ariga *et al.*, 2001). Proinflammatory cytokines are supposed to regulate the synthesis of cathepsins (Deiss *et al.*, 1996; Wang *et al.*, 2000). Furthermore, proinflammatory cytokines circulate in blood or macrophage lineage cells (Starkie *et al.*, 2000). Thus, synthesis of CTSD in degenerative discs may be regulated by the cytokines secreted by disc cells and/or macrophages infiltrated into discs by neovascularisation.

There are multiple reasons (location, tissue harvesting/preparation, *etc*.) to explain the relatively low ratio of tRAS positive samples among all analysed samples at gene expression level. Firstly, connective tissue such as IVD tissue has, in general, a very low expression of tRAS components compared with other tissues such as the gastrointestinal system (Web ref. 1). Furthermore, the state of degeneration and/or the type of tissue (NP or AF) that is harvested might influence the expression of tRAS in tissue. Morimoto and Price *et al*. (Morimoto *et al.*, 2013; Price *et al.*, 2007), as well as the current study, confirm the existence of an independent tRAS system in cartilage and disc tissue. If tRAS components are expressed by NP cells and are mostly located in NP tissue an underrepresentation might be observed, especially in the degeneration subgroup in the present study when the IVDs were highly fibrotic with almost no remaining NP tissue. Alternatively, low cell density in IVD tissue as well as variable mRNA stability may impair the detection of tRAS genes with low expression level by conventional qRT-PCR. Interestingly, immunohistochemistry results showed that AngII protein was present in all the analysed samples, including samples with negative tRAS gene expression status. This finding may be explained by the nature of the qualitative assessment of Ang II expression and/or alternative origin (other than ACE) and transport into the disc (Kramkowski *et al.*, 2006). Previous research provides evidence, that AngI may be converted to Ang-(1-9) by angiotensin-converting enzyme-related carboxypeptidase (ACE-II) followed by AngII in some tissues (Donoghue *et al.*, 2000; Drummer *et al.*, 1988; Kramkowski *et al.*, 2006; Singh *et al.*, 2005; Tipnis *et al.*, 2000). However, the enzymes catalysing this reaction are still unknown. Furthermore, several groups have indicated the existence of alternative pathways of

AngII biosynthesis such as *via* chymostatin-sensitive enzyme or chymase (Kinoshita *et al.*, 1991; Okunishi *et al.*, 1987).

In summary, the tRAS encompasses a highly complex and largely undiscovered system involving multiple pathways of AngII synthesis. RNA isolation from tissue samples with high proteoglycan content is particularly challenging. The techniques for RNA isolation from bovine disc tissue have been improved (Caprez *et al.*, 2018). However, these tissue samples were usually from 6-12 months old calves with higher cellularity than human surgical IVD samples.

The adult IVD is typically an avascular structure. However, in patients suffering discogenic pain and presenting with severely degenerated discs, the IVDs display neovascularisation around the outer AF. It is hypothesised that the genesis of discogenic pain, as well as the expression of proinflammatory cytokines, are influenced by the state of neovascularisation. The present study sought to determine if an independent tRAS is expressed in the human IVD tissue and if the expression of tRAS is associated with IVD inflammation. Hence, the aim was to reduce the risk of blood contamination of the tissue samples by systemic RAS components (by washing tissue with blood-cell-lysis buffer) to avoid false-positive results. On the other hand, intense intradiscal neovascularisation may be needed to detect tRAS components within IVD tissue. Finally, other alternative pathways such as the PPARγ pathway were shown to be involved in the expression of tRAS components in human IVDs (Liu *et al.*, 2019). tRAS driven inflammation is not solely influenced by Angiotensin II receptor type 1a but also regulated by the PPARγ receptor; which, when activated leads to an inhibition of the proinflammatory nuclear factor-kappa-β. Various inflammatory cytokines are expressed by disc cells themselves (mostly NP cells) (Rand *et al.*, 1997). However, CTSD was also detected in disc cells in areas without degeneration, neovascularisation or inflammation which is another argument for the PPAR-γ pathway (Liu *et al.*, 2019; Nachemson, 1969).

The current results also provide evidence for a correlation of tRAS with inflammatory and degenerative changes in IVD tissue. Hence, the study suggests that AngII may have the potential to influence IVD matrix degradation. The presence and contribution of the ACE and AT1 receptors to the inflammatory processes has recently been demonstrated in synovium samples from patients with RA (Price *et al.*, 2007; Walsh *et al.*, 2000; Walsh *et al.*, 1993). Furthermore, the current findings are supported by previous work showing increased tRAS activity found in blood monocytes, nodules, synovial fluid and synovial tissue of patients with RA (Goto *et al.*, 1990; Goto *et al.*, 1992; Veale *et al.*, 1992). Price *et al.* identified elevated expression of Ang I/II protein and AngII type 1 receptor, by Western blot and immunohistochemistry, in synovium from animals with induced acute and chronic joint inflammation (Price *et al.*, 2007).

Additionally, Morimoto *et al.* examined the biological role of AngII in rat annulus fibrosus cells in monolayer culture by real-time polymerase chain reaction (Morimoto *et al.*, 2013; Price *et al.*, 2007). Stimulation of rat IVD cells with AngII did not significantly alter mRNA expression of *IL-1β*, *MMP3*, and *MMP13* compared with the control group. However, mRNA expression of *ADAMTS-5* was significantly upregulated by stimulation due to AngII (Morimoto *et al.*, 2013).

In summary, along with other recent investigations on musculoskeletal tissues, the present work provided evidence that factors of the renin-angiotensin-system are likely to contribute to the inflammatory processes that are operant in IVD degeneration and therefore provide a novel therapeutic target to combat the disabling symptoms.

The imbalance between proinflammatory and anti-inflammatory cytokines plays an important role in the pathogenesis of IVD degeneration and many chronic inflammatory diseases such as RA. Recent data suggest that RAS inhibitors have anti-inflammatory effects in various tissues and organs and show specific beneficial effects in arthritis (Agha and Mansour, 2000; Dalbeth *et al.*, 2005; Refaat *et al.*, 2013; Sagawa *et al.*, 2005; Silveira *et al.*, 2013; Tang *et al.*, 2015; Wang *et al.*, 2013). In rats, administration of the AngII blocker, PD123319 was sufficient to block the mechanical hypersensitivity and the hyperinnervation caused by induced inflammation in the hind paw (Cote *et al.*, 1999). In the kidney, RAS inhibitors completely suppressed LPS-induced IL-6 and TNF-α mRNA levels (Niimi *et al.*, 2002). Therefore, tRAS may be a potential therapeutic target in IVD degeneration induced by inflammatory triggers. Certainly, the current observations are indicative and associated with several limitations such as limited sample size and lack of healthy controls. For this proof of concept trial, human IVD tissue from patients undergoing spinal fusion was used. Study inclusion was conducted by principal diagnosis and not by the state of degeneration. The lack of a healthy control group is therefore not an issue in this study but an intrinsic limitation of surgical research. A scientifically correct control group would be IVDs harvested from young and healthy spines. However, for multiple reasons (mostly ethical), problems in finding true control groups in surgical research are a permanent, intrinsic, but well-known clinical limitation whenever trials are performed on humans. On the other hand, this research is much more relevant than any animal model. The best available control groups in our study were trauma and scoliosis patients as these patient groups were typically younger and presented less signs of IVD degeneration.

Therefore, as an MRI was available the state of endplate/IVD degeneration was assessed and results correlated with all other parameters. There were no

significant correlations between endplate and/or IVD degeneration and other clinical, radiological, laboratory or biochemical parameters. Furthermore, the disc tissue could include neighbouring structures. Specifically, the function of tRAS in the pathological process of human IVD degeneration needs to be further clarified.

Conclusions

For the first time, the existence of tRAS in the human IVD was confirmed. The present study provided evidence that tRAS is likely to contribute to the inflammatory process that is instrumental in IVD degeneration. The absence of renin expression strengthened the hypothesis that CTSD is a renin-like-enzyme. RAS components may serve as a novel target for inhibition of inflammation and degeneration in IVD tissue. Future studies will investigate whether a local inhibition of tRAS components might slow down the inflammatory progression of IVD degeneration and relieve pain.

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Discussion with Reviewers

Reviewer: Within the degeneration group, there is a very high variability of relative gene expression levels of tRAS factors. Is there any correlation with the degree of degeneration within this group?
Authors: A correlation test was performed on the samples, which has a Modic score, Pfirrmann grade and pain level, in order to investigate if any of these degeneration scores are correlated with the tRAS gene expression level. No significant differences were observed between tRAS expressing and non-expressing patients on the Modic score, Pfirrmann grade or pain level.

Reviewer: Will all the patients with IVD degeneration benefit from potential therapies developed around the renin-angiotensin system in the IVD? Not all degenerating discs are tRAS positive in the study.
Authors: As already stated in the Discussion, the imbalance between proinflammatory and anti-inflammatory cytokines plays an important role in

pathogenesis of symptomatic disc degeneration and many other chronic inflammatory diseases such as RA. Various studies have shown that drugs blocking the renin–angiotensin–aldosterone system may offer beneficial effects in addition to decreasing blood pressure on several TNF-α-driven inflammatory diseases such as IDD (Agha and Mansour, 2000; Dalbeth *et al.*, 2005; Refaat *et al.*, 2013; Sagawa *et al.*, 2005; Silveira *et al.*, 2013; Tang *et al.*, 2015; Wang *et al.*, 2013). RAS inhibitors belong to the most commonly prescribed drugs globally (Stagnitti, 2001). A couple of years ago an attempt was made to extract data from German health insurances to analyse whether patients taking RAS inhibitors were less likely to be affected by discogenic back pain. Unfortunately, due to data security issues, it was not possible to access the data. Probably, centralised Scandinavian health-care registries might offer the opportunity to find answers to this question. We do believe that it is unlikely, that all patients taking RAS inhibitors do not experience discogenic back pain considering the high numbers of patients taking RAS inhibitors. Consequently, local inflammation triggered by tRAS might also be enhanced by alternative intradiscal pathways. The absence of renin expression in the present study strengthened the hypothesis that CTSD was a renin-like-enzyme.

Reviewer: In the tRAS positive group, the degenerative disc group show lower expression of all cytokines and proteolytic enzymes. Any thoughts on the mechanism of disc degeneration affecting this; low natural degeneration *vs.* single high impact force traumatic injury?
Authors: This is an interesting point. The stronger proinflammatory response of trauma discs is most likely explained by the single event of trauma. It is known that expression of pro-inflammatory cytokines and proteolytic enzymes can be up-regulated as a response of post-traumatic changes in a one strike injury model (Additional reference: Alkhatib *et al.*, 2014).

Reviewer: Will the tRAS system in adjacent critical tissue structures, such as endplates, be of interest for future studies to elucidate the involvement of this system in the overall mechanism of IVD degeneration?
Authors: Absolutely. It is insufficient to spotlight the disc without targeting the endplates, in order to combat discogenic pain. Capillaries penetrating the permeable endplate guarantee nutrient delivery and waste exchange by means of diffusion in a healthy IVD. However, in the process of degeneration, waste exchange is typically altered, causing enhanced degeneration through accelerated ECM catabolism, inflammation and neovascularisation, which ultimately leads to discogenic pain. The exact mechanisms of the above-mentioned phenomenon, including the involvement of the tRAS, need to be elucidated in future research.

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