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Inflammaging in cervical and lumbar degenerated intervertebral discs: Analysis of proinflammatory cytokine and TRP channel expression

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Abstract

Purpose To investigate and compare the occurrence of inflammatory processes in the sites of disc degeneration in the lumbar and cervical spine by a gene array and subsequent qPCR and to investigate the mechanistic involvement of transient receptorpotential channels TRPC6 and TRPV4.

Methods The gene expression of inflammatory cytokines and TRP channels was measured in human disc samples obtained from patients undergoing discectomy at the cervical (n = 24) or lumbar (n = 27) spine for degenerative disc disease (DDD)
and disc herniation (DH) and analyzed for differences with regard to spinal level, IVD degeneration grade, Modic grade, age, sex, disc region and surgical extent.

Results Aside from genes with known implication in DDD and DH, four previously unreported genes from the interferon and TRP families (IFNA1, IFNA8, IFNB1, TRPC6) could be detected. A correlation between gene expression and age (IL-15)
and IVD degeneration grade (IFNA1, IL-6, IL-15, TRPC6), but not Modic grade, was identified. Significant differences were detected between cervical and lumbar discs (IL-15), nucleus and annulus (IL-6, TNF-α, TRPC6), single-level and multi-level surgery (IL-6, IL-8) as well as DDD and DH (IL-8), while sex had no effect. Multiple gene-gene pair correlations, either between different cytokines or between cytokines and TRP channels, exist in the disc.

Conclusion This study supports the relevance of IL-6 and IL-8 in disc diseases, but furthermore points toward a possible pathological role of IL-15 and type I interferons, as well as a mechanistic role of TRPC6. With limited differences in the inflammatory profile of cervical and lumbar discs, novel anti-inflammatory or TRP-modulatory strategies for the treatment of disc pathologies may be applicable independent of the spinal region.

Keywords Cervical and lumbar discs · Degenerative disc disease (DDD) and disc herniation (DH) · Inflammaging ·Inflammation · Intervertebral disc · Transient receptor potential (TRP) channel

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Introduction

The intervertebral disc (IVD) is a unique structure that lies between adjacent vertebrae in the vertebral column. It consists of an outer fibrous ring, the annulus fibrosus (AF), rich in collagen type I, a gel-like nucleus, the nucleus pulposus the nucleus pulposus (NP), rich in collagen type II, and cartilaginous end plates [1].

Disc degeneration (DDD) is an age-related process that occurs early in life and is associated with dehydration and fibrosis of the NP, as well as formation of tears and clefts in the AF. Factors known to be associated with DDD include mechanical loading, genetics and nutritional deprivation [2]. Certain individuals experience age-related chronic inflammation of the IVD, termed “inflammaging”, which is characterized by up-regulation of proinflammatory cytokines such as Interleukin-6 (IL-6) [3]. Furthermore, osteophytes and spondylosis can develop, and the posterior longitudinal ligament and the ligamentum flavum may bulge into the spinal canal [1, 4]. On the molecular level, degenerative processes in the disc are characterized by a shift in the collagen synthesis profile and increased expression of catabolic enzymes, such as matrix metalloproteinases (MMP), with a subsequent loss of proteoglycans [1]. The proteolytic disintegration of aggrecan, which is promoted by cytokine-regulated aggrecanases (ADAMTS-4 and -5) [4, 5], reduces the disc’s barrier function to nerve ingrowth [6, 7]. Microvessels can also invade the degenerating disc, increasing the production of the neutrophin nerve growth factor (NGF) and thus the incidence of neural structures within the tissue [7].

While disc degeneration is asymptomatic in the majority of cases [8], it is associated with low back and neck pain in a subpopulation. In fact, 84% of the population suffers from low back pain at some point in their lifetime [9] and vertebral endplate changes, so-called Modic changes, are thought to contribute to pain development [10]. The occurrence of Modic changes is not only related to traumatic injury, low-grade bacterial infection and genetics, but possibly also to localized inflammation, with a recently identified pro-inflammatory crosstalk between bone marrow and IVDs [10-12]. The importance of IVD inflammation is further supported by research describing the nociceptive role of abnormal levels of proinflammatory molecules secreted by NP and AF cells as well macrophages, T cells and neutrophils [13]. Proinflammatory cytokines not only irritate invading nerve endings directly, but can also promote the expression of NGF from IVD cells, thus potentially explaining the higher neutrophin levels in symptomatic disc degeneration [14]. NGF is known to stimulate the expression of acid-sensing ion channel 3 in dorsal root ganglion (DRG), thus further promoting ischemic and inflammatory pain [15, 16]. Additionally, secreted cytokines can worsen disc pathology through initiation of autophagy, senescence, apoptosis and induction of catabolic processes [13, 15].

Based on these findings, it has recently been hypothesized that inflammatory cytokines have a potential impact on DDD and the subsequent symptomatology [17-19]. Interestingly, a superfamily of cation selective transmembrane receptors, the so-called Transient Receptor Potential (TRP) channels, have emerged as potential contributors to DDD and discogenic pain. TRP channels, which we have previously shown to be expressed in the IVD, are multimodal ion channels regulated by a diverse range of stimuli. Dysregulation of several TRP channels, including TRPV4 and TRPC6, was shown to affect inflammatory, mechanical and osmotic sensitivity in different cell types, with a pathological and nociceptive role in numerous tissues and organs [20, 21]. Although little information exists to date on TRP channels in the IVD, changes in their expression or activity may promote degeneration, inflammation and pain and hence be of mechanistic relevance in disc inflammaging.

As the majority of research on disc pathology and molecular treatment options has been conducted on lower back pain and lumbar disc tissue, very limited data exist on the molecular mechanisms of symptomatic cervical DDD. Furthermore, the mechanisms leading to IVD inflammaging are in general not well understood. However, to promote the development of novel, molecular therapies for lumbar and/or cervical disc disease, a better insight into both aspects is crucial. Therefore, the purpose of this study was to analyze and compare the occurrence of inflammatory processes and the possible involvement of TRP channels in the sites of disc degeneration in the lumbar and cervical spine.

Materials and methods

Sample Collection

A total of 51 disc samples were obtained from 45 patients [18 men, 27 women, mean age = 52 (age 16–79 years)] undergoing elective spinal surgery in the cervical (n = 24) or lumbar (n = 21) region. Cervical samples were collected as entire discs due to the tissue size. Lumbar discs were intraoperatively excised as NP (n = 15) and/or AF (n = 12) samples, followed by macroscopic tissue evaluation. 22 patients suffered from disc herniation and 23 from degenerative disc disease. 26 individuals underwent one level discectomy and 19 multi-level discectomy. Assessment of the disease state was performed using Pfirrmann grading (IVD degeneration) and Modic grading (endplate changes). Informed consent for sample collection was obtained from each patient and the study was approved through the local ethics committee (Ethics Committee of the Canton Lucerne/Switzerland, #1007). Detailed patient information is given in Table 1.

Table 1 Patient Information

Nr.	Level	Age	Sex	Pathology	Pfirrmann Grade	Modic Grade	Extent	Experiment
1	C6/7	33	m	DH	3	0	SL	qPCR
2	C5/6	50	m	DH	4	0	SL	qPCR
3	C5-7	40	f	DDD	4	1	ML	Array
4	C4-7	78	f	DDD	5	2	ML	Array
5	C5-7	64	m	DH	4	0	ML	qPCR
6	C4/5	74	m	DDD	4	1	SL	qPCR
7	C4/5	68	m	DDD	4	0	SL	qPCR
8	C5-7	75	f	DDD	5	1	ML	qPCR
9	C7/Th1	45	m	DDD	2	0	SL	qPCR
10	C4-7	71	f	DH	3	2	ML	Array
11	C4-7	54	f	DDD	4	1	ML	qPCR
12	C5-7	52	f	DDD	4	1	ML	qPCR
13	C3/4, C6/7	65	f	DDD	4	1	ML	qPCR
14	C6/7	60	f	DDD	4	0	SL	qPCR
15	C5-7	46	m	DDD	3	0	ML	qPCR
16	C6/7	49	f	DH	2	0	SL	qPCR
17	C5-7	66	m	DH	5	1	ML	qPCR
18	C5-7	57	f	DH	4	0	ML	qPCR
19	C4-6	58	f	DH	4	0	ML	qPCR
20	C4/5	47	f	DDD	3	0	SL	qPCR
21	C4-6	52	f	DDD	4	1	ML	qPCR
22	C5-7	77	f	DDD	4	0	ML	qPCR
23	C4-7	61	f	DDD	5	0	ML	qPCR
24	C3-7	79	m	DH	4	2	ML	qPCR
25*	L4/5	30	m	DDD	2	0	SL	qPCR
26*	L5/S1	46	f	DH	3	1	SL	Array
27*	L5/S1	34	m	DH	3	1	SL	qPCR
28*	L5/S1	46	f	DH	5	3	ML	qPCR
29*	L4/5	46	f	DH	3	0	SL	Array
30*	L5/S1	59	f	DDD	5	0	SL	Array
31	L5/S1	62	f	DDD	5	0	SL	qPCR
32	L4/5	66	f	DH	2	2	SL	qPCR
33	L5/S1	53	m	DH	2	1	SL	qPCR
34	L4/L5	59	m	DH	2	1	SL	qPCR
35	L4/L5	52	m	DDD	3	1	SL	qPCR
36	L4/L5	64	f	DDD	4	2	ML	qPCR
37	L4/L5	76	f	DH	3	2	SL	qPCR
38	L4/L5	16	f	DH	3	1	SL	qPCR
39	L4/5, L5/S1	31	m	DDD	4	2	ML	qPCR
40	L5/S1	54	f	DH	2	1	SL	qPCR
41	L5/S1	33	m	DH	2	1	SL	qPCR
42	L4/5	70	f	DDD	4	2	SL	qPCR
43	L5/S1	39	m	DH	3	1	SL	qPCR
44	L5/S1	28	m	DH	2	1	SL	qPCR
45	L4/5	21	f	DDD	3	2	SL	qPCR

C = cervical, L = lumbar, f = female, m = male, DDD = degenerative disc disease, DH = disc herniation, SL = single level surgery, ML = multi-level surgery, * = two samples (NP and AF) collected from the patient. The grade of disc degeneration is indicated as Pfirrmann Grade.

RNA Isolation

RNA was extracted by TRIzol/chloroform method, followed by affinity-based purification. Briefly, samples were shock frozen in liquid nitrogen and pulverized using custom-made grinders. The tissue powder was transferred into TRIzol (1 ml per 200 mg tissue, 15596018, Thermo Scientific) and the sample was further homogenized with a Polytron three times for 20 s (POLYTRON® PT 10/35 GT), with cooling in between. After 5 min of incubation, homogenized samples were vortexed, centrifuged (4 °C, 12'000g, 10 min) to remove tissue debris and the supernatants were supplemented with chloroform (1 part chloroform to 5 parts sample). After vortexing, phase separation was allowed (RT, 5 min), samples were centrifuged (4 °C, 12'000g, 15 min), the aqueous phase was transferred, mixed with 70% ethanol (1:1 ratio) and RNA was subsequently purified by the RNeasy Mini Kit (74104, Qiagen) following the manufacturer's recommendation. RNA was eluted in 30 µl of RNAsefree water. The quality and quantity of RNA was quantified using a Nanodrop (Thermo Fisher), specifically controlling the 260/280 and 260/230 ratio.

Gene Expression Analysis

Two micrograms of RNA was used to synthesize cDNA in a total volume of 60 µl, using the reverse transcription kit (4374966, Applied Biosystems). For samples with lower yields, the reverse transcription was conducted at reduced concentrations. To identify the most relevant cytokines, three cervical samples and three lumbar samples (2× AF, 1× NP) were used for the gene expression screening with the TaqMan Array Human Cytokine Network (4418769, Applied Biosystems) according to the protocol provided by the manufacturer. Briefly, 5 µl of TaqMan Fast Universal PCR Master Mix (4352042, Applied Biosystems) and 5 µl of cDNA (10 ng, diluted in RNAse-free water) were added to 96-well plates precoated with the respective TaqMan primers/probes and gene expression was measured by the real-time qPCR (CFX96 Touch™ Detection System, Biorad). Each array constituted of 28 cytokines: IFNA1, IFNA16, IFNA17, IFNA2, IFNA6, IFNA7, IFNA8, IFNB1, IFNG, IL-1A, IL-1B, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL- 12A, IL-12B, IL-13, IL-15, IL-16, IL-17A, IL-18, LTA and TNF-α, and four housekeeping genes: 18S, GAPDH, HPRT1 and GUSB.

Based on the array gene expression results, IFNA1, IFNA8, IFNB1, IL-1B, IL-6, IL-8, IL-15, TNF-α, as well as the housekeeping gene GAPDH were selected for further gene expression analysis on the large set of samples. Additionally, two candidates of the TRP family, TRPC6 and TRPV4, were also included due to a potential mechanistic role in disc disease that has emerged recently. Gene expression was quantified using human TaqMan primers on all remaining 45 samples. Briefly, 5.5 µl of TaqMan Fast Universal PCR Master Mix and TaqMan primers (Table 2) were mixed with 4.5 µl of cDNA and quantified using qPCR as previously described [16]. Importantly, to ensure comparability to initially

measured samples identical primers were used as in the gene array. The qPCR and the array data were pooled and the obtained Ct values were analyzed by comparative method (gene of interest relative to GAPDH) and displayed as 2^{-dCt} values.

Table 2 TaqMan primers used for qPCR analysis.

Gene	Gene Class	Primer Number
GAPDH	Internal Control	Hs02758991_g1
IFNA1	Interferon	Hs00855471_g1
IFNA8	Interferon	Hs00266883_s1
IFNB1	Interferon	Hs01077958_s1
IL-1B	Interleukin	Hs00174097_m1
IL-6	Interleukin	Hs00174360_m1
IL-8	Interleukin	Hs00358796_g1
IL-15	Interleukin	Hs01003716_m1
TNF-a	Tumor necrosis factor	Hs01045114_g1
TPRC6	Transient receptor potential channel subfamily C, member 6	Hs00989190_m1
TRPV4	Transient receptor potential channel subfamily V, member 4	Hs01099348_m1

Statistical Analysis

Data consistency was checked and data were screened for outliers by using quantile plots and normality using Kolmogorov–Smirnov test. Due to the given distributions, generalized linear models were not applicable and, hence, Wilcoxon-matched pairs test, Mann–Whitney U test and Spearman correlation were used to analyze continuously distributed data. Cross tabulation tables with Fisher's exact test, Pearson's Chi square test, marginal homogeneity test and McNemar's test were used to analyze cross tabulation tables. One-factorial ANOVA with unpaired and two-sided Student's *t* tests as post hoc tests were used to test means among different groups. Whisker plots with medians and 25% and 75% quantiles as well as scatter plots were used to illustrate the results. All reported tests were two sided, and p values < 0.05 were considered to be statistically significant. For the comparison of lumbar and cervical gene expression patterns, the entire data set as well as the patient matched data set was analyzed. Matching was conducted with regard to IVD degeneration grade, age, surgical extent and pathology, with the highest focus on IVD degeneration grade and subsequently decreasing in importance. All statistical analyses in this report were performed using STATISTICA 13 (Hill, T. & Lewicki, P. Statistics: Methods and Applications. StatSoft, Tulsa, OK) and PASW 22 (IBM SPSS Statistics for Windows, Version 21.0., Armonk, NY) and StatXact 10 (Cytel Software 2013, Cambridge MA, USA).

Results

Expression levels: Overview

In the initial experiment, six IVD samples were analyzed for 28 cytokines by gene array. Based on the results, 12 out of 28 cytokines (IL-3, IL-4, IL-5, IL-9, IL-13, IL-17A, IL-1A, IL- 12B, LTA, IFNA16, IFNA17, INFA2) were excluded from further analysis due to low or undetectable gene expression in the majority of samples. Table 3 shows the measured dCt values for the remaining 16 cytokines in the six tested samples normalized to GAPDH. The cytokines IFNA1, IFNA8, IFNB1, IL-1B, IL-6, IL-8, IL-15 and TNF-α, and the calcium channels TRPC6 and TRPV4, were selected for qPCR on all remaining samples. The selection was made based on indicative differences in the IFNA8 and IFNB1 gene expression between the cervical and lumbar samples, the novelty of the candidates IFNA1, IFNA8, IFNB1, IL-15, TRPC6 and TRPV4, as well as the relevance of IL-1B, IL-6, IL-8 and TNF-α in the scientific literature.

Table 3 Results of the gene array study (dCt values) of the 16 well expressed cytokines and indication of those genes selected for subsequent qPCR analysis

Gene	**Patient 3** *Cervical*	**Patient 4** *Cervical*	**Patient 10** *Cervical*	**Patient 25** *Lumbar AF*	**Patient 28** *Lumbar AF*	**Patient 26** *Lumbar NP*	Final Choice
IFNA1	9.07	9.10	7.98	9.81	10.96	9.47	x
IFNA6	7.79	8.10	7.72	13.66	11.95	14.14	
INFA7	6.58	6.93	7.25	13.02	11.79	13.11	
IFNA8	8.12	8.45	8.28	11.32	11.81	12.31	x
IFNB1	8.60	8.71	8.07	13.19	11.75	14.56	x
IFNG	10.64	10.55	11.53	11.26	16.17	14.52	
IL-1B	6.02	6.00	6.59	6.33	10.04	8.28	x
IL-2	12.19	10.96	12.26	15.31	13.62	16.15	
IL-6	5.59	6.77	8.16	5.64	7.27	10.67	x
IL-8	1.43	4.54	8.05	4.61	9.88	6.35	x
IL-10	6.06	7.04	9.11	5.08	8.46	9.97	
IL-12	9.28	8.89	9.40	9.83	11.15	11.28	
IL-15	11.85	7.37	8.51	6.90	7.44	11.43	x
IL-16	3.93	3.73	3.27	5.06	4.41	5.98	
IL-18	6.80	5.23	6.24	6.86	8.19	10.51	
TNF-α	8.42	8.39	9.62	9.67	10.10	11.57	x

When combining data from all 51 samples IL-8 and TRPV4 were found to have the highest expression, followed by IFNA8. The three previously unreported interferons IFNA1, IFNA8 and IFNB1, and the mechanosensitive channel TRPC6 were detected at levels similar to IL-6 and IL-1B ($p > 0.05$), and were higher than that of TNF-α ($p < 0.001$). Additionally, significant differences were found between the interferons themselves. Interestingly, all investigated genes except IL-15 ($p = 0.71$) were expressed at significantly higher levels than TNF-α ($p < 0.001$), which has

been extensively investigated in the published literature and is often used for cell stimulation experiments. Although a high variability was observed in the mRNA levels of IL-15, possibly due to expression differences between the cervical and the lumbar spine (see Fig. 4), its expression was significantly different from numerous other genes, including TRPV4 (p>0.001). Supplementary Table 1 summarized the statistical results.

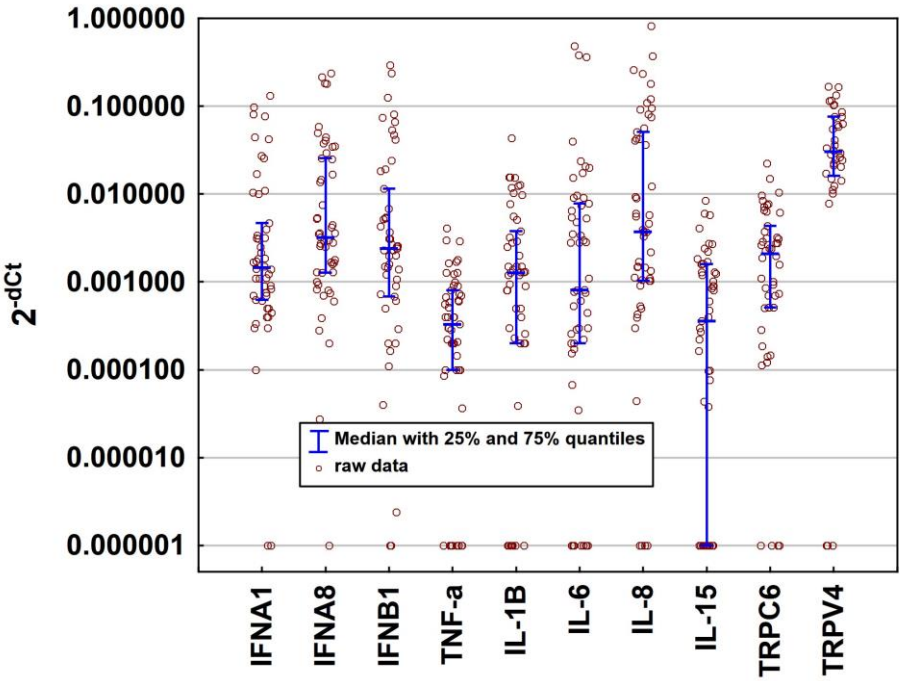

Fig. 1 Gene expression of the selected cytokines and TRP channels in all specimens (n = 51, 24× cervical, 15× lumbar AF, 12× lumbar NP), calculated as 2^{-dCt} values (relative to GAPDH)

Expression levels: correlation with age, degeneration grade and modic grade

In a subsequent step, we tested for correlations between gene expression and age, IVD degeneration grade and Modic grade on the entire set of samples. IL-15 mRNA levels were shown to positively correlate with age (n = 51, r = 0.49, p = 0.0003, not shown) and the IVD degeneration grade (n = 51, r = 0.29, p = 0.038, Fig. 2a). Additionally, a significant correlation was found between the IVD degeneration grade and the expression of IL-6 (n = 51, r = 0.36, p = 0.009, Fig. 2b), IFNA1 (n = 50, r = − 0.28, p = 0.0493, Fig. 2c) and TRPC6 (n = 46, r = 0.30, p = 0.045, Fig. 2d). None of the investigated genes correlated with the Modic grade (data not shown). Furthermore, age and IVD degeneration grade were correlated (n = 51, r = 0.49, p = 0.0003, Supplementary Fig. 1).

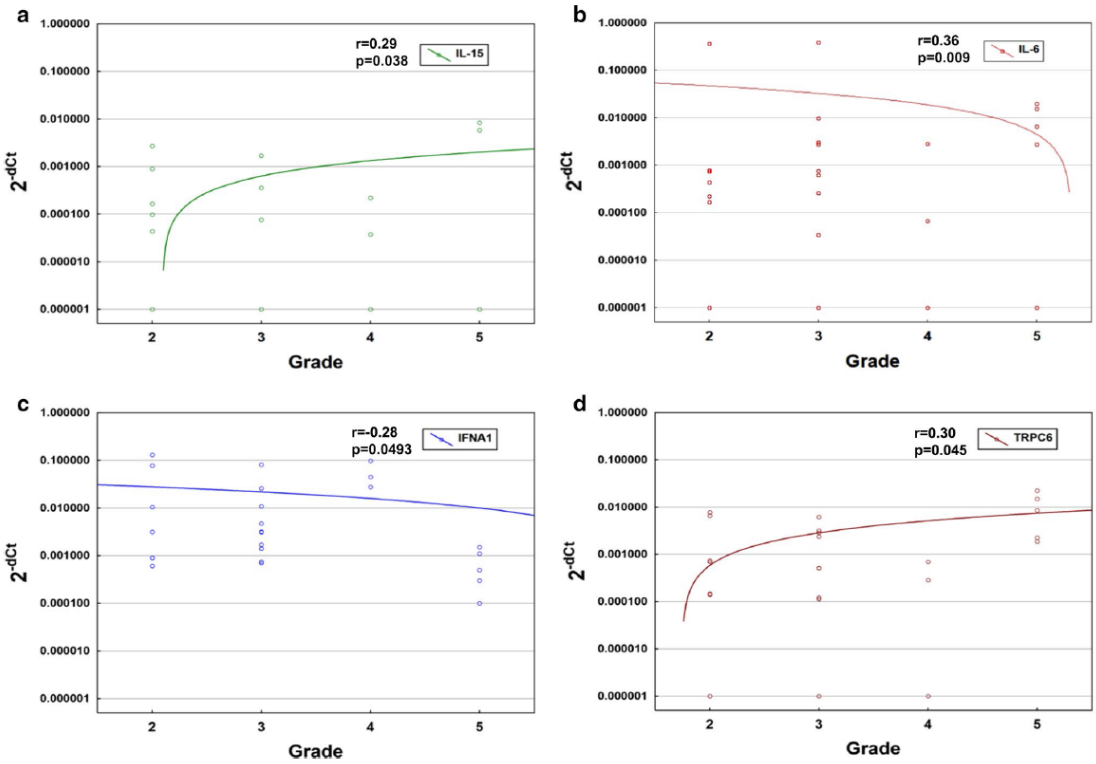

Fig. 2 Correlations between the gene expression of IL-15 (a), IL-6 (b), IFNA1 (c) and TRPC6 (d) and the IVD degeneration grade. Results were calculated as 2^{-dCt} values (relative to GAPDH). Note: gene expression is plotted on a logarithmic scale

Expression levels: differences with extent of surgery, pathology and gender

The extent of the surgery (single level versus multi-level) was found to affect the expression of the cytokines IL-6 ($p = 0.006$) and IL-8 ($p = 0.041$), with both being expressed to a higher level in the samples obtained from multi-level surgeries (Fig. 3). Furthermore, IL-8 expression was influenced by the underlying pathology (DDD versus DH), with higher levels in the DDD samples ($p = 0.016$, not shown). However, analyzing gene expression in relation to pathology is hampered by the fact that it is difficult to clearly distinguish between DDD and DH in the included patients. None of the investigated genes differed between males and females (data not shown).

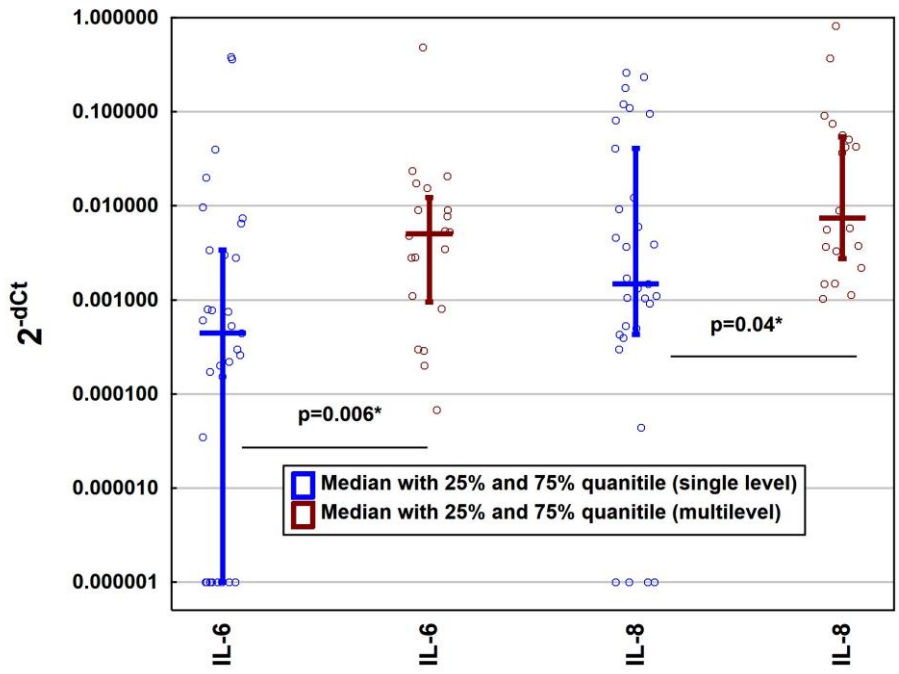

Fig. 3 Statistically significant differences in the gene expression of IL-6 and IL-8 between patients undergoing single-level (n = 31) and multi-level (n = 20) discectomy. Results were calculated as 2^{-dCt} values (relative to GAPDH)

Expression levels: differences in the spinal region (cervical/lumbar)

When comparing the gene expression in the cervical and lumbar samples over the entire sample population (n = 51), significant differences were found for the cytokines IFNA1 (cervical < lumbar, $p = 0.038$), IL-6 (cervical > lumbar, $p = 0.0375$), IL-15 ($p = 0.0001$, cervical > lumbar) and the ion channel TRPC6 ($p = 0.045$, cervical > lumbar) (Fig. 4a). As these genes were also found to be regulated by the IVD degeneration grade (IL-15, IL-6, IFNA1, TRPC6), age (IL-15) and surgical extent (IL-6), we aimed to further elucidate whether differences were truly related to spinal region or confounded by dissimilarities in age and IVD degeneration grade in the cervical and lumbar group, using a sample-matching approach. When analyzing 28 well-matched samples (14× cervical and 14× lumbar), we were able to confirm the difference in IL-15 expression in the cervical and lumbar IVDs ($p = 0.013$, Fig. 4b), whereas the difference in IFNA1 expression did not reach significance in this grouping ($p = 0.056$, data not shown), possibly due to lower statistical power arising from the reduced sample size. IL-6 and TRPC6 showed no differences after matching was conducted.

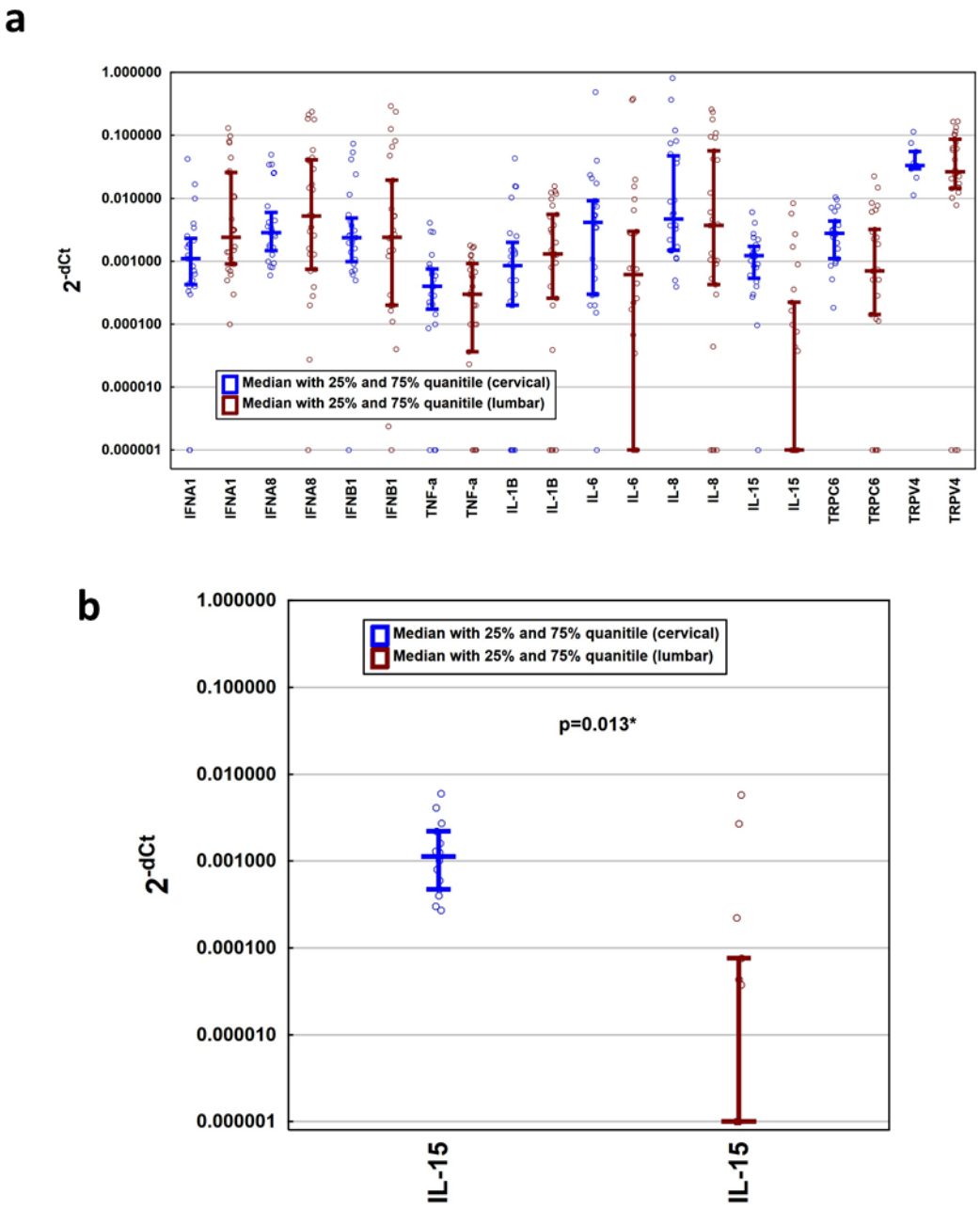

Fig. 4 Differences in the gene expression between cervical (n = 24) and lumbar (n = 27) samples (entire sample population), with statistically significant results for IFNA1, IL-6, IL-15 and TRPC6 (a) and confirmation of statistical difference in the gene expression of IL-15 between well-matched cervical (n = 14) and lumbar (n = 14) samples (b). Results were calculated as 2^{-dCt} values (relative to GAPDH)

Expression levels: differences in the disc region (NP/AF)

In lumbar samples, a comparison between AF and NP tissue revealed significantly higher expression of IL-6 (p = 0.0236, Fig. 5a), TRPC6 (p = 0.014, Fig. 5b) and TNF-α (p = 0.047, Fig. 5c) in the AF. Due to the small size of cervical tissue, NP and AF could not be separately analyzed.

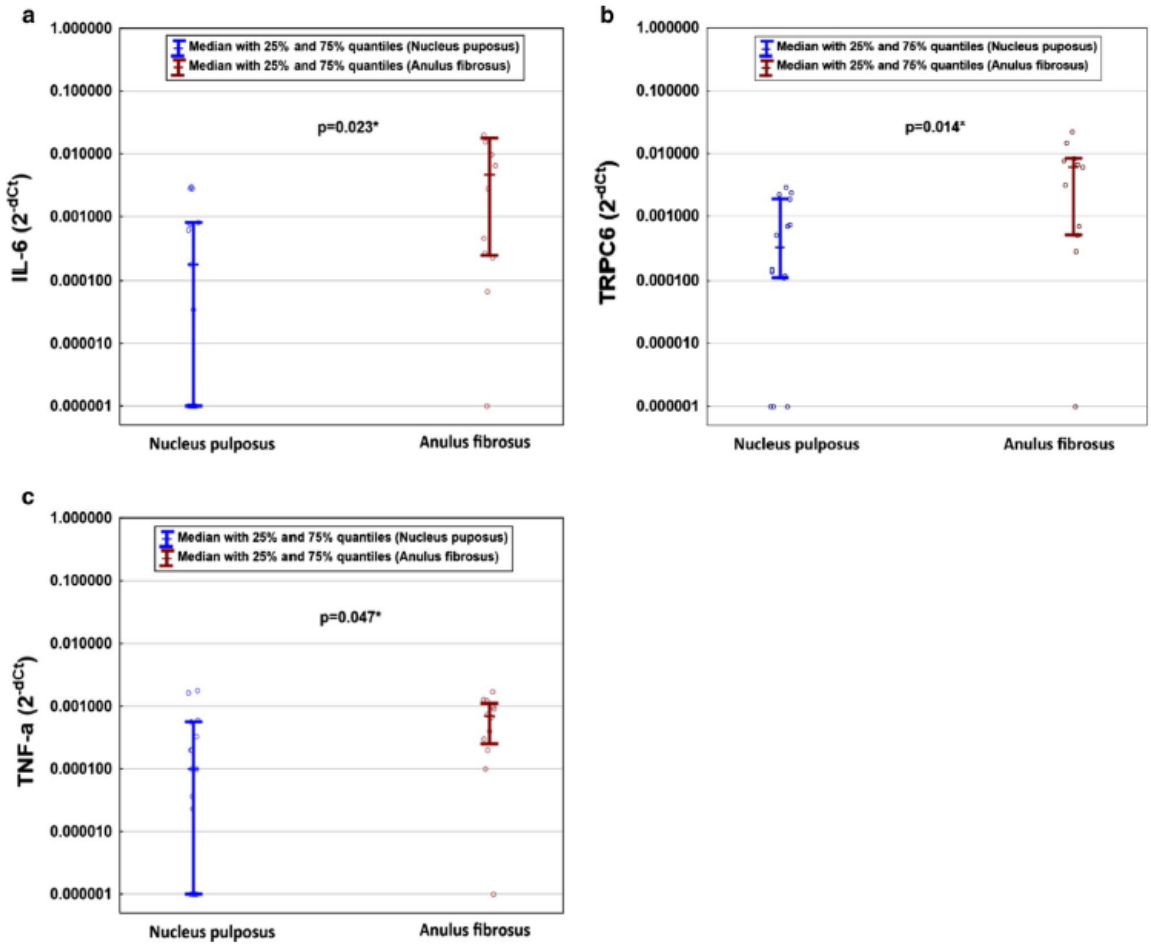

Fig. 5 Statistically significant differences in the gene expression of IL-6 (a), TRPC6 (b) and TNF-α (c) between AF (n = 12) and NP (n = 15) samples in lumbar discs. Results were calculated as 2^{-dCt} values (relative to GAPDH)

Expression levels: correlation between genes

To test whether the expression of target genes is intra-correlated, a Spearman correlation test was conducted on the entire data set. Numerous genes were found to be correlated (for all results see Table 4), with the strongest correlation for the gene pairs IFNA8 and IFNB1 (n = 51, $r = 0.93$, $p < 0.0001$, Fig. 6a), IL-6 and IL-8 (n = 51, $r = 0.81$, $p < 0.0001$, Fig. 6b), IFNA1 and IFNB1 (n = 50, $r = 0.78$, $p < 0.0001$, Fig. 6c), IL-1B and TNF-α (n = 51, $r = 0.78$, $p < 0.0001$, Fig. 6d), IFNA1 and IFNA8 (n = 50, $r = 0.74$, $p < 0.0001$, Fig. 6e), IL-15 and TRPC6 (n = 46, $r = 0.64$, $p < 0.0001$, Fig. 6f), IL-6 and TRPC6 (n = 46, $r = 0.59$, $p < 0.0001$, Fig. 6g), IFNA8 and TRPC6 (n = 46, $r = -0.57$, $p < 0.0001$, Fig. 6h), and IL-1B and IL-8 (n = 51, $r = 0.56$, $p < 0.0001$, Fig. 6i).

Table 4. Significant intra-correlations between candidate genes, calculated by Spearman correlation test.

Gene pairs	Sample number	Spearman's *r*	p-value
IFNA1 and IFNA8	50	0.78	< 0.00001
IFNA1 and IFNB1	50	0.74	< 0.00001
IFNA1 and TRPC6	45	-0.46	0.002
IFNA1 and TRPV4	35	-0.46	0.005
IFNA8 and IFNB1	51	0.93	< 0.00001
IFNA8 and TNF-α	51	-0.30	0.03
IFNA8 and TRPC6	46	-0.57	< 0.00001
IFNB1 and IFNA1	50	0.74	< 0.00001
IFNB1 and TRPC6	46	-0.44	0.002
IFNB1 and TRPV4	36	-0.34	0.04
TNF-α and IL-1B	51	0.78	< 0.00001
TNF-α and IL-6	51	0.53	0.00007
TNF-α and IL-8	51	0.48	0.0004
TNF-α and IL-15	51	0.49	0.0002
TNF-α and TRPC6	46	0.44	0.002
IL-1B and IL-6	51	0.44	0.001
IL-1B and IL-8	51	0.56	0.00002
IL-1B and TRPC6	46	0.39	0.008
IL-6 and IL-8	51	0.81	< 0.00001
IL-6 and IL-15	51	0.38	0.006
IL-6 and TRPC6	46	0.59	0.00002
IL-8 and TRPC6	46	0.44	0.002
IL-15 and TRPC6	46	0.64	< 0.00001

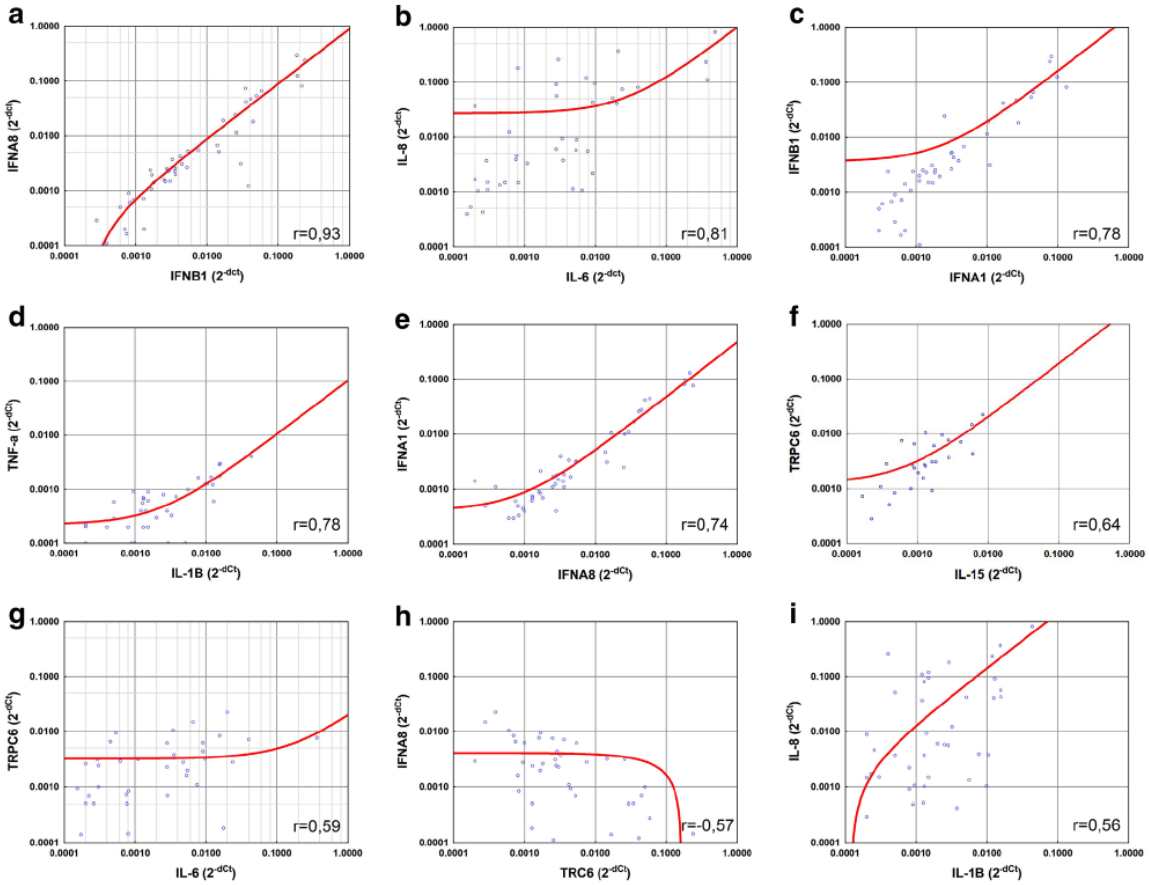

Fig. 6 Statistically significant correlations between the expression of IFNA8 and IFNB1 (a), IL-6 and IL-8 (b), IFNA1 and IFNB1 (c), IL-1B and TNF-α (d), IFNA1 and IFNA8 (e), IL-15 and TRPC6 (f), IL-6 and TRPC6 (g), IFNA8 and TRPC6 (h) as well as IL-1B and IL-8 (i). Results were calculated as 2^{-dCt} values (relative to GAPDH)

Discussion

Although a major effort has been made over the past decade to better understand degenerative disc disease, the underlying pathogenesis is still poorly understood. Recent findings point toward a possible role of inflammation in the development of discogenic back pain [19, 22]. However, this is - to our knowledge - the first study to compare the inflammatory process and mechanisms in the cervical and lumbar spine. Importantly, a large number of cytokines, partially with thus far unidentified markers, was employed to not only provide novel insights for cervical, but also lumbar disc pathology.

According to our results, the most highly expressed genes in the pathogenic lumbar and cervical samples were the cytokine IL-8 and the TRPV4 channel, whereas the cytokines IL-1B and specifically TNF-α, which are commonly investigated in disc pathologies, were expressed at significantly lower mRNA levels. The TRPV4 channel is

one of the six member of the TRP Vanillin (TRPV) family. In IVD cells and chondrocytes, TRPV4 administers transduction of osmotic and mechanical signals and may play a role in inflammatory joint swelling [23]. Furthermore, it was recently shown that degeneration of the IVD, with an associated drop in proteoglycan content and hence tissue osmolarity, not only enhances the expression of the cytokines IL-1B and IL-6, but also of the TRPV4 channel [24]. The other highly expressed gene in our dataset, IL-8, is a chemokine that belongs to the CXC subfamily and is secreted by multiple cell types in response to inflammatory stimuli especially during acute phases [25]. IL-8 is known to induce hyperalgesia by evoking the local production of sympathetic amines that sensitize nociceptors [26]. In the disc, oxidative/nitrosative stress and lesions due to mechanical loading result in higher levels of IL-8 [11, 27]. Interestingly, not only local IL-8 expression, but also serum and cerebrospinal fluid concentrations are affected by spinal pathologies [28, 29]. In detail, patients with higher lumbar radicular pain and or more pronounced disc herniation possess higher IL-8 levels in cerebrospinal fluid [28] and serum [29], respectively.

Although a diagnostic differentiation between DDD and DH can be difficult, we found a significantly higher expression of IL-8 in DDD biopsies compared to DH biopsies, a result that is in line with previous studies [30]. Patients undergoing multi-level surgeries also demonstrated enhanced IL-8 levels compared to single-level surgery patients. Both findings underline the pathophysiological relevance of this chemokine in IVD diseases.

Aside from IL-8, IL-6 was also expressed to a higher level in multi-level surgery patients in the herein presented study. IL-6 is an inflammatory cytokine with multiple biological effects [25]. After tissue injury, it promotes monocyte differentiation into macrophages and activates maturation of B- and T-lineage lymphocytes [31], thereby stimulating the production of immunoglobulin via B-lymphocytes [32]. By binding to the non-signaling membrane-bound IL-6 receptor (mIL-6R) and subsequently interacting with membrane protein gp130, it activates a variety of intracellular signaling pathways, including the Janus-activated kinase/signal transducer activator of transcription (JAK/STAT), the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), and the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) [33]. Importantly, IL-6 is believed to play a major role in the pathogenesis of spinal neuropathic pain, specifically in symptomatic radiculopathy (by inducing PGE2- mediated allodynia in experimental rat models) and peripheral nerve injury [25, 31, 34, 35]. This notion is supported by studies demonstrating that lumbar radicular pain induced by disc herniation is associated with elevated IL-6 serum levels [29, 36]. The functional role of IL-6 in spinal pain is further supported through therapeutic studies. A recent study demonstrated that epidural injection of an anti-IL-6R monoclonal antibody, tocilizumab, onto the spinal nerve alleviated radicular

leg pain, numbness, and low back pain without causing adverse events in 60 patients with lumbar spinal stenosis-induced sciatica [37]. Interestingly, we could demonstrate that the expression of IL-6 was dependent on the disc degeneration grade, the zonal region of the IVD (AF versus NP) and the spinal level (cervical versus lumbar). However, the latter effect was no longer present in matched samples, indicating that non-uniform distribution of the degeneration grade may have been the underlying cause of the difference seen in the entire (non-matched) dataset.

A significant correlation to the grade of degeneration was furthermore observed for TRPC6, an emerging target in the studies of pain and inflammation. In the lumbar DRG neurons from rats, TRPC6 (as well as TRPV4) seems to contribute to mechanical hyperalgesia, whereas in cartilage and the IVD, this channel is hypothesized to regulate the *in vitro* phenotypic stability and cellular ageing of resident cells [38, 39]. To our knowledge, this is the first study to describe the expression of TRPC6 in IVD tissue and its relevance in disc inflammaging. Aside from demonstrating that TRPC6 expression is degeneration dependent (with degeneration being an age-related process), we could also demonstrate that TRPC6 expression differs between NP and AF. This zonal difference in TRPC6 expression, with higher levels in the more fibrotic AF, is supported by the finding that TRPC6 may be involved in the expression of fibrosis-associated molecules and was hence shown to be expressed at a higher level in fibrotic stenosis areas in the intestine than in non-fibrotic gut areas of Crohn's disease patients [40]. Furthermore, we found found TRPC6 to be more highly expressed in the cervical spine, but only in the entire dataset. Once matching was conducted, no significant difference was observed. Overall, TRPC6 has a similar expression pattern as IL-6, with which it moderately correlates. While no studies exist to date that investigate the interaction of TRPC6 with IL-6 in the IVD, a decreased expression of IL-6 was found in TRPC6-null mice in a study of lung inflammation [41]. Aside from IL-6, we could also demonstrate mild to moderate correlations between TRPC6 and other cytokines, such as TNF-α.

TNF-α is a type II transmembrane protein secreted by macrophages, but also other cell types (including disc cells [42]), which binds its receptors TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR. In the disc, TNF-α is thought to participate in the initiation of the inflammatory cascade [43], as it is associated with an increase in the levels of other cytokines, such as IL-1B and IL-6 [44]. These reports are in agreement with our results that show a positive correlation between the expression of TNF-α and the cytokines IL-1B, IL-6, IL-8 and IL-15. In our study, relatively low levels of TNF-α were measured, possibly due to the advanced stage of degeneration of many samples [45]. Our results indicate a higher expression of TNF-α in the AF than the NP. In fact, previous reports have provided contradictory findings on the zonal expression of TNF-α, describing it to be either higher or

lower in the AF compared to the NP [46-48]. As TNF-α is implicated in neurogenic, radicular and low back pain [49], correlating the duration of symptoms with TNF-α expression would have been of interest, but this information was not available in the patient history. Importantly, recent evidence suggests therapeutic effectiveness of TNF-α inhibitors for disc herniation-associated radicular pain through inhibition [50]. Hence, although the role of TNF-α in disc herniation is established, its implication in disc degeneration is still somewhat unclear [48].

Similar to TNF-α, IL-1B is secreted by immune cells and also IVD cells, but at higher levels as shown by us and others [48]. IL-1B, together with IL-1A, is the most studied member of the IL-1 family. Both cytokines are first synthesized as a precursor protein before being processed to shorter active peptides [48, 51, 52]. While IL-1 is expressed in balance with its antagonist IL-1Ra in the healthy disc, catabolic disruption of this balance occurs during degeneration. Importantly, IL-1B furthermore contributes to matrix degradation and reduced matrix synthesis [48, 51]. Consequently, therapeutic targeting of IL-1B has been conducted, with positive results [53, 54]. We were able to demonstrate weak to moderate correlations between IL-1B and several other proinflammatory cytokines, such as TNF-α, IL-6 and IL-8, suggesting a possible interplay between these molecules. While existing knowledge clearly points towards involvement of IL-1B in disc disease, we could not observe any significant differences for any of the investigated parameters.

Aside from the well-investigated cytokines discussed above, inflammatory candidate genes that can be considered as novel in disc research, namely IL-15, IFNA1, IFNA8 and IFNB1, were also analyzed.

IL-15 is fundamental in the immune response as it modulates the activation and proliferation of natural killer- T- and B cells [55-57]. While little evidence exists on is relevance in disc disease, it is essential in cancer pathology, with promotion of malignancies like multiple myeloma, cutaneous T-cell lymphoma and large granular lymphocytes leukemia [58, 59]. Moreover, IL-15 is found in skeletal muscles where studies have suggested its involvement in autoimmune myositis progression [60]. IL-15 also plays a crucial role in autoimmune diseases by promoting the effect of cytotoxic $CD8^+$ T cells, including rheumatoid arthritis, inflammatory bowel disease, and multiple sclerosis, and is hence suggested as a therapeutic target [61, 62]. We found IL-15 to be expressed in a degeneration- and age-dependent manner, but with relatively high variability over the entire dataset. We found IL-15 to be expressed in a degeneration- and age-dependent manner, but with relatively high variability over the entire dataset. However, rather than being coincidental, the observed variability is likely related to the fact that IL-15 expression is significantly lower in lumbar disc samples compared to cervical samples. Importantly, even after matching samples with regard to the IVD degeneration grade and age—to exclude

these factors as confounders—the significant difference in IL-15 expression between the cervical and lumbar disc was maintained, indicating that IL-15 is indeed degeneration, age and region dependent. Interestingly, it was hypothesized that mechanical stress (related to obesity and resistance training) may regulate IL-15 expression and activity in skeletal muscle fibers [63, 64]. Therefore, the different stress magnitudes in lumbar and cervical discs may have affected the expression of IL-15—a mechanism that could be further explored in in vitro loading experiments. Similarly, IL-15 was recently shown to have a significant role in the pathogenesis of osteoarthritis, with expression levels being dependent on the disease stage (albeit with different pattern than in the IVD) [65, 66]. While our study only provides descriptive evidence for the potential relevance of IL-15 in degenerative disc disease, mechanistic investigations are required to demonstrate its pathological relevance.

Interferons are a multigene family of inducible cytokines that are divided in two groups, type I IFN (IFN-α, IFN-β, and IFN-ω) and type II (IFN-γ). Although interferons are generally considered to be important modulators of the immune system, no data currently exists with regard to degenerative disc disease, apart from IFN-γ [13, 67]. We present first data on the occurrence of members of the IFN-α (IFNA1, IFNA8) and IFN-β (IFNB1) subfamily in diseased discs. Interestingly, IFNB1 (similar to IL-1B and TNF-α) has been suggested to promote autophagy for certain cell types [68] and elevated autophagy has recently been described in degenerated AFs [69]. Our results demonstrate that IFNA1, IFNA8 and IFNB1 are intra-correlated and are expressed at significantly higher levels than TNF-α, yet at levels similar to IL-6 and IL-8. However, when cervical and lumbar samples were matched with regard to the IVD degeneration grade and age, no significant difference in IFNA1 expression (with $p = 0.056$) was found. This may be due to the reduction in sample size and hence lowered statistical power. On the other hand, it could also indicate that IFNA1 expression is influenced primarily by degeneration and age and only secondarily by spinal region and its associated factors, such as altered biomechanical loading situations and differences in diffusion distances and hence cellular nutrition. Although our study has provided first indications of a significant role of investigated members of the interferon type I family in disc diseases, further studies are warranted to determine their therapeutic potential [70].

In summary, this study demonstrated clear differences in mRNA expression for several of the analyzed parameters and genes. Future studies could focus on either confirming results on the protein level or on analyzing (on a larger set of NP and AF samples) whether the investigated genes are affected by increasing age, degeneration and/or Modic grade when using a zone-specific evaluation approach. Furthermore, investigating the proinflammatory crosstalk between end plates, vertebral bone marrow and the IVD could

provide further insight into the role of Modic changes in disc inflammaging and the development of low back pain. Clear technical limitations that weaken the implications of this study are the lack of (1) healthy (non-degenerated) disc tissue of (2) patient-specific pain scores as well as of (3) collection of data on additional environmental factors, such as smoking or body weight, which were previously shown to be of importance in the degenerative processes of the disc.

Conclusion

Our study unveiled a potentially crucial role of Ca^{2+} -permeable cation channels, specifically of TRPC6, in disc inflammaging. Furthermore, we confirmed the presence of the proinflammatory cytokines IL-1B, TNF-α, IL-6 and IL-8 in DDD and highlighted the expression and relevance of cytokines that have previously gained little or no attention in disc research (INFA1, IFNA8, INFB1, IL-15). Importantly, we were able to demonstrate that the expression of IL-15, INFA1, IL-6, IL-8 and TRPC6 was affected by relevant patient/tissue characteristics, such as the IVD degeneration grade, age, spinal level and/or pathology. These molecules may hence constitute targets to modulate the process of disc degeneration and pain development. However, larger-scale and more mechanistic studies are required to confirm these results, to investigate the specific function of these cytokines and to evaluate their therapeutic potential.

Compliance with ethical standards

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Conflict of interest All authors declare that they have no conflict of interest

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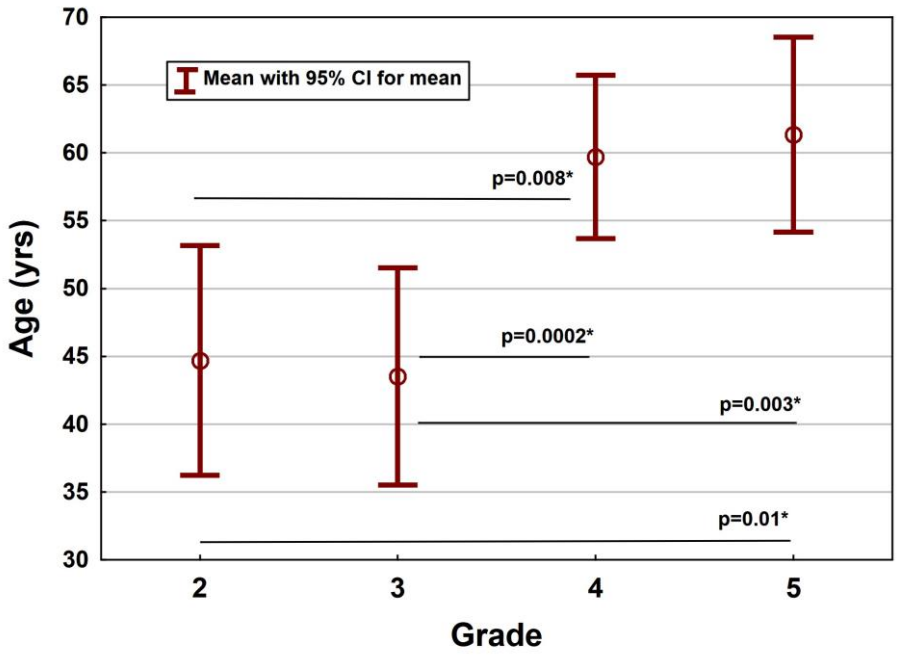
Supplementary Materials

Supplementary Table 1. Differences in gene expression between selected pairs of genes

Genes	p-value*	Genes	p-value*
IFNA8 - IFNA1	< 0.00001	TRPV4 - IFNB1	.016
IFNB1 - IFNA1	.006	IL6 - IL1B	.125
IL1B - IFNA1	.253	IL8 - IL1B	.00001
IL6 - IFNA1	.862	IL15 - IL1B	.002
IL8 - IFNA1	.015	TNF-α - IL1B	< 0.00001
IL15 - IFNA1	.001	TRPC6 - IL1B	.247
TNF-α - IFNA1	.00001	TRPV4 - IL1B	. < 0.00001
TRPC6 - IFNA1	.900	IL8 - IL6	.0001
TRPV4 - IFNA1	.003	IL15 - IL6	.0001
IFNB1 - IFNA8	.011	TNF-α - IL6	< 0.00001
IL1B - IFNA8	.013	TRPC6 - IL6	.965
IL6 - IFNA8	.294	TRPV4 - IL6	.001
IL8 - IFNA8	.197	IL15 - IL8	< 0.00001
IL15 - IFNA8	< 0.00001	TNF-α - IL8	< 0.00001
TNF-α - IFNA8	< 0.00001	TRPC6 - IL8	.003
TRPC6 - IFNA8	.120	TRPV4 - IL8	.110
TRPV4 - IFNA8	.028	TNF-α - IL15	.071
IL1B - IFNB1	.129	TRPC6 - IL15	< 0.00001
IL6 - IFNB1	.892	TRPV4 - IL15	< 0.00001
IL8 - IFNB1	.078	TRPC6 - TNF-α	< 0.00001
IL15 - IFNB1	.00005	TRPV4 - TNF-α	< 0.00001
TNF-α - IFNB1	< 0.00001	TRPV4 - TRPC6	< 0.00001
TRPC6 - IFNB1	.516		



Supplementary Fig. 1 Correlation between the age and IVD degeneration grade (n = 51, r = 0.49, p = 0.0003)