**UNIVERSITY OF COPENHAGEN**

FACULTY OF HEALTH AND MEDICAL SCIENCES

PhD Thesis

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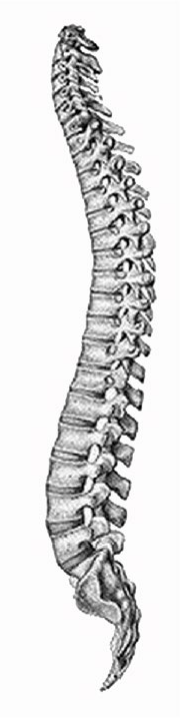
# MOLECULAR BIOLOGY IN THE PAIN GENERATION IN LUMBAR INTERVERTEBRAL DISCS



This thesis has been submitted to the Graduate School of Health and Medical Sciences, University of Copenhagen on 2nd January 2022

*To all the parents who helped make their children's dreams come true.*

PhD Thesis

# MOLECULAR BIOLOGY IN THE PAIN GENERATION IN LUMBAR INTERVERTEBRAL DISCS

By

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**Title:** Molecular biology in the pain generation in lumbar intervertebral discs

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**PREFACE**

The Present PhD thesis is based on my work at the Neurobiology Research Unit, Copenhagen University Hospital Rigshospitalet, Denmark, which was carried out in January 2019 – December 2021 under Professor Jens Damsgaard Mikkelsen, and my co-supervisor Associate Professor Rachid Bech-Azeddine and guidance of Jørgensen LM.

This Thesis is based on the following manuscripts, which we followingly are referred to by their roman numerical

**I.** **Aripaka, S.S**., Bech-Azeddine, R., Jørgensen, L.M. *et al*. Low back pain scores correlate with the cytokine mRNA level in lumbar disc biopsies: a study of inflammatory markers in patients undergoing lumbar spinal fusion. European Spine Journal, 2021. 30(10): p. 2967-2974.

**II.** **Aripaka, S.S.,** Bech-Azeddine R, Jørgensen LM, Mikkelsen JD. The expression of metalloproteinases in the lumbar discs correlates strongly with Pfirrmann MRI grades in lumbar spinal fusion patients. The manuscript was submitted to *Brain and Spine Journal,* and it is under review.

**III.** **Aripaka S.S.,** Bech-Azeddine R, Jørgensen LM, Mikkelsen JD. Transient Receptor Potential (TRP) Channels mRNA Transcripts in the Lumbar Intervertebral Discs: Biomarkers for inflammation, pain, disability, and clinical outcome. Manuscript submitted *European Spine Journal.*

**SUMMARY**

Low back pain (LBP) refers to pain and stiffness of the lower portion of the back; the primary cause of disability globally. About 85-90% of patients are diagnosed with essential LBP, with no documented course behind the pathology. LBP is associated with degeneration of the intervertebral disc (IVD) (visualized using magnetic resonance imaging), and lumbar spinal fusion of the degenerated level may alleviate pain for the patient. However, the patients present heterogenicity in chronic back pain intensity, treatment outcome, and prognosis. We hypothesize that LBP has a vital inflammatory component and that inflammatory mediators such as TNF-*α,* IL-1, and IL-6 are expressed in the annulus fibrosus and nucleus pulposus in a higher concentration in patients relative to their grading of pain intensity and disability. Furthermore, the degeneration is also associated with dysregulation of extracellular matrix synthesis due to increased expression of matrix cleaving proteases, such as matrix *metalloproteinases* (MMPs) and *A Disintegrin and Metalloproteinase with Trombospondin motifs* (ADAMTSs). Cytokines have also been shown to upregulate the expression of these proteases. Similarly, Transient Receptor Potential (TRP) ion channels also emerged as receptors for inflammatory molecules involved in the processing of inflammatory mediated pain. Previous studies have shown dysregulation of TRP ion channels in IVD and other joint diseases.

The aim of this thesis is (I) to evaluate the association of inflammatory mediators with low back pain, (II) to examine the association of several MMPs and ADAMTSs subtypes in IVD with degeneration and (III) to investigate the expression of TRP ion channels in IVD and its association with inflammatory mediated pain.

We studied the expression of TNF-α, IL-1β, and IL-6 in IVD biopsies from 34 patients and found a highly statistically significant positive correlation between pain intensity (Visual Analog Scale score) and disability (Oswestry Disability Index (ODI) score) with the expression of cytokines. We further studied the role of different metalloproteinases in the lumbar discs because the loss of extracellular matrix is observed in degenerative disc disease (DDD). We studied the expression of different metalloproteinases and correlated them to the Pfirrmann magnetic resonance imaging classification system (grade I-V) of lumbar DDD. We found a highly significant positive correlation between Pfirrmann grades and the gene expression of several metalloproteinases like MMP-1, ADAMTS-1&5. A post hoc statistical analysis (uncorrected p-

values) also demonstrated a positive correlation between the expression of TNF-α, IL-6, and ADAMTSs/MMPs and the Pfirrmann grades. These findings show that disc degradation in DDD is strongly associated with the expression of some metalloproteinases. We further studied the role of TRP ion channels that have recently emerged as contributors to disc pathology and plays an important role in pain processing. Hence, the purpose of our overall study was to determine the expression of different TRP ion channel subtypes (TRPV1, TRPV4, TRPC6, TRPM2 and TRPM8) in IVD biopsies from patients with chronic low back pain. We found a statistically significant positive correlation between pain intensity and expression of TRPV1, TRPC6, TRPM2, TRPM8 in the annulus fibrosus. Moreover, we showed a significant positive correlation between disability score (ODI) and expression of both TRPV1 and TRPM8. Interestingly, postoperative follow-up of treatment response after 12-months demonstrated a significant positive correlation between TRPV1 expression at baseline and the follow-up ODI score, which might be a potential marker for predicting the effect of surgery. Overall, our results suggest the association between TRP ion channels expression and chronic low back pain.

The present PhD thesis creates a basis for future research on other cytokines, proteases and TRP ion channels. Our studies help to characterize biomarkers of DDD that may serve as potential predictors of treatment response in the future. However, broad multi-tiered design approaches are necessary to develop effective therapies and interventions to delay the onset of disc degeneration and prevent associated chronic low back pain.

## DANSK RESUMÉ

Lænderygsmerter referer til smerter og stivhed i den nederste del af ryggen; den primære årsag til invaliditet på verdensplan. Omkring 85-90% af patienterne er diagnosticieret med uspecifikke lænderygsmerter uden noget dokumenteret forløb bag patologien. Lænderygsmerter er forbundet med massiv degeneration af den intervertebrale disk (IVD) (visualiseret ved hjælp af magnetisk resonans billedefremstilling), og lumbal spinal fusion af det degenererede område kan lindre patientens smerter. Dog repræsenterer patienterne en heterogen gruppe både hvad angår sværhedsgraden af kroniske rygsmerter, behandlingseffekten og prognose. Vores hypotese er, at lænderygsmerter har en stærk inflammatorisk komponent og at inflammatoriske mediatorer, såsom TNF-α, IL-1 og IL-6 udtrykkes i *annulus fibrosus* og *nucleus pulposus* i højere koncentrationer hos patienterne relativ til styrken af deres smerteintensitet og invaliditet. Derudover er degenerationen også forbundet med dysregulering af ekstracellulær matrixsyntese på grund af stigning i ekspressionen af matrix spaltende proteaser, som fx *matrix metalloproteinase* (MMP) og *A Disintegrin and Metalloproteinase with Trombospondin motifs* (ADAMTS). Cytokiner har også vist sig at opregulere ekspressionen af disse proteaser. Ligeledes er *Transient Receptor Potential* (TRP) ionkanalerne receptorer for inflammatoriske molekyler som er involveret i processering af inflammatorisk-medieret smerte. Tidligere undersøgelser har vist dysregulering af TRP ionkanaler i IVD og andre ledsygdomme.

Formålet med nærværende afhandling er (I) at undersøge sammenhængen mellem inflammatoriske mediatorer og lænderygsmerter, (II) at undersøge sammenhængen mellem forskellige MMP og ADAMTS subtyper i IVD med degeneration og (III) at undersøge ekspressionen af TRP-ionkanaler i IVD og deres forbindelse med inflammatorisk-medieret smerte.

Vi undersøgte ekspressionen af TNF-α, IL-1β og IL-6 i IVD-biopsier fra 34 patienter. Vi fandt en meget høj statistisk signifikant positiv sammenhæng mellem ekspressionen af cytokiner og patientens graduering af smerteintensitet (Visuel Analog Skala score) og invaliditet (Oswestry Disability Index (ODI) score). Vi undersøgte yderligere rollen af forskellige metalloproteinaser i

lændeskiverne, da tab af ekstracellulær matrix ses ved degenerativ diskussygdom. Vi undersøgte ekspressionen af forskellige metalloproteinaser og disses korrelation med Pfirrmann klassificering (grad I-V) fra magnetisk resonans billedefremstilling af lumbal degenerativ diskussygdom. Vi finder en stærk statistisk signifikant positiv korrelation mellem Pfirrmann karakterer og genekspression af flere metalloproteinaser, såsom MMP-1 og ADAMTS-1&5. En post-hoc statistisk analyse (ukorrigerede p-værdier) viste også en positiv sammenhæng mellem ekspressionen af TNF-α, IL-6 og ADAMTS/MMP og Pfirrmann karakterer. Resultaterne viser, at disknedbrydning i degenerativ diskussygdom er stærkt forbundet med ekspressionen af flere metalloproteinaser. Vi undersøgte yderligere TRP ionkanalers rolle. Disse har for nylig vist sig at bidrage til diskuspatologien, og de spiller en essentiel rolle i smerteprocessering. Formålet med vores overordnede undersøgelse var derfor at bestemme ekspressionen af forskellige TRP ionkanalsubtyper (TRPV1, TRPV4, TRPC6, TRPM2 og TRPM8) i IVD-biopsier fra patienter med kroniske lænderygsmerter. Vi fandt en statistisk signifikant positiv sammenhæng mellem smerteintensitet og udtryk af TRPV1, TRPC6, TRPM2 og TRPM8 i *annulus fibrosus*. Desuden viste vi en statistisk signifikant positiv sammenhæng mellem ODI-score og udtryk af TRPV1 og TRPM8. Et interessant fund var, at postoperativ opfølgning af behandlingsrespons efter 12 måneder viste en signifikant positiv sammenhæng mellem TRPV1 ekspression ved baseline og den opfølgende ODI-score som kan være en potentiel markør til at forudsige effekten af operationen. Samlet set tyder vores resultater på en sammenhæng mellem TRP ionkanalers udtryk og kroniske lænderygsmerter.

Nærværende Ph.d.-afhandling skaber grundlag for fremtidig forskning i andre cytokiner, proteaser og TRP ionkanaler. Vores undersøgelse hjælper med at karakterisere biomarkører for degenerativ diskussygdom der tjener som potentielle prædiktorer for behandlingsrespon i fremtiden. Imidlertid er brede flerlagede designtilgange nødvendige for at udvikle effektive behandlinger og interventioner der kan forsinke påbegyndelse af diskus-degeneration og forhindre associerede kroniske lænderygsmerter.

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**ABBREVIATIONS**

| | |
|---|---|
| AF | Annulus Fibrosus |
| ADAMTS | A disintegrin and metalloproteases with thrombospondin motifs |
| BMP | Bone morphogenetic protein |
| cDNA | Complementary DNA |
| CEP | Cartilagenous End Plate |
| CGRP | Calcitonin gene-related peptide |
| DRG | Dorsal root ganglion |
| DMEM | Dulbecco's modified Eagle medium |
| DDD | Degenerative disc disease |
| ECM | Extra cellular matrix |
| FBS | Fetal bovine serum |
| GAG | Glycosaminoglycan |
| HIF1 | Hypoxia-inducible factors |
| IVD | Intervertebral disc |
| IL | Interleukin |
| LBP | Low back pain |
| MRI | Magnetic resonance imaging |
| MAPK | Mitogen-activated protein kinase |
| MMP | Matrix metalloproteases |
| NP | Nucleus Pulposus |
| NF-κB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NGF | Nerve growth factor |
| ODI | Oswestry disability index |
| PBS | Phosphate-buffered saline |
| PG | Proteoglycan |
| PLIF | Posterior Lumbar Interbody Fusion |

| | |
|---|---|
| RT-qPCR | Quantitative reverse transcription-polymerase chain reaction |
| RNA | Ribonucleic acid |
| Shh | Sonic hedgehog |
| TGFβ | Transforming growth factor-beta |
| TNF- α | Tumor necrosis factor |
| TRP | Transient receptor potential |
| TLIF | Transforaminal Lumbar Interbody Cage |
| VAS | Visual analogue scale |

Where the mind is without fear and the head is held high;

Where knowledge is free;

Where the world has not been broken up into fragments by narrow domestic walls;

Where words came out from the depth of truth;

Where tireless striving stretches its arms towards perfection;

Where the clear stream of reason has not lost its way into the dreary desert sand of dead habit;

Where the mind is led forward by thee into ever widening thought and action;

Into that heaven of freedom, my Father, let my country awake."

*Gitanjali 35*

***Rabindranath Tagore***

# 1. INTRODUCTION

According to the Global Burden of Diseases, Injuries and Risk Factors Study 2017 (GBD 2017), Low back pain (LBP) is the leading cause of years lived with disability in both females and males among 354 causes in 195 countries and territories from 1990 to 2017[1] (Fig 1).

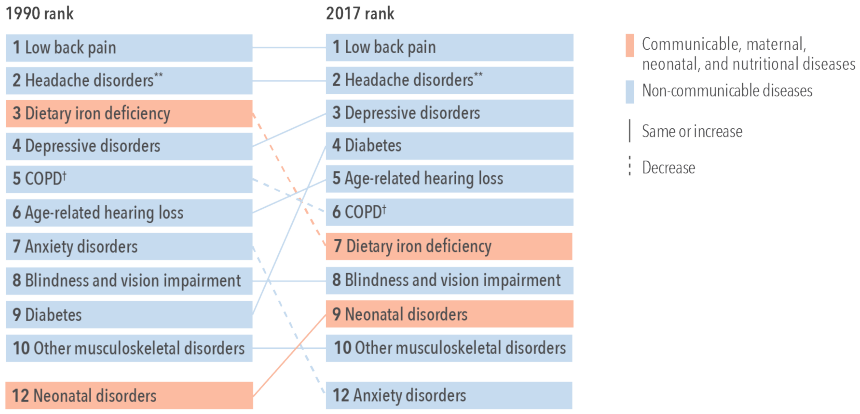



***Fig 1.*** *Leading causes of global all-age disability, 1990 and 2017[1]*

It is estimated that 80% of the population suffers from LBP at some point in their lives, while 10% are chronically disabled [2]. Despite these frequencies, LBP contributes to an enormous socio-economic burden to western societies and a huge drain on medical resources worldwide [3, 4]. About 85-90% of patients experiencing LBP is labelled as non-specific LBP, indicating the origin of the pain is unknown as the pathophysiology producing these symptoms can be multifactorial and often difficult to identify specific cause[5]. Degenerative changes that occur with age include intervertebral disc degeneration (IVDD), facet arthropathy, hypertrophy and ligamentum flavum hypertrophy in the lumbar spine. These processes can manifest as discogenic low back pain, disc herniation (leakage of IVD material and subsequent compression of a nerve root and lead to severe radicular symptoms), lumbar stenosis (narrowing of the neural canal and neuroforaminal spaces of lumbar spine), and/or spondylolisthesis (slippage of one vertebra in relation to the vertebra below it). Although LBP is commonly diagnosed with disc degeneration, the actual cause is still not known [6, 7]. So far, it is very unclear why some patients develop chronic low back pain, but some are asymptomatic even though their X-ray and magnetic resonance imaging (MRI) show similar changes [8]. The purpose of this chapter is to provide a

comprehensive insight into the anatomy, physiology and pathophysiology of an intervertebral disc.

## 1.1. Human intervertebral disc: Relevant anatomy and physiology

### 1.1.1. Vertebral column:

The human vertebral column comprises 33 vertebrae, which connects the skull to the pelvis. It is divided into five regions cervical (7 ), thoracic (12), lumbar (5), sacral (5), and coccygeal (4) [10]. The motion of the vertebral column is varied within these regions, with the greatest freedom of movement for cervical and lumbar regions. Each vertebral segment has a spinal canal and two vertebral foramina, which protects the passage of the spinal cord and nerve roots. IVD separates the upper 24 vertebral bodies and lowers 9 IVD fused in adults. IVD are fibrocartilaginous structures connecting two adjacent vertebral bodies and confers flexibility and mechanical stability, which are about 7 to 10 mm thick and have a diameter of 4 cm in diameter in the lumbar region spine height [9].

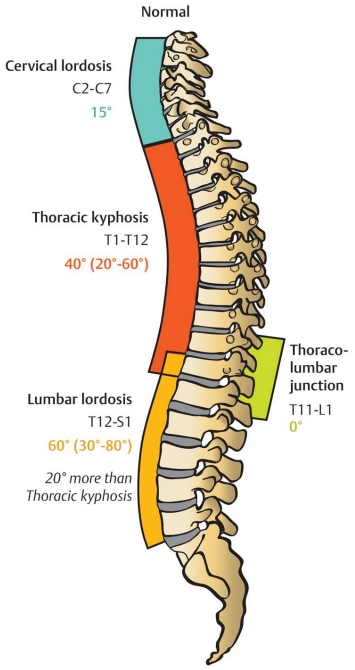



*Fig 2: Schematic representation of anatomical regions of the human spine[10]*

### 1.1.2. Disc development: embryogenesis and postnatal growth

The macroscopically internal structure of IVD has distinct anatomical regions of varied developmental origins. It is divided into outer annulus fibrosus (AF) surrounding centrally located gelatinous nucleus pulposus (NP) and cartilaginous endplates which surrounds the IVD cranial and caudal ends [11]. Embryonic development of the spinal column originates from the notochord and sclerotome of the rod-like mesoderm derived structure[12, 13]. For the development of IVD, notochord plays an important role as a signal center by mediating cell migration, differentiation, and cell survival, which eventually gives rise to NP [14-16]. The vertebra column is formed by the aggregation of somatic mesenchymal cells around

the notochord, resulting in a continuous perichordal tube. After differentiation, ventral somites de-epithelialize and form mesenchymal sclerotomes, giving rise to pre-cartilaginous structure and connective tissue [17]. The axial mesenchyme gains an alternating pattern of condensed and non-condensed areas, eventually developing into AF and vertebral bodies. Notochord disappears within the forming vertebral bodies while expanding simultaneously within intervertebral regions to form NP[18, 19]. Embryogenesis of disc is orchestrated by series of molecular signals from notochord cells and neural tube [20].

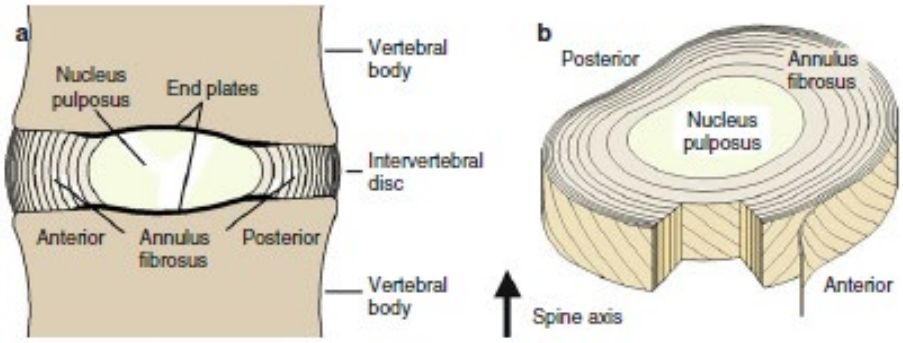



*Fig 3: Illustration of adult intervertebral disc (a) Midsagittal cross-section. (b) 3d view showing central NP surrounded by lamellar AF.[21]*

The important players in molecular signaling of IVD embryogenesis are Sonic hedgehog (Shh), transforming growth factor-beta (TGF$\beta$). Noggin, Pax, Sox and. Shh is a signaling molecule with diverse roles, but it plays an important role in skeletal morphogenesis and directs cell differentiation[22, 23]. Noggin works synergistically with Shh and is initially expressed in notochordal cells before becoming localized to developing AF, where it remains until birth [23]. Noggin is an antagonist for Bone morphogenetic protein (BMP), so it potentially blocks the BMP signaling originating from vertebral bodies.[24] Pax genes encode transcription factors that regulate critical cellular functions like proliferation, differentiation, apoptosis and stem cell maintenance [21]. Specifically, Pax expression is important for maintaining tissue boundaries [25], like delineating more and less condensed regions of cells, which eventually give rise to IVD and vertebral bodies, respectively [26]. *Pax1* and *Pax9* play important role in the development of IVD and vertebral bodies[27]. The Sox (Sry-related high-mobility-group) gene family members perform diverse functions during development; among those, *Sox5, Sox6* and *Sox9* are implicated in chondrogenesis [28]. Mice lacking both *Sox5* and *Sox6* failed to form

notochordal sheath due to associated downregulation of collagen II and aggrecan genes which encodes cartilage matrix, which ultimately results in failure of NP to form. TGF$\beta$ [29] *is* implicated in linear patterning of the discs and vertebral bodies, and its signalling is important in the regulation of cell differentiation and proliferation, skeletal development and extracellular matrix production [21]. Conditional deletion of TGF$\beta$ receptor 2 results in defects in formation of NP and inner AF and partial mineralization of disc region [30-32].

During early natal life, the inner annulus is richly supplied by blood vessels, but the blood vessel penetration is confined to outer AF and the cartilaginous end plates [33]. During the early postnatal year, in humans, disc blood vessels are limited only to the connective tissue surrounding the AF and endplates, eventually leaving the disc completely avascular structure[33, 34]. The loss of blood vessels limits the flow of nutrients reaching NP; this supports the idea that the disc has a poor ability to remodel and repair [35]. Nerves follow a similar pattern with blood vessels confined only to outer AF in non-degenerated adult discs [35].

### 1.1.3. Cell types in the intervertebral disc

The cell density is considerably high in the fetal stage but significantly decreases by age. Unlike CEP and AF, in which the cell types remain relatively constant throughout life, cells in NP undergo substantial changes in their cellular composition. During fetal/ early natal, the cell population in NP exhibit morphological similarities to its notochord precursor[15, 36], hence these cells are classically referred to as 'notochordal-like', these cells are large (30-40 µm in diameter) and contain large vacuoles that occupy 25% of the cell area [37] which is the trait common to cell of the embryonic notochord. The vacuoles of notochord like cells carry anabolic factors suggested to induce matrix synthesis [38]. The notochordal cell count gradually declines and eventually disappears by the juvenile stage, i.e., in the first ten years after birth [21, 36]. The NP becomes populated by a new cell population of smaller size appears which are classically referred as 'chondrocyte-like' with apparent similarities with cartilage chondrocytes. To avoid the confusion with cartilage chondrocytes, they are referred to as 'mature NP cells'[34], these cells are relatively small (~10 µm in diameter), lack vacuoles[39], In adults these cells sparsely and randomly distributed within NP with a cell density of about 4,000 cells/ $mm^3$ [40]. The origin of mature NP cells is subject to debate [41]; some studies support that these cells are recruited from adjacent tissue such as CEP [42]. However, growing evidence suggests that the

cells are descended directly from embryonic notochord [41]. Embryonic expression of notochordal cell marker brachyury (T) by notochordal-like cells and mature NP cells supports their common notochordal lineage[43].

AF cells originate from the sclerotome, and morphologically similar to fibroblast appears thin and elongated spindle-shaped and arranged following the lamellae's orientation. The AF is densely cellularized, with about 9,000 cells per $mm^{3,}$ and the diameter varies between 15 and 30µm [40, 44].

Cells in the endplate are mesenchymal chondrocytes with rounded morphology, similar to articular chondrocytes. Compared to AF and NP, the endplate has the highest cellularity with approximately 15,000 cells/$mm^3$ with an average diameter of 20µm [40].

### 1.1.4. The phenotype of distinct IVD cell types

Recent transcriptional profiling of healthy IVD cells identified distinctive cell type-specific markers to distinguish NP and AF cells, leading to recommendations for NP markers by the ORS Spine Research Interest Group[45]. Based on the criteria of specificity, function and expression across different species, the proposed list of primary NP markers that included is the signalling factor sonic hedgehog (SHH), hypoxia-inducible factors (HIF1α and HIF2α), the transcription factor Brachyury (T), the glucose transporter (GLUT1), the carbonic anhydrases (CA3 and CA12), the CD24 antigen, and the keratins KRT8, KRT18, and KRT19 [17]. They also proposed secondary markers, a subset of which validated the transcription factors PAX1 and FOXF1, the cadherin CDH2. Few of these secondary markers, including PAX1 and FOXF1, have been validated in human cells [46].

Compared to NP, only a few studies have proposed AF-specific markers, Glypican-3 (*GPC3*), which is a heparin sulfate proteoglycan whose gene expression in human is higher in AF compared to NP cells [47], the gene for type V collagen (*COL5A1*) [48, 49]. RNA microarray analysis on bovine IVD expression of *CXCL1, EMCN,* and *KERA* in AF cells is higher than NP and endplates[43]. *CXCL1* encodes a member of chemokines whose expression has been associated with disc degeneration.[50].

### 1.1.5. Disc organization and composition

Distinct anatomical regions in the disc have different mechanical properties resulting from specific tissue microstructures and distinct cell populations; maintenance of IVD homeostasis and structural integrity depends on multiple factors like cellular responses to mechanical load [17]. The disc ECM is rich in collagen and proteoglycan. Collagen is an ECM protein present in mammalian connective tissue, different types of collagens express in the disc, but the most prominent are type I and II, other collagens present in mature IVD are types III, V, VI, IX, XI, XII, and XIV [51]. Collagen provides the disc cells attachment to 3D mesh confining other matrix elements, such as proteoglycans (PG) [52]. Proteoglycans are either bound to hyaluronic acid or unbound, and the most abundant PG is aggrecan. Most of the aggrecan is bound to hyaluronic acid during the early development stages, but later it shifts to unbound form during later stages of development. Other PG's in the disc are versican and members of the small leucine-rich protein (SLRP) family: decorin, fibromodulin, chondroadherin, and lumican [53].

PGs consist of a core protein to which one or more glycosaminoglycan (GAG) chains of highly sulphated repeating disaccharide units are covalently attached. Typically, most PG's consist of 1 or 2 GAG chains, but aggrecan consists of up to 150 GAG chains [52].

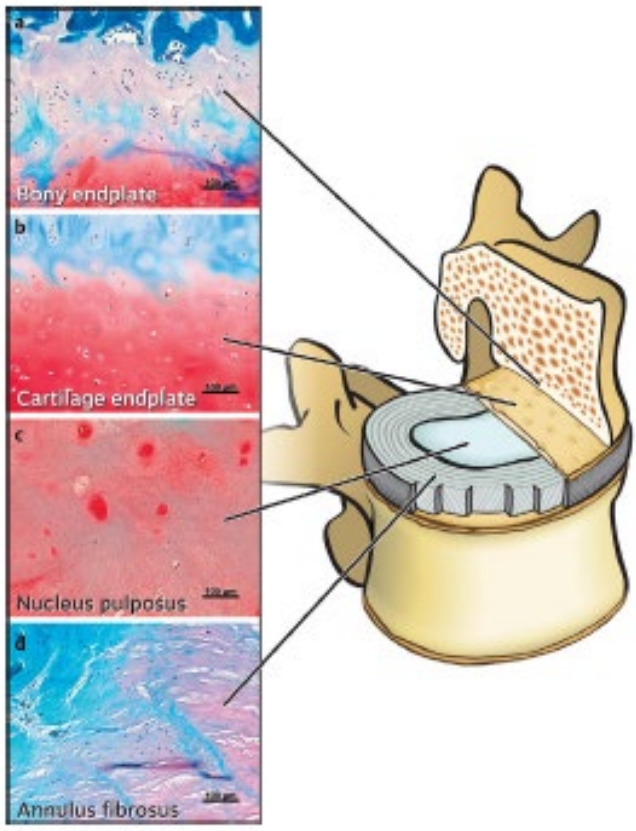



*Fig 3. Human intervertebral disc histological images. (a) bony endplate, (b) cartilaginous endplate of a young, healthy patient. (c) nucleus pulposus. (d) annulus fibrosus. Stained with Safranin-O, fast green FCF, and Weigert's hematoxylin[52].*

The IVD is composed of three structurally different yet interdependent types of tissue: the nucleus pulposus (NP), the annulus fibrosus (AF) and the cartilaginous endplates

### 1.1.6. The nucleus pulposus (NP)

The NP is a heterogenous structure mainly composed of collagen type II, which provides a scaffolding mesh that entraps matrix elements like aggrecan, hyaluronic acid, and other molecules [54]. NP structure is gelatinous, and aggrecan is abundant PG which contains many GAG chains which contribute to water retention and generate a swelling pressure through fixed negative charges that help NP in its ability to resist compression and maintain disc height [55]. Collagen type II and aggrecan make up to 20% and 50% of the NP's dry weight, respectively [35]. Even though the same molecules are responsible for mechanical properties in hyaline cartilage, the ratio of aggrecan to collagen is 27:1 in NP, whereas, in cartilage, it is 2:1 [56]. About 70 to 90% of the NP's wet volume is occupied by water bound to aggrecan [35]. Previous studies have shown that high proteoglycan content with its negative charge is an important factor in preventing nerve ingrowth into anural and avascular adult, non-degenerated IVD [57]. Because of the avascular nature of the tissue, IVD cells tune their metabolism to low oxygen availability, and NP cells are almost entirely dependent on the glycolytic pathway to generate metabolic energy [58]. These cells are specialized to survive in a hypoxic environment where Hypoxia inducible transcription factors-1 and -2 are active in NP cells [59]. Indeed, NP cells have very few mitochondria and extensive endoplasmic reticulum (ER), and cells generate lactic acid as the metabolic end product. So, any changes in oxygen tension in tissue that leads to aberrant cell functions and altered biochemical activities would be subverted [41]. Peripherally, the NP is encircled by the AF.

### 1.1.7. The Annulus fibrosus (AF):

AF is divided into two distinctive areas inner and outer AF. The inner AF is which is a transition zone, and its composition is similar to NP and mainly contains Type II collagen and aggrecan [17]. The outer AF mainly contains collagen I fiber bundles arranged in 25 concentric lamellae organized in layers of alternating diagonal orientation, approximately about $30^{o}$ to the longitudinal axis of the spine [52, 60]. Aggrecan is present throughout the AF, but its concentration is much lower than NP, whereas collagen makes up to 50 to 70% of AF dry weight

and PG makes up 10 to 20% [61]. This composition of collagen fibers contribute tensile strength to AF, which provides resilience to lateral motion and prevent the extrusion of central nuclear material [52].

### 1.1.8. Vertebral Endplate (VEP):

IVD is bordered by endplates which separate the vertebral bone from the IVD itself. The vertebral endplate has two functions, it anchors the disc to vertebrae, and the other function is to provide the route for nutrient and water exchange for the disc. Hence it should be resilient as well as porous. VEP consist of two layers, the cartilage endplate(CEP) consists of hyaline cartilage composited of Type II collagen and aggrecan at a ratio of 2:1, and the bony endplate consists of cortical bone [62].

### 1.1.9. Blood supply and nerve innervation of the Intervertebral Disc

The vascularity of IVD is limited to the endplate, and it is supplied by the basivertebral vessel bundles [11]. The non-degenerated adult IVD is an avascular structure with some cells being up to 8mm from the nearest blood supply [63]. In humans, during early postnatal life, the density of blood vessels that have penetrated outer AF and CEP begins to decrease and eventually, the disc becomes avascular [33].

Following the similar pattern of blood vessel non-degenerated adult IVD is largely aneural, only innervated by nerves fibers consist primarily of unmyelinated (C) fibers and small myelinated (A- δ ), which mostly innervate outer AF accompanied by blood vessels or independent nerve fibers [64]. Most of the nerves innervating IVD arise primarily from dorsal root ganglions that are nociceptive [64]; these nerve fibers are positive for acetylcholinesterase, substance P, calcitonin gene-related peptide, C-flankin Peptide, neuropeptide Y, neurofilament protein and synaptophysin [65]. Nerve innervation is an important distinction between the disease and aged disc. However, nerve fibers deep inside the disc have been demonstrated in the degenerated disc [64].

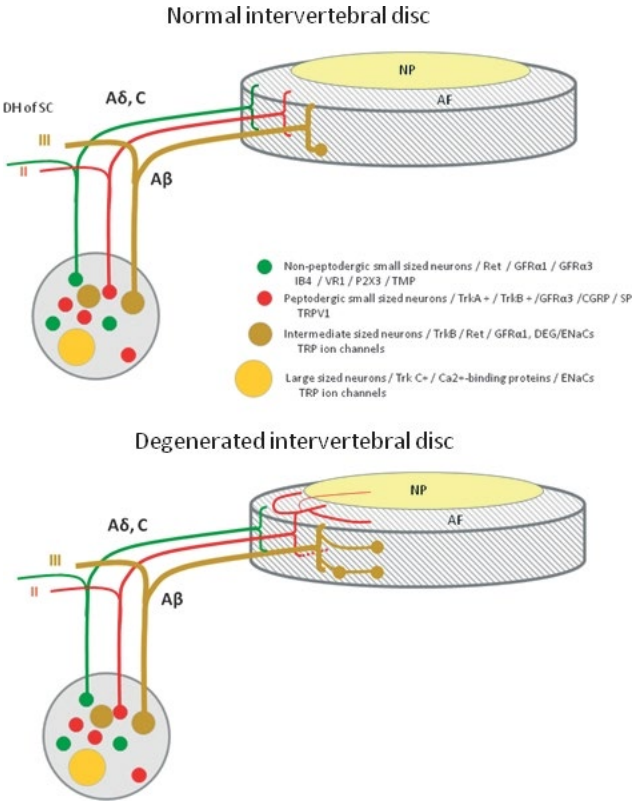



*Fig 5: Schematic representation of the innervation of normal (top) and degenerated (bottom) intervertebral disc(IVD). Thin myelinated Aδ fibers and unmyelinated C fibers arise from small neurons (red and green) originate from small peptidergic neurons expressing TrkA/TrkB (red) or non-peptidergic neurons expressing glial cell-derived neurotrophic factor family of neurotrophic factors (Ret) (green) they mediate nociception. The myelinated Aβ fibers (brown) arise from intermediate neurons; they mediate touch, pressure, and vibration sensations. Neurons in DRGs can be differentiated on expression of receptors for neurotrophic factors, the pattern of expression of different ion channels primarily of the degeneration/epithelial sodium channels (DEG/ENaCs) (ENaCα, β and γ; acid-sensing ion channel (ASIC)1, ASIC2 and ASIC3) and transient receptor potential (TRP) (TRPA1, TRPC1, TRPC6 and TRPV1-4) families, and peptide content. glial cell-line-derived neurotrophic receptor subtypes α1 and α3; ATP-gated ion channel subtype P2X3, CGRP, calcitonin gene-related peptide; GFRα1 and GFRα3, P2X3,; SP, substance P; TMP, thiamine monophosphatase; VR1, vanilloid receptor subtype 1.*[64]

### 1.1.10. Biomechanics of healthy IVD

The important functions of IVD are to facilitate the capacity to absorb the shock, transfer loads and allow the movement in the vertebral column. The tensile and elastic properties of AF allow a state of equilibrium between stability and flexibility [52]. A moderate dynamic loading is beneficial for discs because it promotes nutrient exchange with endplates [66, 67]. Similarly, the physiological strain also affects various factors such as nutrient and waste product transport within IVD. Similarly, the mechanical strain of the disc affects the extracellular environment, which indirectly effects the cell. For example, the strain will change water content, pressure and pH [68]. Chronic loading and traumatic loads cause damage to ECM, which eventually changes the disc integrity and can cause herniation or annular tears [69].

## 1.2. Pathophysiology of Disc Degeneration Disease (DDD)

The hallmarks of disc degeneration are loss of disc height, proteoglycan and disorganized ECM, and initiating a cascade of non-reversable cell response, further disruption of structure. So a degenerated IVD is a structure with structural failure combined with accelerated signs of ageing [70]. Multiple studies have identified risk factors associated with disc degeneration, including genetic predisposition, smoking, abnormal biomechanics, decreased nutrient transport [54, 71-73]. Degeneration develops as a natural part of disc ageing, but the contribution of these risk factors may induce aberrant responses and lead to the pathological breakdown of IVD. The following sections discuss the key changes observed in disc degeneration and known mechanisms that drive these changes.

### 1.2.1. Composition and Architectural changes:

Degenerated discs exhibit a loss of transition zone between AF and NP caused by changes in collagen synthesis within IVD [52]. During the early stages of degeneration, there is an increase in the synthesis of collagen II as an early compensation mechanism [74] but ultimately shifts from decreased collagen II to increased collagen I in the inner AF and NP [75]. These changes in collagen reorganization are accompanied by a decrease in aggrecan content and an increase in expression of small PG's like versican, biglycan and decorin, resulting in dehydration and fissures that extend into the AF [54, 74, 75]. These changes decrease the disc height and induce fibrosis results in deleterious effects on disc mechanical properties. Similarly, changes in ECM of outer AF involve a slight increase in Type II collagen, which compromises the ability of AF to contain NP during axial compression [76]. Hence, weakened outer AF lamellae result in an increased likelihood of NP leakage. Furthermore, these defects in outer AF also allow the neoinnervation and angiogenesis into IVD [54, 77, 78]

### 1.2.2. Neoinnervation and Neovascularization

The non-degenerated adult IVD is innervated primarily by afferent nerve fibers limited to external lamellae [64], but in a degenerated disc, the nerve fibers extend to non-neuronal regions accompanied by neovascularization [79]. In degenerated discs, an increased expression of nerve growth factors and loss of chondratin sulphate creates an environment that favors neoinnervation. Furthermore is has been shown that degenerated IVD have increased levels of

inflammatory cytokines like tumor necrosis factor (TNF)- α, interleukin (IL)-1 β, IL-6, which sensitize nociceptive neurons to noxious stimuli including heating [80] and mechanical loading [81] suggesting the role of cytokines in low back pain. Overall, these interactions between nociceptive neurons, changes in disc morphology and inflammatory milieu released by native cells and invading immune cells due to neovascularization are believed to contribute to discogenic back pain [79, 82].

### 1.2.3. Immune Cells and Inflammatory Cytokines

Inflammatory mediators are naturally involved in the immune response to tissue injury, infection or disease by producing pro-inflammatory cytokines. These cytokines recruit other immune cells to the site of injury and helps in tissue repair, followed by the secretion of anti-inflammatory cytokines to restore equilibrium in tissue [74, 83]. Multiple studies have established elevated pro-inflammatory cytokines and chemokines in degenerative and herniated discs [84-86]. This increase in cytokines level is a cellular response to decreased nutrients and invading immune cells [79]. Pro-inflammatory cytokines are secreted by NP cells, AF cells, T cells, neutrophils and macrophages. To once immune-privileged IVD results in numerous pathological changes like increased cellular senescence and apoptosis [87, 88], increased breakdown of ECM [86], further increase in expression of cytokines and neurotropic factor[77], angiogenesis, neoinnervation, and increase in sensitization of nerve fibers to noxious stimuli [39]. Furthermore, the immune cells stimulate the expression of nerve growth factor (NGF) [89]; this leads to dorsal root ganglion (DRG) neurons expressing pain-associated cation channels, which might provide a link between disc degeneration and discogenic low back pain [90, 91].

Multiple cytokines are elevated in the degenerative disc; the two most common cytokines studied about DDD are TNF-α and IL-1β, which induce apoptosis and inflammatory signaling through their respective receptors TNFR and IL1R1. Signaling through these receptors induce the activation of the transcription factors mitogen-activated protein kinase (MAPK) and nuclear factor κB (NF-κB) pathways [92], resulting in phosphorylation proteins involved in inflammatory and catabolic genes such as IL-6, IL-8, MCP-1, COX-2 [93]. Even though a large amount of evidence suggests the role of TNF-α and IL-1β in degeneration, an increased level of TNFR1 expression was only found in herniated discs which indicate the disc-specific role for of TNF-α and IL-1β [94].

Along with TNF-α and IL-1β, additional cytokines showing an important role in degeneration are IL-6, IL-17, IL-8 , and IFN-γ [52]. Furthermore, IL-6 increases the catabolic activity of TNF-α and IL-1β in NP cells [95], and it has been implicated in the sensitization of nociceptive neurons noxious stimuli [96]. Therefore IL-6 is believed to have a dual role in catabolic activity and nociception.

### 1.2.4. Matrix Breakdown and Disk Resorption

Disc resorption results when the catabolic activity of enzymes outweighs anabolic activity [97]. Matrix metalloproteases (MMP's), a disintegrin and metalloproteases with thrombospondin motifs (ADAMTS's) are families of enzymes that cleave collagens and proteoglycans, respectively. Disc degeneration is associated with the increase in expression of these proteases, including MMP-1, MMP-2, MMP-3, MMP-9, MMP-13, ADAMTS-4, ADAMTS-5, ADAMTS-7, ADAMTS-12 [98]. Cytokines levels in degenerative discs play an essential role in the upregulation of MMP's and ADAMTS's. The previous study has shown that NP cells stimulated with TNF-α, IL-6, IL-1β showed significant upregulation of MMP-13 [95]. Furthermore, as described above, NF-κB mediated pathways also increase multiple MMP's and ADAMTS's; in fact, an invitro study showed that inhibition of NF-κB mediated pathways decreased IL-1β induced upregulation MMP-3, MMP-9, MMP-13, ADAMTS-4, and ADAMTS-5 [99]. Similar results are also observed after TNF- α stimulation which increased ADAMTS-4 and ADAMTS-5 expression and subsequent degradation of aggrecan and collagen type II [100]. This interplay between proteases and cytokines explains the pathways of disc degeneration, where inflammation is the driving factor for initiating events and further breakdown of ECM.

### 1.2.5. Mechanotransduction as a regulator of IVD cell activity

NP's osmotic properties enable the compressive loads on the spine, and consequent intradiscal pressure exerted by NP is then transferred by the tension that developed between lamellae of the AF [54]. Mechanical loading is a key regulator in IVD tissue homeostasis, and several recent reviews described the biological response of NP and AF cells to mechanical loading [67]. Either underloading or overloading results in a shift of cellular responses to catabolism with increased expression of proteases, inflammatory cytokines, and cell apoptosis [17]. These inflammatory mediators can stimulate the release of different which laters the expression and function of

different ion channels like transient receptor potential (TRP) ion channels. Sodium channels, nociceptors causes sensitization for noxious stimulus and pain hypersensitivity [101]. TRP channels are a family of transmembrane receptors cation-selective with diverse structures, activation mechanisms, and physiological functions [102]. TRP channels function as sensors for various chemical , physical stimuli like temperature, pH, metabolites of plant origin, osmolarity, mechanical stress and regulate the cellular response to stimuli. Nociceptors can adjust their sensitivity following continuous noxious stimuli or tissue injury [103]. Sensitization causes an increase in spontaneous receptor activity, lower activation threshold, and action potential firing [104], which changes the protective behavior response. Hence these changes in local nociceptors and dorsal horn sensitizations causes the initiation and maintenance of hyperalgesia [103]. Therefore TRP channels, especially TRPV1, is a molecular site of sensitization where both inflammatory mediators and noxious stimuli(heat) are required for nociceptor activation [103]. These TRP channels are described for their role in mechanosensing and their active role in transmission of inflammation and pain, recently detected in IVD [102, 105, 106]. Hence the significance of TRP channels as a therapeutic target should be assessed

### 1.3. Imaging Modalities for studying disc pathology:

The critical objective of IVD imaging is to provide a classification scheme for the physician that gives an overview concerning treatment options. The ideal classification system of degeneration is quantitative, avoids observing bias, detect early changes, region-specific evaluation and correlation with clinical symptoms.

**Radiographic Imaging:** X-ray radiographic imaging of disc degeneration using the Thompson grading scale [107] is commonly used to detect overall morphological deformities. However, it cannot depict soft tissue clearly and requires contracting agent injections [86].

**Computed Tomography:** Computed tomography (CT) discography is introduced to improve surgical outcomes [108]. CT is also an invasive technique due to radiation and sometimes requires an injection of the contrasting agent through AF; a further drawback is that its use has high incidence of false positives [109], and it fails to distinguish symptomatic findings incidental. However, Dynamic CT can be employed to measure a range of motions in vivo when a load or torque is applied[110].

**MRI Methods to Investigate Spinal Morphology:** Schneiderman et al. first introduce an MRI-based scale for disc degeneration using T2 signal intensity as a radiological parameter, as T2 signal intensity correlated with disc hydration [111]. With its good soft tissue contrast, MRI has a great potential to characterize disc morphological and functional parameters[112]. Usually MR (T2 weighted) images, the nucleus pulposus appears brighter, and the annulus fibrosus is invisible due to its short T2 signal in normal discs. Degeneration can be quantified by measuring NP reduction in signal intensity [113], with an increase in degeneration is no distinction between AF and NP can be seen in MRI. Pfirrmann introduced the most commonly used grading system, which besides taking T2 signal intensity, also takes disc structure (homogenous or inhomogenous), disc height, the distinction between AF and NP into five grades [114]. Later, it is modified into the Eight grade system by further quantifying the loos of disc height (<30%, 30-60% and >60%) to increase better discrimination in the older population [115]. Further, Riesenburger et al. introduced a classification that includes modic changes that grade end plate changes by considering the signal intensity changes in the vertebral bone morrow , where T2 hyperintense signal at vertebral endplates represents oedema formation [52]. Similar to histological classification, Pfirrmann grading correlates to biochemical changes of IVDs such as MMPs, and BMP expression in animal and human discs [116, 117]. Even though biochemical changes correlate with MRI changes, the Pfirrmann grading system cannot analyze tissue composition, given that biochemical changes occur prior to morphological changes captured by MRI [118]. Recently, quantitative MRI was introduced, and it is based on T1 and T2 relaxation time measurements, which allows direct assessment of biochemical composition and mechanical behavior of IVD [119, 120]. It has been shown that T2 relaxation time positively correlates with disc water content and negatively to disc degeneration [121]. Similarly, T1-rho relaxation time positively correlates with proteoglycan content and negatively degenerative changes [118, 122].

The clinicians are mainly dependent and guided upon by patient history and signs and symptoms about radiological imaging, mainly using MRI. Since neurosurgeons and orthopedic spine surgeons are mainly trained by mechanistic understanding of the pathology, surgical approaches are usually offered to patients suffering from disc pathologies that include surgical removal of disc material, decompression of spinal canal and fusion surgery for degenerated segment [123]. Hence, it is very important to understand other factors like local inflammation that play a crucial

role in disc degeneration. This study is designed to understand the pathological mechanisms depending on the pro-inflammatory profile and other downstream cascade mechanisms that are activated by inflammation in disc degenerated patients and helps provide a change in clinical practice from diagnosis to treatment

# 2. AIMS

**This study aims to understand the molecular mechanisms in pain generation in lumbar intervertebral discs from patients with low back pain and lumbar degenerative changes on MRI undergoing lumbar spinal fusion.**

**The specific aims of the present thesis are threefold:**

1. To evaluate the association of low back pain (LBP) to inflammatory mediators as indexed by mRNA gene expression in the intervertebral disc (IVD). We hypothesize that increased expression of inflammatory mediators (TNF-α, IL-1 and IL-6) in patients with disc degeneration and LBP correlates with pain intensity and disability.
2. To examine the correlation between gene expression of several MMPs and ADAMTSs subtypes in IVD obtained from patients with low back pain (LBP) undergoing lumbar fusion surgery. We hypothesize that increased intervertebral MMPs and ADAMTSs expressions are correlated with a higher degree of tissue degeneration.
3. To investigate the expression of TRP channel subtypes (TRPV1, TRPV4, TRPC6, TRPM2, TRPM8) in intervertebral disc (IVD) biopsies and examine its role in inflammatory pain.

# 3. MATERIALS and METHODS

## 3.1 Inclusion criteria

*Patients with degenerated discs*

We included 48 patients (36F, 12M) of 18-65 years of age with low back pain (LBP) and radiculopathy scheduled to undergo surgery with Posterior Lumbar Interbody Fusion (PLIF) and placement of a Transforaminal Lumbar Interbody Cage (TLIF) and presenting with disc degeneration on MRI at the surgical level at Center of Rheumatology and Spine Diseases, Rigshospitalet – Glostrup. The preoperative magnetic resonance image (MRI) and lumbar X-rays evaluated spondylolisthesis, instability, foraminal stenosis.

**Exclusion criteria**

Patients with prior lumbar surgery, other known rheumatoid disease, infections and severe systemic diseases such as liver- or kidney diseases, hematologic diseases or known cancer and currently in treatment with steroids are not included in the study

## 3.2 Rating Scales

One to two weeks prior to surgery, patients participated in preparation for surgery where patients were asked to fill out the questionnaires accessing the duration of back and leg pain, including separate scores of back and leg pain on a 100mm visual analogue scale (VAS) where 0 indicated "no pain" and 100 represented "the worst imaginable pain"[124]. In addition, Oswestry Disability Index (ODI) (ODI 0–100) questionnaires were used to assess the disability due to LBP (ODI 0–100), which ranges from (0 = no disability–100 maximal possible disability)[125]. Furthermore, EQ-5D-3L was also used which access the general health status of patients based on five dimensions mobility, self-care, usual activities, pain/discomfort and anxiety/depression, with three levels for each scale, and where the summary index score range from 1 (no problems/perfect health) to 0 (health status equivalent to death) [126]. The questionnaires mentioned above will also be sent to the included patients one year after the surgery for follow-up.

### 3.3 Pfirrmann MRI Grading System

Pfirrmann MRI grading system is broadly used for disc degeneration and regeneration studies in the human and animal spine, and it showed higher inter and intraobserver agreement [114, 127, 128]. The classification is based on disc height, structure, signal intensity, and the distinction between AF and NP on T2 weighted images. A Pfirrmann grades I and II indicates a non-degenerative disc, whereas grade III, IV, and V signify an increasingly degenerative disc

| Grade | Structure | Distinction of nucleus and annulus | Signal intensity | Height of intervertebral disc |
|---|---|---|---|---|
| I | Homogeneous, bright white | Clear | Hyperintense, isointense to cerebrospinal fluid | Normal |
| II | Inhomogeneous with or without horizontal bands | Clear | Hyperintense, isointense to cerebrospinal fluid | Normal |
| III | Inhomogeneous, gray | Unclear | Intermediate | Normal to slightly decreased |
| IV | Inhomogeneous, gray to black | Lost | Intermediate to hypointense | Normal to moderately decreased |
| V | Inhomogeneous, black | Lost | Hypointense | Collapsed disc space |

Abbreviations: MRI, magnetic resonance imaging.

Table 1: MRI-based Pfirrmann grades scales [52]

### 3.4 Quantitative reverse transcription-polymerase chain reaction (RT-qPCR)

RT-qPCR addresses the requirement for quantitative data analysis in molecular medicine, biotechnology, and diagnostics and becomes the choice method for mRNA quantification [129]. Using PCR, a specific sequence with in DNA or complementary DNA (cDNA) template can be copied and amplified to a million-fold using sequence-specific oligonucleotides, heat-stable DNA polymerases, and thermal cycling. While using the traditional endpoint PCR, detection and quantification were performed after the amplification by gel electrophoresis and image analysis. However, in real-time or quantitative PCR (real-time PCR or qPCR), the PCR product is measured and quantified at the end of each cycle.

Real-time PCR (qPCR) uses fluorescent reporter dyes, and assay relies on measuring the increase in fluorescent signal, which is directly proportional to the amount of DNA produced during each PCR cycle. Individual reactions are characterized by a PCR cycle at which fluorescence rises above a defined threshold background fluorescence, a parameter known as threshold cycle (Ct) or

crossing point (Cp). The higher the target template the lower the Ct. Fluorescence is plotted against the cycle number, the system generates an amplification plot that represents the accumulation of product during the PCR run.

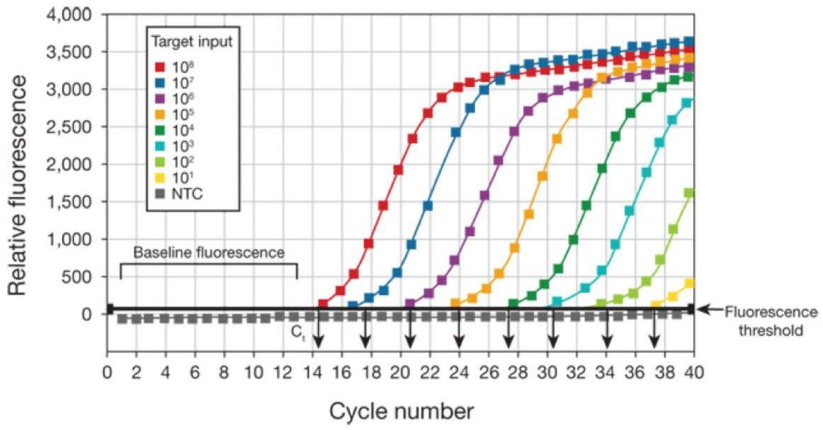


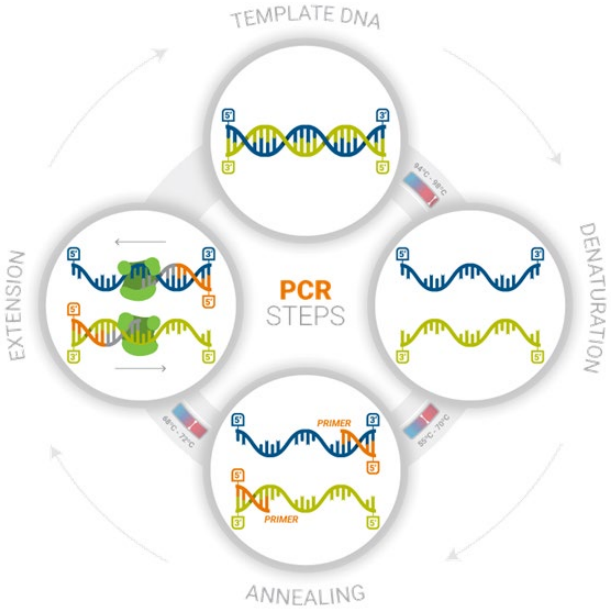

***Fig 7. Relative fluorescence vs cycle number.*** *An illustration showing the fluorescent signal from each sample is plotted against the cycle number. Adopted from https://www.rhenium.co.il/curveshapes/*

Three essential steps make up each cycle in the real-time PCR reaction and generally run for 40 cycles.

**1. Denaturation**: Incubation at higher temperature is used to "melt" dsDNA into single strands (ssDNA) and loosen the secondary structure in ssDNA. Usually, the DNA polymerase can withstand without losing activity is typically around 95°C. By increasing the GC content the denaturation temperature can be increased.

**2. Annealing:** Complementary sequences can hybridize during this step, so an appropriate temperature is used based on the primers' melting temperature (Tm). Usually, five °C below the ($T_m$) of the primer will be selected.

**3. Extension:** The activity of DNA polymerase is optimal at 70–72°C, and primers extension occurs at a rate of up to 100 bases per second [129].




508

509

510 ***Fig 7: Illustration showing PCR steps in each cycle, adopted from https://rise.articulate.com/share/Real-time PCR***
511 ***Handbook 2.0***

512 Quantitative reverse transcription PCR (RT-qPCR) is used when the starting material is RNA,
513 where RNA is first transcribed into cDNA by reverse transcriptase, then it is used as a templet for
514 qPCR reaction.

515 **3.4.1 RNA Extraction Protocol for IVD Biopsy's (AF And NP)**

516 1. AF and NP biopsies were weighed (80mg±20) and were further used for RNA extraction.

517 2. Initially, the tissue was digested at 37°c on a waving shaker set at 40rpm for 1 hour in
518 DMEM containing 2mg/ml pronase (Qiagen). 5ml of pronase solution for 100mg of
519 tissue.

520 3. Digestion was terminated by adding 10% FBS (0.5ml FBS/5ml solution) when the tissue
521 appeared nearly digested, and it was washed with PBS twice.

4. Leftover PBS was aspirated, and 1ml TRI reagent was added and homogenized using T 10 basic ULTRA-TURRAX®(IKA) for 1 min until all the tissue was homogenized completely.

5. The homogenate was incubated for 5 min at room temperature and centrifuge at 12000g for 10 min at 4°c. Then, the supernatant was carefully removed and used for phase separation.

6. 0.2ml of chloroform per ml of TRI reagent was added to the supernatant and shaken vigorously for 15 sec and allowed to stand for 15min at room temperature. The resulting mixture was centrifuged at 12000g for 15min at 4°c.

7. The upper aqueous phase was transferred to a fresh tube, and 0.5ml 2-propanol per ml of TRI reagent was added and mixed properly and allowed to stand for 30min on dry ice and centrifuged at 12000g for 10 min at 4°c.

8. Remove the supernatant and wash the RNA pellet by adding 1 ml of 75% ethanol per 1 ml of TRI Reagent. Vortex the sample and then centrifuge at 7,500 x g for 5 minutes at 4°C. If the pellet floats, then centrifuge at 12,000 *x g*.

9. Remove ethanol and put samples in the water bath/heat block at 65°C for 10 minutes.

10. Take samples from the water bath/heat block and add 20 ul of DNase and RNase free water.

11. Take samples to Nanodrop and determine their concentrations. Use DNase and RNase free water as blank.

### 3.4.2 Assessment of Nucleic Acid Purity and concentration

RNA content was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific). Nucleic acid and proteins have maximum absorbance at 260nm and 280nm, respectively. Historically, the absorbance ratio is used to measure purity in nucleic acid and protein extraction. A ratio of ~2.0 is generally accepted as "pure" for RNA. Similarly, the absorbance at 230nm is accepted as the result of other contamination; therefore, the ratio of A260/A230 is frequently also calculated and is commonly in the range of 2.0–2.2. However, residual chemical contaminations from extraction procedures may overestimate nucleic acid concentration and influence the downstream analysis. For example, a low ratio of A260/A280 may result from contamination absorbing at 280nm or less; this can be due to residual phenol contamination associated with reagents used in the extraction process. Similarly, a low ratio of A260/A230 may result in contamination absorbing at 230nm or less, and this can be due to carbohydrate carryover, residual phenol, residual guanidine and glycogen used for precipitation in some protocols.

### 3.4.3 cDNA synthesis

cDNA was synthesized from 0.2µg of RNA using ImProm-II™ Reverse Transcription System (Promega, USA). After the concentration was measured using a spectrophotometer (NanoDrop, USA), samples were mixed with 1 µl Oligo(dT)15 primer (0.5 µg/reaction) and incubated at 60 °C for 5 minutes in an iCycler (Bio-Rad) and placed on ice for 5 min after the incubation. Then reverse transcription reaction mix (ImProm-II™ 5X Reaction buffer, MgCl2, dNTP mix, recombinant RNasin ribonuclease inhibitor, ImProm II™ Reverse transcriptase, nuclease-free water) was added to each sample. Finally, the samples are placed in the iCycler for cDNA synthesis. The cycles used for the reverse transcription were: 5 min at 25 °C, 60 min at 42 °C and 15 min at 70 °C the cDNA samples were placed on ice for 2 minutes, then added 80 µl 0.5xTris-EDTA buffer (Fluka 93302) and stored at -20 °C.

Primers were designed using Primer-BLAST [130], and following criterias are considered: Primer length 18-25bp, product size 120-200bp, melting temperature (Tm) 62-65 °C, exon-intron junction spanning, GC content 50-60% [131] and purchased from LGC Biosearch technologies (Copenhagen, Denmark). In addition, primers were accepted if no primer-dimers were present and had an efficiency between 1.8-2.1.

### 3.4.4 RT- qPCR

RT-qPCR was performed in 20µl of reaction mixture consisting of 5 µl cDNA and 15 µl master mix (iQ SYBR® Green Supermix (BioRad, California, USA) and 15pmol of each primer using a Light Cycler 480 Real-Time PCR System (Roche Diagnostics, IN) and Quantstudio™ 5 (Thermofisher) for 40 to 50 cycles in a fixed sequence at 94 °C for 30 seconds, 60 °C for 15 seconds, and 72 °C for 15 seconds followed by a melting curve programme. All samples, no templet control (NTC), were run in triplicates, and an inter-run calibrator (IRC) was also run on each plate, enabling us to compare the plates. Amplicons were generated using the primer sets listed in Table:2. Then, we started performing the gene expression analysis by validating five housekeeping genes, a detailed validation analysis attached in the annexure. We have selected three stable expressing genes in IVD, i.e., Succinate Dehydrogenase Complex Flavoprotein Subunit A (*SDHA*), Lactate dehydrogenase A (*LDHA)* and Beta-Actin (*ACTB*) and expression of all the cytokines was measured relative to the geometric mean of 3 housekeeping genes.

| Gene | Forward (5`- 3`) | Reverse (5`- 3`) |
|---|---|---|
| **TNF- α** | TTC CTG ATC GTG GCA GGC | GCT GAT TAG AGA GAG GTC CCT G |
| **IL-6** | TTC GCT CTT CCA GTT GGA CT | CAC CAG GGG AAG AAT CTG AG |
| **IL-1β** | ACC AAG TGC CAC AAA GGA AC | CTG CAA TTG AAG CAC TGG AA |
| **SDHA** | CGA GCT GCA TTT GGC CTT TC | TTG ATT CCT CCC TGT GCT GC |
| **LDHA** | GCC TGT ATG GAG TGG AAT GAA | CCA GGA TGT GTA GCC TTT GAG |
| **β-Actin** | TGG AAC GGT GAA GGT GAC AG | AAC AAC GCA TCT CAT ATT TGG AA |
| **TRPV1** | CTG CCC GAC CAT CAC AGT C | CTG CGA TCA TAGAGC CTG AGG |
| **TRPV4** | CTA CGG CAC CTA TCG TCA CC | TTA GGC GTTT TCT TGT GGG TCA |
| **TRPC6** | ATC TTG ACG GAG AGT GCG G | CTG GTG GTA GCA AAG CGT A |
| **TRPM2** | ACC CTT TTA CAC GGC AGA GA | TAC TGG ATC GTG GAC AGT GG |
| **TRPM8** | GCA AGT GTG GCT ATG CCC A | CCA AAG GCG TCG GTA GGA A |
| **Aggrecan** | GTG CCT ATC AGG ACA AGG TCT | GAT GCC TTT CAC CAC GAC TTC |
| **MMP- 1** | GCC ATC ACT TAC CTT GCA CT | AGA CAC CAC ACC CCA GAA CA |
| **MMP-3** | TCC TAC TGT TGC TGT GCG TG | AGG TTC ATG CTG GTG TCC TC |
| **MMP-2** | TAC AGG ATC ATT GGC TAC ACA CC | GGT CAC ATC GCT CCA GAC T |
| **MMP-10** | TGA GTT TGA CCC CAA TGC CA | GTC TTC CCC CTA TCT CGC CT |
| **MMP-13** | GGC TTA GAG GTG ACT GGC AA | ATC AGG AAC CCC GCA TCT TG |
| **ADAMTS-1** | AGG ATG AAA CGC CGG AAC AA | CCC CAC CAC AAG ACA AGT GA |
| **ADAMTS-4** | ACT GGT GGT GGC AGA TGA CA | TCA CTG TTA GCA GGT AGC GCT TT |
| **ADAMTS-5** | TCG GGA GGA TTT ATG TGG GC | TGG AAT CGT CAT GGG AGA GG |

***Table:2 List of primers used in this study***

### 3.5. Statistical analysis

Analysis was performed with the GraphPad Prism 8.2 software. Data are expressed as mean ± SD. An unpaired student t-test evaluated group differences in mRNA expression. The correlation of mRNA levels and clinical data was assessed using the Pearson (r) or Spearman's correlation (ρ) coefficient. Similarly, a p-value of $< 0.05$ is considered statistically significant for all comparisons and for posthoc analyses, the p-value is uncorrected for multiple comparisons.

# 4. RESULTS

In the following chapter, the main findings from three studies are presented. Further results are contained within each paper in Appendix

## 4.1. Study I

Study 1 evaluated the association between expression of inflammatory cytokines (TNF-α, IL-1β and IL-6), clinical symptoms (pain intensity and disability) in 34 LBP patients [9 M, 25 F] undergoing 1, 2 levels posterior lumbar instrumented spinal fusion surgery. All the patients reported pain intensities for back pain and leg pain, and the mean (SD) reported scores by VAS were 63 ± 28 and 60 ± 25, respectively. The mean (SD) disability reported by ODI scores by all patients is 41 ± 15. We successfully extracted RNA from 33 AF to 32 NP biopsies and showed that TNF-α, IL 1β and IL-6 were expressed in these tissues. In addition, the expression of TNF-α and IL-6 was significantly higher in AF than in NP.

First, our RT-qPCR analysis showed a significant correlation between pain intensity (VAS) and the expression of TNF-α (Fig. 8a) and IL-1β (Fig. 8b) in AF. Similarly, in NP, the correlation observed for TNF-α (Fig. 8d), and IL-6 (Fig. 8e) with VAS scores supports inflammatory mediators' involvement in pain generation.

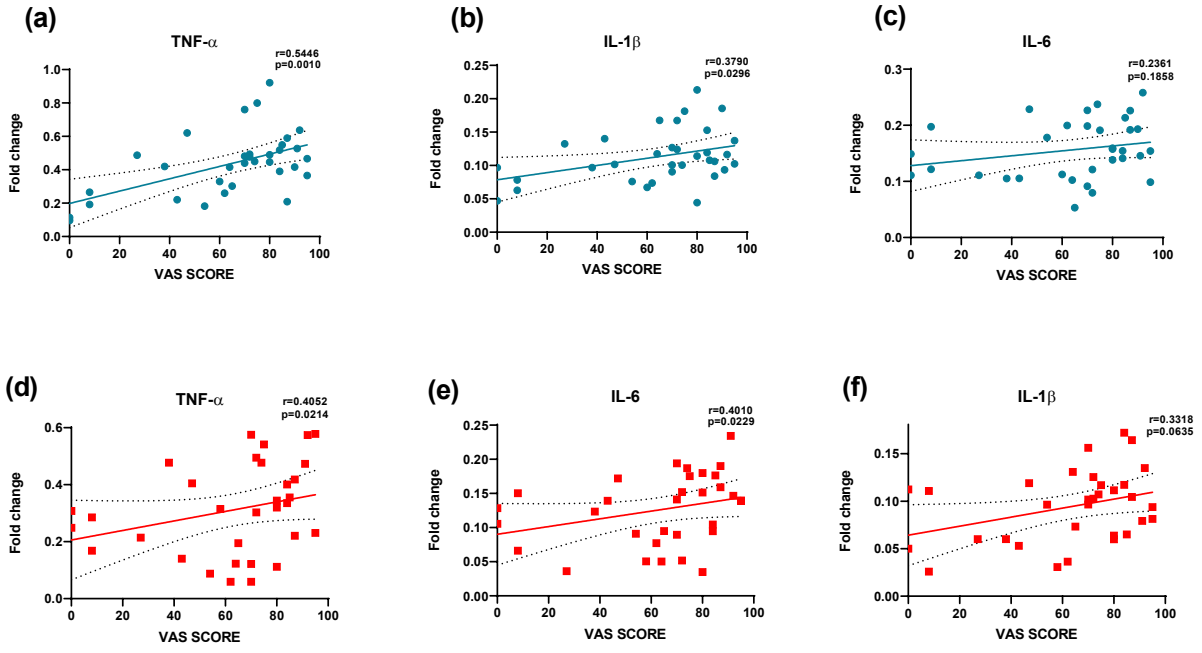


***Fig 8: Association between pro-inflammatory cytokine expression and pain intensity in the AF and NP.*** *In AF, VAS score (1 day before surgery) showed positive correlation with (a) TNF-α (r = 0.54, p = 0.001), (b) IL-1β (r = 0.37, p = 0.029) and (c) no correlation observed with IL-6. Similar trends were observed in NP, VAS score (1 day before surgery) showed positive correlation with (d) TNF-α (r = 0.40, p = 0.02), (e) IL-6 (r= 0.40, p = 0.02), (f) no correlation observed with IL-1β. p < 0.05 is considered statistically significantly for all comparisons. r = Pearson correlation coefficient*

Second, we found a significant positive correlation between IL-6 and ODI scores in AF and NP. By contrast, TNF-α and IL-1β expression were not correlated with ODI scores in both regions.

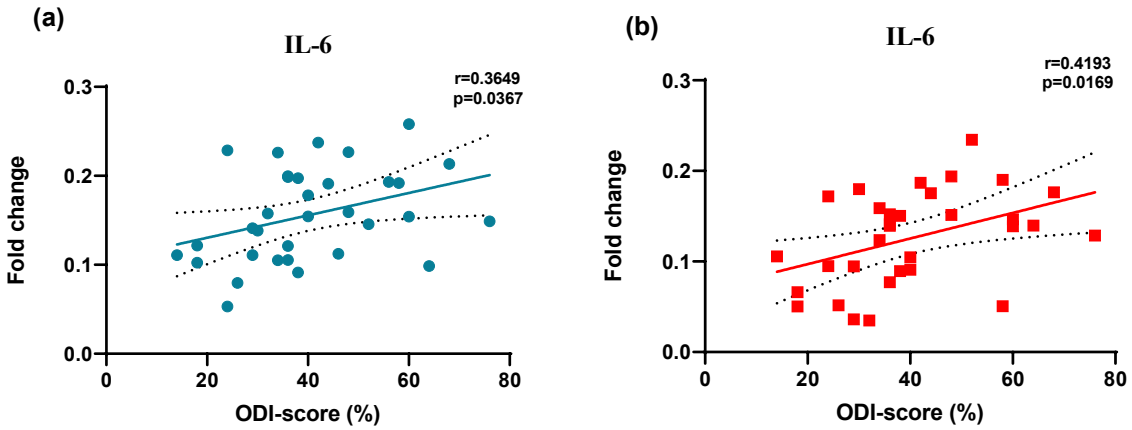



***Fig 9: Association between pro-inflammatory cytokine expression and Oswestry disability index (ODI) in the AF and NP:*** *(a, b) In AF and NP, ODI score (1 day before surgery) showed a significantly positive correlation with IL-6 (r = 0.36, p = 0.03), (r = 0.41, p = 0.01) respectively. No other pro-inflammatory cytokine correlated with ODI scores in both AF and NP. p < 0.05 is considered statistically significant for all comparisons. r = Pearson correlation coefficient*

Third, we conducted a post-hoc analysis of the intercorrelation between mRNA expression of three cytokines (TNF- α, IL-1β and IL-6) in both the tissue samples; we found a significant positive correlation between TNF- α and IL-1β in AF, the similar correlation observed between TNF- α and IL-6 in NP.

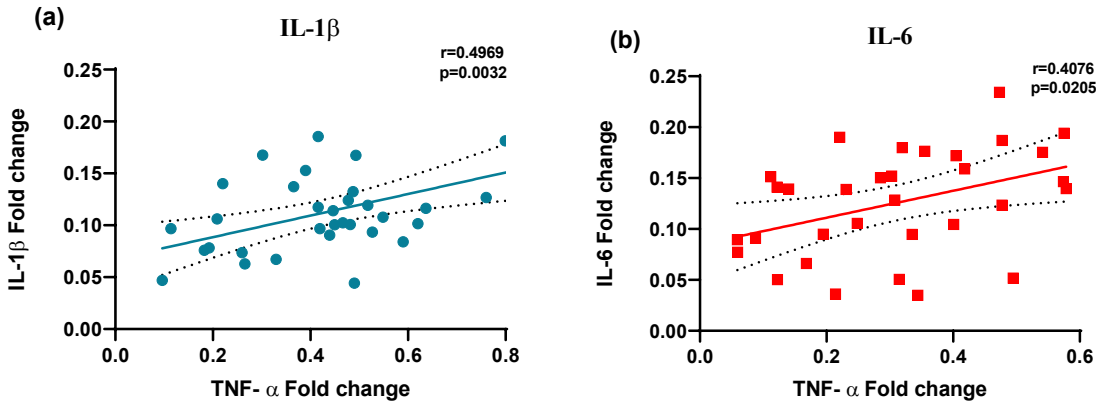



***Fig 10: Association between pro-inflammatory cytokine expression in AF and NP:*** *(a) In AF, TNF-α showed a significant positive correlation with IL-1β (r = 0.49, p = 0.003), but no correlation was observed with IL-6. (b) In NP, TNF-α showed a significant positive correlation with IL-6 (r = 0.40, p = 0.02), but no correlations observed with IL-1 β. p < 0.05 is considered statistically significantly for all comparisons. r = Pearson correlation coefficient*

## 4.2. Study II

In study II, we evaluated the association of MMP and ADAMTS subtypes expression with disc degeneration disease in 34 LBP patients [9 M, 25 F] of 18-65 years of age with low back pain (LBP) and radiculopathy scheduled to undergo surgery with Posterior Lumbar Interbody Fusion (PLIF). Gene expression was demonstrated for different MMP transcript subtypes (1, 2, 3, 10, and 13) and ADAMTS subtypes s (-1, -4, and -5) in NP.

First, We found a significant positive correlation between the degree of degeneration assessed using the 5-level Pfirrmann MRI grading system and expression of MMP-1, MMP-3, MMP-10, MMP-13, ADAMTS-1, ADAMTS-5 (Fig 11 a-f). Furthermore, a posthoc analysis showed a positive correlation between degeneration grade and expression of pro-inflammatory cytokines like TNF-α and IL-6 (Fig 11 g-h): We did not find any significant correlations for MMP2, ADAMTS-4, IL-1β, NGF or BDNF.

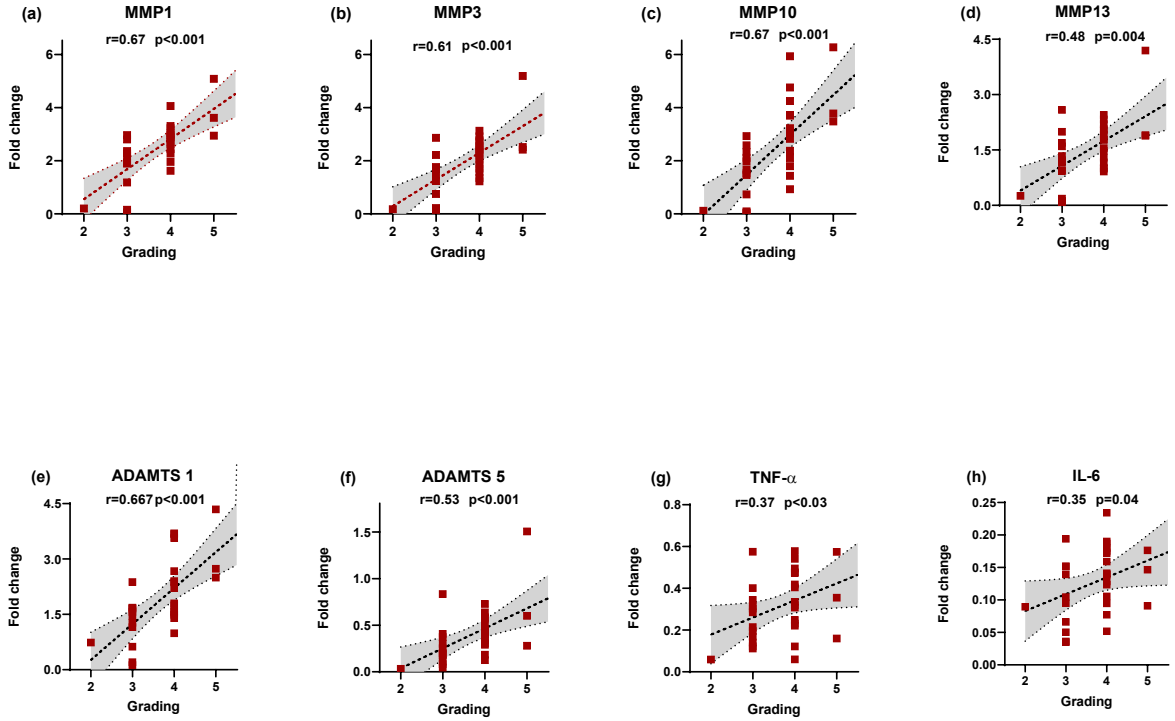



***Fig 11: Expression of MMPs and ADAMTSs correlate with Pfirrmann MRI grades:*** *Significant positive correlation observed between Pfirrmann MRI grades and expression of (a) MMP-1 (ρ=0.67, p<0.0001) , (b) MMP-3(ρ=0.61, p=0.0002), (c) MMP-10(ρ=0.671, p<0.0001), (d) MMP-13 (ρ=0.48, p=0047), (e) ADAMTS-1(ρ =0.679, p<0.0001) , (f) ADAMTS-5 (ρ=0.53, p=0.001), (g)TNF-α (ρ=0.37, p<0.03) and (h) IL-6 (ρ=0.35, p=0.04) . p<0.05 (two tailed) are considered statistically significant for all comparisons. ρ=spearmen's correlation coefficient.*

Second, we found significant positive correlations in our posthoc pair-wide correlation analysis between the expression of MMPs and ADAMTSs (Fig 12 a&b)

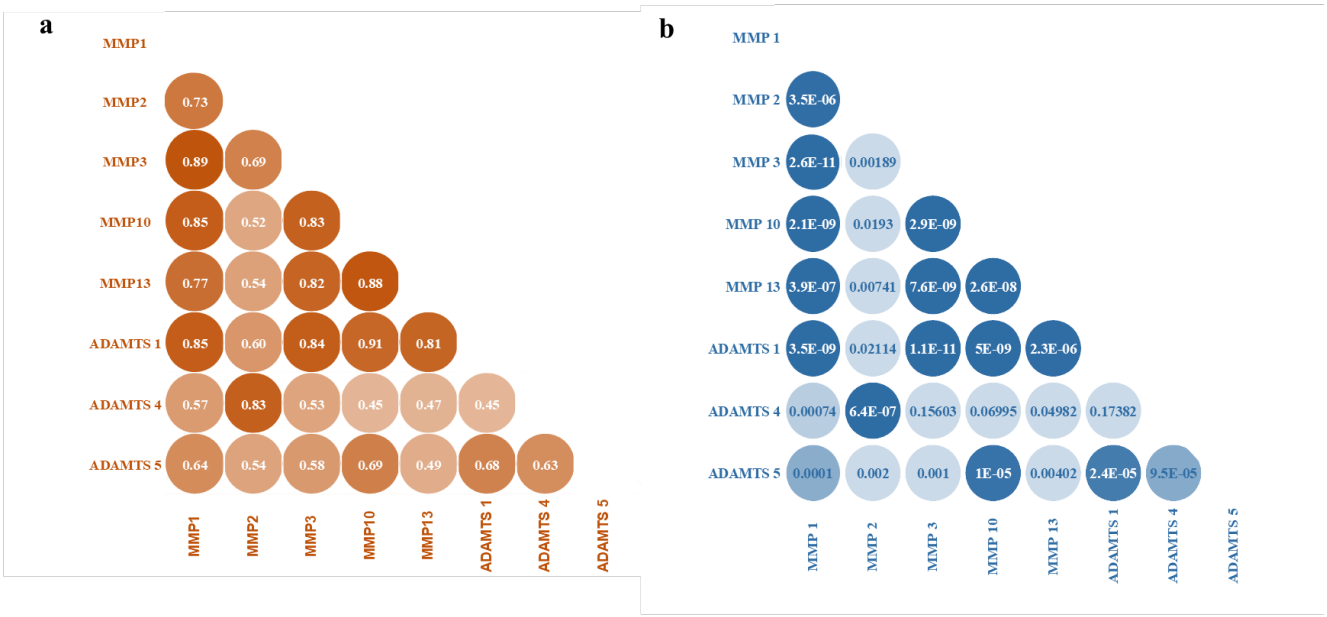
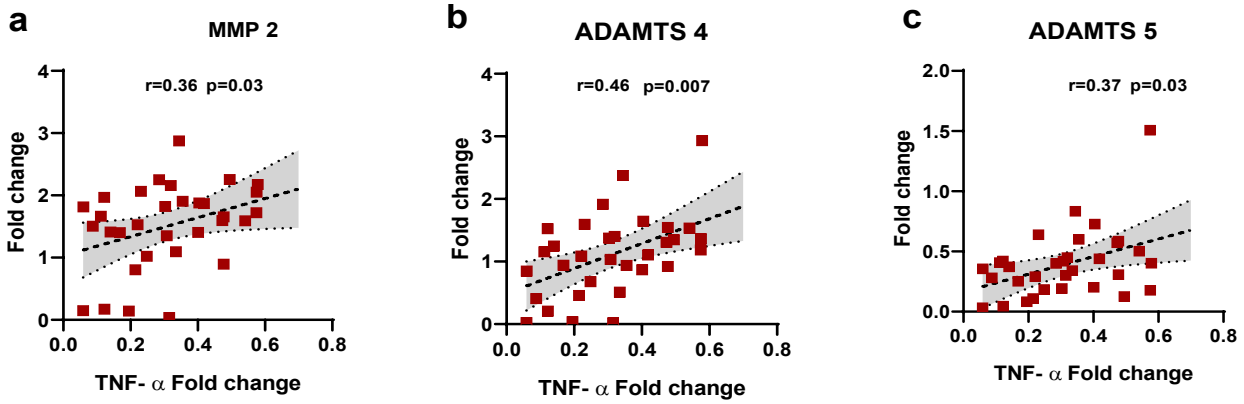



***Fig 12: Significant inter-correlations between expression MMPs and ADAMTSs:*** *(a) correlations(ρ) between MMPS and ADAMTSs, and (b) corresponding significance values(p). p<0.05 (two-tailed) are considered statistically significant for all comparisons. ρ=spearmen's correlation coefficient*

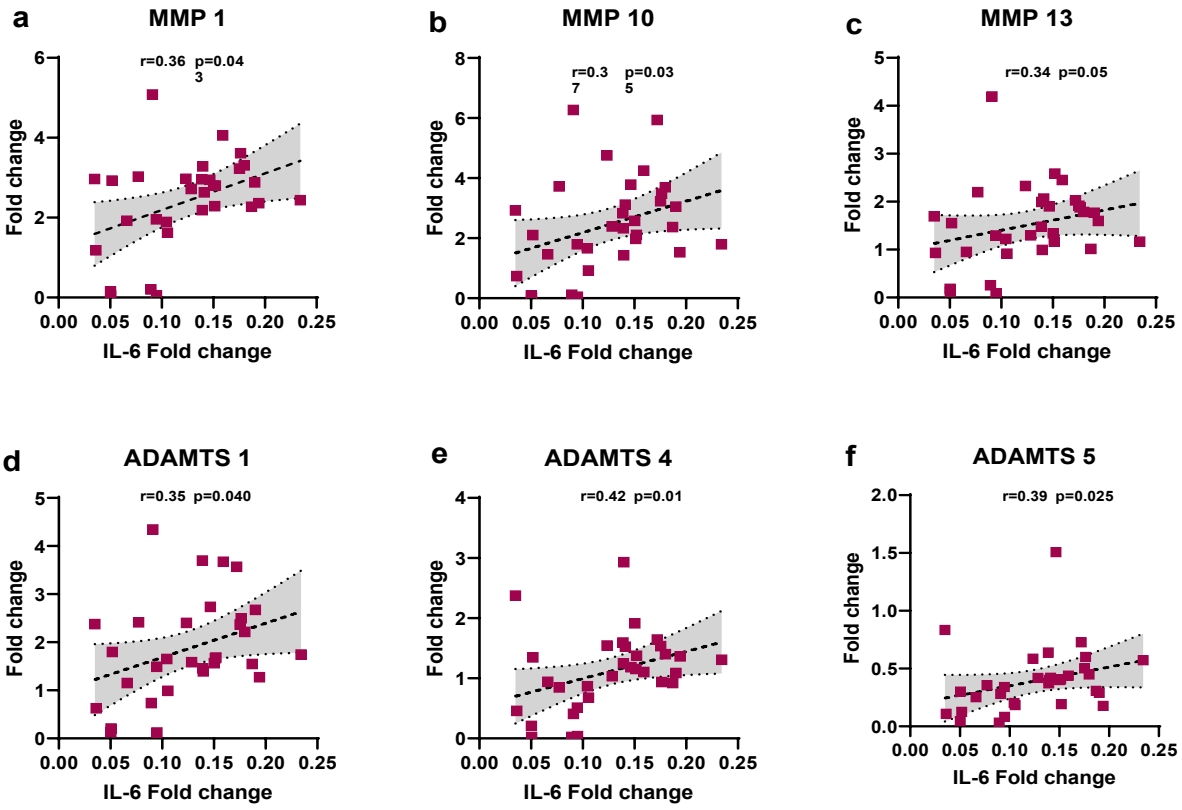
Third, we found an association between the expression of individual cytokine mRNA's expression and the expression of different MMPs and ADAMTSs. We found a strong positive correlation between the expression of TNF-α and MMP-2, ADAMTS-4, and ADAMTS-5 (Fig. 13a-c), but not to the other proteases. Similarly, we found significant positive correlations between IL-6 and MMP-1, MMP--10 MMP-13, ADAMTS-1, ADAMTS-4, (Fig. 14a-f) but no correlation with MMP (-2, -3, -13). Interestingly, we found a significant negative correlation between the expression of aggrecan and age.




678

679 ***Fig 13: Association between pro-inflammatory cytokine TNF-α expression and MMP and ADAMTS:*** *A post hoc*
680 *analysis of the intercorrelation between TNF-α mRNA expression to the expression of different MMPs and ADAMTSs*
681 *and we found significant positive correlations between TNF-α and (a) MMP2 (r=0.36, p=0.039), (b) ADAMTS*
682 *4(r=0.46, p=0.007), and (c) ADAMTS5 (r=0.33, p=0.03). p<0.05 (two-tailed) are considered statistically significant*
683 *for all comparisons.p=spearmen's correlation coefficient.*

684



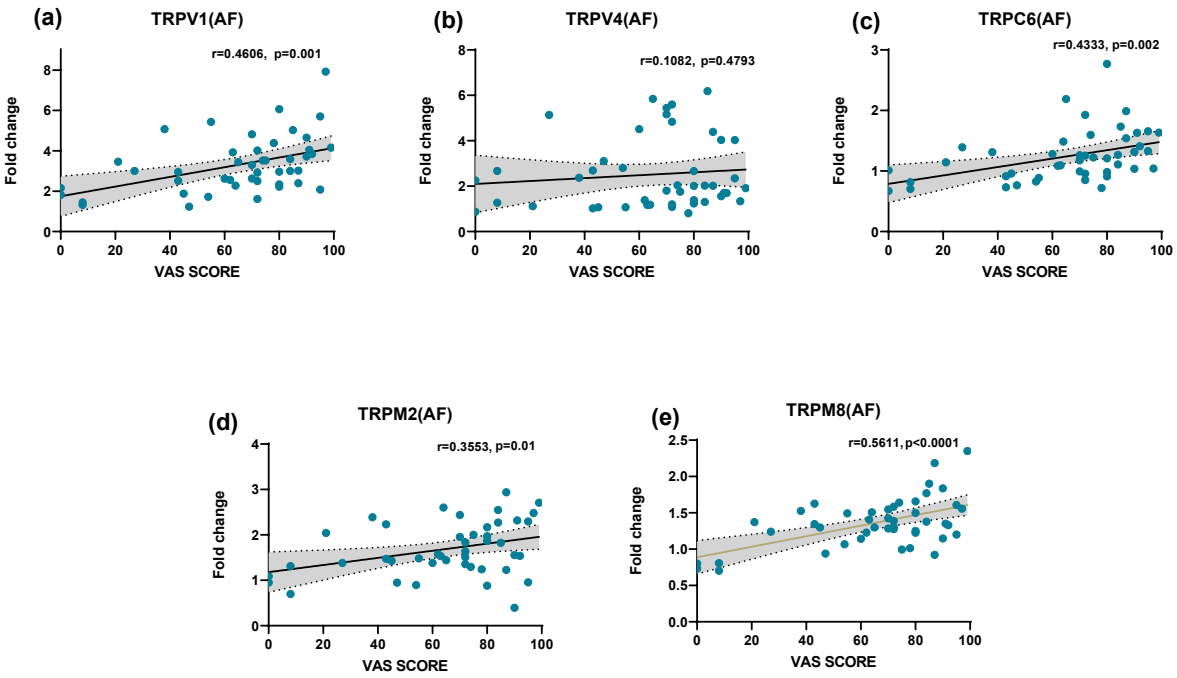

685

***Fig 14: Association between pro-inflammatory cytokine IL-6 expression and MMP and ADAMTS:*** *A post hoc analyses of the intercorrelation between IL-6 mRNA expression to the expression of different MMPs and ADAMTSs and we found significant positive correlations between IL-6 and (a) MMP1(r=0.36, p=0.04) , (b) MMP10(r=0.36, p=0.037), (c) MMP13 (r=0.34, p=0.05), (d) ADAMTS 1 (r=0.35, p=0.049), (e) ADAMTS4 (r=0.42, p=0.016), (f) ADAMTS5 (r=0.39, p=0.024). p<0.05 (two tailed) are considered statistically significantly for all comparisons. ρ=spearmen's correlation coefficient.*

### 4.3. Study III

In Study III, we investigated the expression of TRP channel subtypes (TRPV1, TRPV4, TRPC6, TRPM2, TRPM8) in 48 patients (36F, 12M) with low back pain (LBP) and radiculopathy scheduled to undergo Posterior Lumbar Interbody Fusion (PLIF) surgery and placement of a Transforaminal Lumbar Interbody Cage (TLIF). The patient's average age is 52 ±9 (Ranging from 18-65). Ratings of LBP with VAS for the included subjects was 65 ± 26 (mean ±SD), and the ODI score was 42 ± 17. Our RT-qPCR analysis found the expression of all five TRP channels in both AF and NP biopsies, but an expression of TRPV1 and TRPV4 is higher in AF when compared to NP.

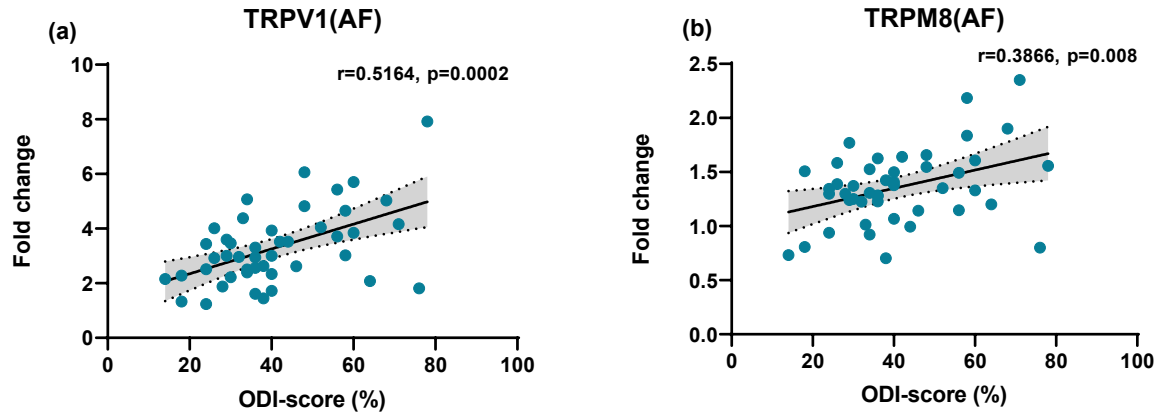
First, we found an association between pain intensity and expression of all but one TRP channel in the AF: TRPV1 (Fig. 15a); TRPC6 (Fig. 15c); TRPM2 (Fig. 15d); and TRPM8(Fig. 15e). No such correlation was found for TRPV4(Fig.15b). The VAS scores for low back pain did not correlate with any TRP channels' expression in NP.




705

706 ***Fig 15: Association between TRP channel expression in AF and VAS score for pain intensity:*** *VAS score (1 day*
707 *before surgery) showed a significantly positive correlation with (a) TRPV1(r=0.46, p=0.001), (b) No correlation*
708 *observed with TRPV4, (c) Positive correlations demonstrated with TRPC6(r=0.43, p=0.002), (d) TRPM2(r=0.35,*
709 *p=0.01), (e) TRPM8 (r=0.56, p<0.0001). No correlations were observed in NP. p < 0.05 is considered statistically*
710 *significant for all comparisons. r = Pearson correlation coefficient*

711 Second, we also observed a significant positive correlation between the ODI score and expression
712 of TRPV1 (Fig. 16a); TRPM8 (Fig. 16b) in AF, but not with TRPV4 TRPC6, and TRPM2. The
713 TRP channels expression does not correlate with ODI scores in NP.




714

***Fig 16: Association between TRP channel expression in AF and Oswestry disability index (ODI):*** *ODI score (1 day before surgery) showed a significantly positive correlation with (a) TRPV1 (r=0.51, p=0.0002), (b) TRPM8(r=0.386, p=0.008). TRPV4, TRPM2, and TRPC6 did not show any correlations in AF. No correlations were observed in NP. p < 0.05 is considered statistically significant for all comparisons. r = Pearson correlation coefficient.*

Third, we further found an association in a follow-up analysis; we showed that the expression of TRPV1 level at baseline was correlated with the ODI score for disability at one-year follow-up after the surgery (Fig 17) (n= 34). However, the expression of other TRP channels in AF and NP was not associated with the outcome scores for pain and disability at one-year follow-up.

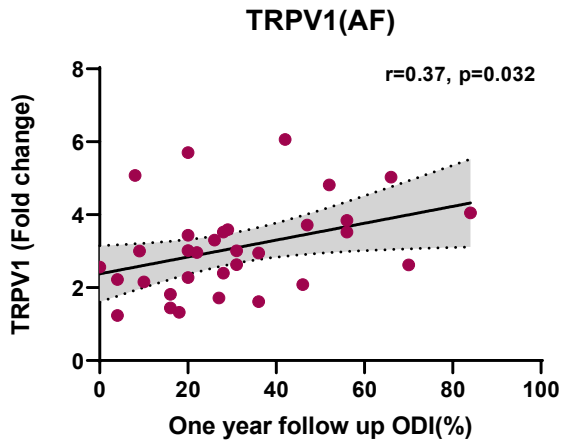



***Fig. 17 Significant positive correlation between TRPV1 channel and one-year follow-up ODI scores (r=0.37, p=0.03).*** *p < 0.05 (uncorrected) is considered statistically significant for all comparisons. r = Pearson correlation coefficient*

Fourth, in order to further elaborate the mechanisms of expression of TRP channels, we conducted a posthoc analysis where we assessed how TRP channels expression correlates to other markers of low back pain and disease, namely pro-inflammatory cytokines, Nerve Growth Factor (NGF), Pfirrmann MRI classification for disc degeneration (grade I-V). The expression of TRPV1 in NP showed a significant positive correlation with Pfirrmann grade (I-V) for disc degeneration; no other markers in the AF nor the NF displayed such correlation. Furthermore, to elucidate the mechanisms of pain generation, we correlated the expression of TRP levels in AF and NP with the most dominant pro-inflammatory cytokines. In the AF, we observed a significant positive correlation between TNF-α and the expression level of TRPV1 (Fig. 18a), TRPM2 (Fig. 18d) and TRPM8 (Fig. 18e) and with TRPV4 (Fig. 18f) in NP. However, even

TRPV4 was not correlated to TNF-α in the AF, it was strongly correlated to IL-1β in the NP (Fig. 19a). With regard to IL-6, only TRPV1 in NP was correlated (Fig. 19b).

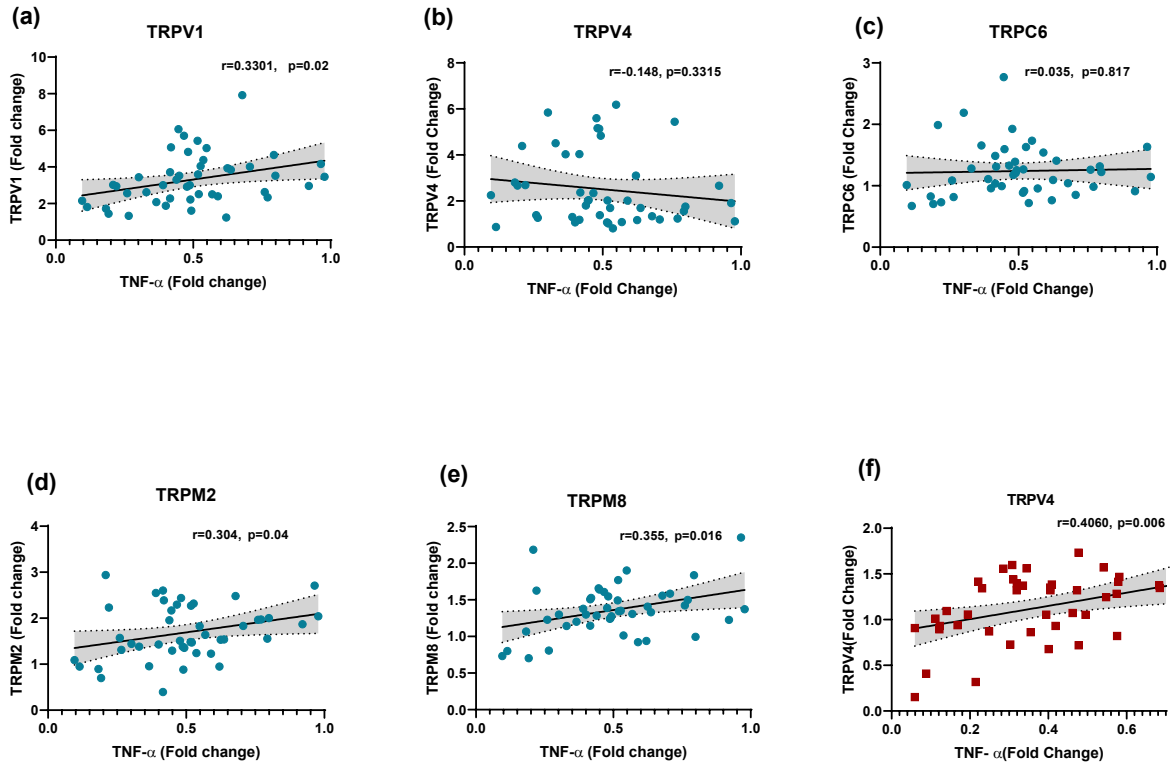



***Fig 18: Association between TNF-α expression and TRP channel expression in AF and NP:*** *In the AF, TNF-α showed significant positive correlation with (a) TRPV1 (r=0.33, p=0.02), (d) TRPM2 (r=0.30, p=0.04), and (e) TRPM8 (r=0.35, p=0.016), and no correlations observed with TRPV4 and TRPC6 (b, c). Furthermore, TRPV4 is the only TRP channel showed correlations with (f) TNF-α (r=0.40, p=0.006) in the NP. $p < 0.05$ (un-corrected) is considered statistically significant for all comparisons. r = Pearson correlation coefficient.*

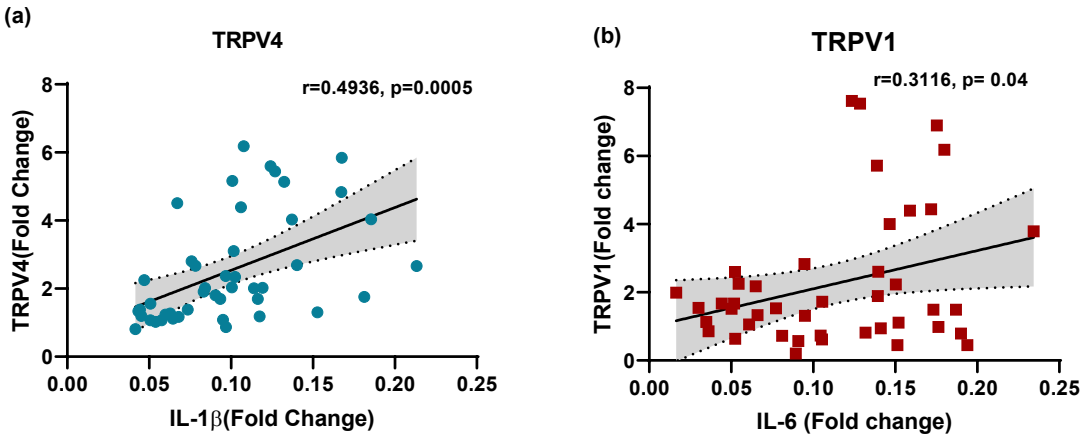



***Fig 19: Association between IL-1β, IL-6 expression and TRP channel expression in AF and NP:*** *In AF, IL-1β showed a correlation with (a) TRPV4(r=0.49, p=0.0005), no other channels showed any correlation with IL-1β in both AF and NP. Further, TRPV1 showed correlation with (b) IL-6 (r=0.31, p=0.04) in NP, and no other channels showed correlation with IL-6 in both AF and NP. p < 0.05 (un-corrected) is considered statistically significant for all comparisons. r = Pearson correlation coefficient.*

Finally, we showed TRP channels expression significantly (uncorrected) correlates with expression of NGF in both AF (TRPV1 and TRPM2) and NP (TRPV1, TRPV4, TRPC6, TRPM2 and TRPM8) (Fig. 20a-g), although the analysis was only performed in a minor portion of the IVD samples (n=34).

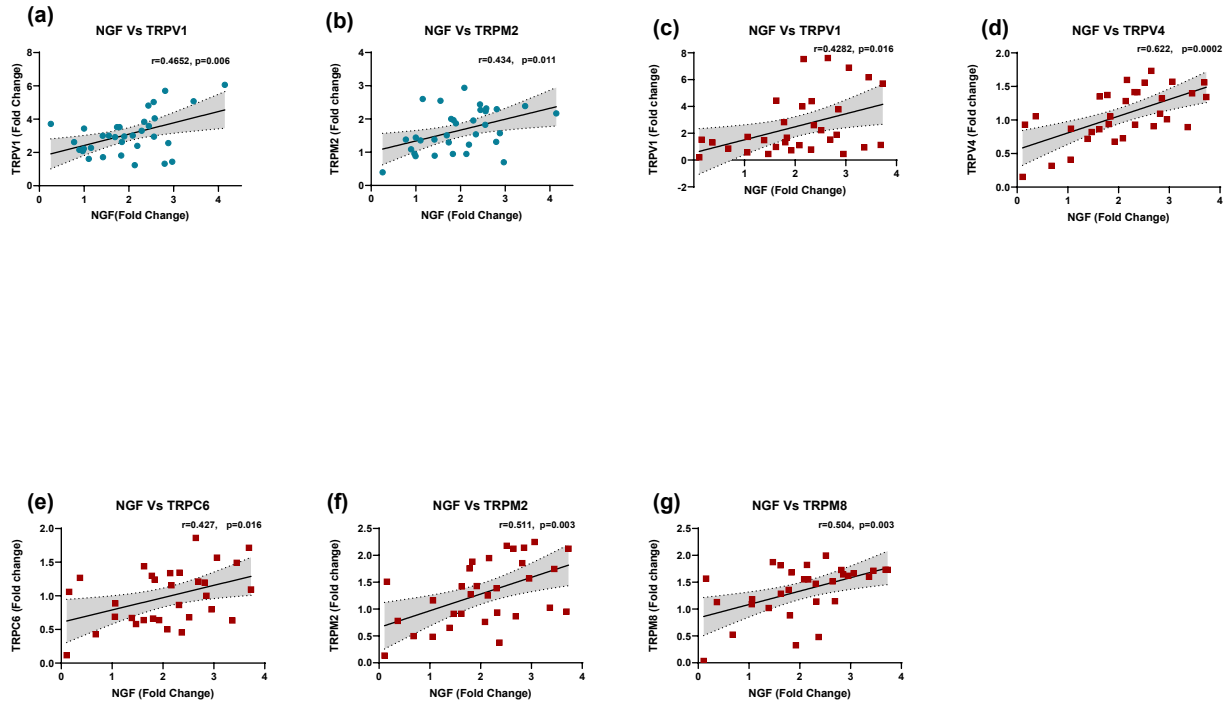



***Fig 20: Association between expression of TRP channels and NGF:*** *Our post-hoc analysis on 34 IVD samples. In AF, expression of NGF showed significant positive correlation with (a)TRPV1 (r=0.46, p=0.006) and (b) TRPM2 (r=0.43, p=0.01), but not with the other channels. In NP, expression of NGF showed significant positive correlation with all TRP channels (c)TRPV1 (r=0.42, p=0.01), (d) TRPV4 (r=0.62, p=0.0002), (e) TRPC6(r=0.42, p=0.016), (f) TRPM2 (r=0.51, p=0.003) and (g) TRPM8 (r=0.50, p=0.003). $p < 0.05$ (uncorrected) is considered statistically significant for all comparisons. r = Pearson correlation coefficient.*

# 5. DISCUSSION

## 5.1. Study I

We observed statistically significant positive correlations between clinical symptoms and expression of pro-inflammatory cytokines in both AF and NP biopsies from LBP patients undergoing surgery for Posterior Lumbar Interbody Fusion (PLIF). Furthermore, TNF-α was the only cytokine among three cytokines that showed positive correlations with pain intensity in both the regions, supporting the previous understanding that TNF-α is implicated in neurogenic, radicular, and low back pain [105, 132]. Furthermore, we found a correlation between TNF-α and IL-1β in AF and IL-6 in NP, which explains the synergistic regulation of IL-1β and IL-6 mRNA expression by TNF-α. These results support previous literature that TNF-α is one of the initiating factors of the inflammatory cascades in IVD [87] and has a significant role in regulating the expression of other pro-inflammatory cytokines like IL-1β and IL-6 [133] which might play an essential role in disc degeneration pathogenesis.

We also observed a significant positive correlation between the expression of IL-6 and the ODI score. Interestingly IL-6 is the only cytokine that showed a positive correction with disability scores, and TNF- α only correlates with pain scores suggests that the pain and disability are not strongly correlated biochemically, which corresponds to what is known from the clinical perspective they are partially overlapping. Our results help to understand the critical role of IL-6 in disability in disc degeneration. Furthermore, we have seen an association between pain intensity and expression of IL-6, suggesting the role of IL-6 in pain generations and modulation as it is shown previously by Koch et al. that circulating IL-6 levels correlated with chronic pain levels [134] and it is an association with neuropathic pain in osteoarthritis (OA) [135].

The molecular mechanisms of disc degeneration are not completely understood, and our results showed the role of cytokines in pain intensity and disability, which further increase the knowledge in disc degeneration aetiology and suggest that cellular cascades caused by inflammatory cytokines can be a link between LBP and inflammation in DDD patients. Understanding the downstream cytokine mechanisms is essential for developing a potential therapeutic approach, but cytokines have complex mechanisms regulating inflammation, so targeting a single cytokine might not work

efficiently. Hence targeting multiple cytokine strategies might help treat the pain associated with inflammation.

### 5.2. Study II

We have found a significant positive correlation between Pfirrmann MRI grades and the expression of several MMPs and ADAMTSs, suggesting that disc degeneration is strongly associated with an increase in the expression of ECM degrading proteases. MMPs and ADAMTSs are a family of enzymes that cleaves collagens and proteoglycans, respectively. In healthy discs, there is careful regulation between growth factors and catabolic enzymes maintenance homeostasis in disc health; any loss of collagen and proteoglycans is the key to developing DDD [98, 136, 137]. Our results support previous findings which have indicated that loss of ECM by proteolytic cleavage is critical in degeneration leading to loss of function and pain generation [75, 138-140]

We observed an association between MMP-1 and degeneration grade. MMP-1 belongs to a subclass of collagenases that break down the fibrillar collagens types I, II, and III [141]. Previous studies also showed that increasing degeneration increases the MMP-1 immunoreactivity in NP [98]. Similarly, MMP-3 and -13 also showed a significant positive correlation with degeneration grade. Furthermore, previous studies have shown more MMP-13 immunoreactive cells within the NP of degenerated discs [140, 142], and a positive correlation between MMP-3 expression and MRI grades in the degenerated disc [116]. Together, these results suggest an increase in expression of MMPs further increase the destruction of ECM, mainly breakdown of collagen II which is a significant component of NP matrix.

We observed a significant positive correlation with the expression of ADAMTS-1 and 5 to the Pfirrmann MRI grading system. ADAMTSs have an essential role in the degradation of aggrecan [143, 144], a significant component of NP and collagen. Increased expression of ADAMTSs increase the loss of aggrecan, resulting in a decrease in water holding capacity of NP, altered diffusion of nutrients, loss of disc height and inability to withstand compressions which leads to DDD [145].

We also observed an association between the expression of pro-inflammatory cytokines and MMPs and ADAMTSs. This association is significant because cytokines like TNF-α induce the activity of MMP-2 mainly through extracellular signal-regulated kinase pathways [146]; previous

studies showed that TNF-α and IL-1β regulate the expression of ADAMTS-4/5 through Mitogen-activated protein kinase (MAPK) and nuclear factor-kappa light chain enhancer of activated B cells (NF-κB) signaling pathways [147]. Together, our findings suggest that the key role of cytokines in ECM metabolism enables us to propose the role of intracellular pathways like MAPK, NF-κB pathways in regulating the expression of protease and cytokines.

### 5.3. Study III

We have demonstrated the expression of TRP channels in IVD tissue and showed the expression is associated with clinical rating scores for pain (VAS) and disability (ODI), Pfirrmann MRI classification for disc degeneration. This study investigated five different TRP channels (TRPV1, TRPV4, TRPC6, TRPM2, TRPM8) in IVD biopsies. Our analysis showed that TRPV1 expression was positively correlated with pain (VAS) and disability (ODI) scores in AF and with degeneration grade in NP, supporting the previous finding. In addition, we found a similar positive correlation between pain intensity and expression of TRPC6. TRPM2 and TRPM8 suggest that TRP channels in nociceptive mechanisms in degenerated discs.

We have also seen that TRPV1 and TRPV4 displayed a correlation with the expression of TNF-α and IL-6, and only IL-1β showed a correlation to TRPV4, which suggests the essential role of pro-inflammatory cytokines in the expression of TRP channels and its regulatory role in pain mechanisms [147, 148]. Previous studies have shown that TRPV1 channels are located on dorsal root ganglion neurons that innervate IVD and involve nociception [149] and non-neuronal cells like chondrocytes and IVD cells [105, 150, 151]. Furthermore, studies have shown that TRPV1 channels are responsive to inflammation, and an increase in inflammatory stimulus increased the expression of TRPV1, causing chronic inflammatory pain in rats. A possible reason for channel activation can be acidic pH(≤5.9), due to accumulation of lactic acid due to poor clearance of fluids in degenerated IVD results in transient increases in intracellular calcium ($iCa^{2+}$), which subsequentially increase in expression of cytokines, matrix metalloproteinases (MMPs), growth factors, and prostaglandins (PG) which are involved in cell hemostasis as well as inflammation [63, 152, 153]. Similarly, we have seen an association between TRPV4 expression and IL-1β and TNF-α in AF and NP, respectively; these results support an in-vitro study conducted by Walter et al. suggested that increase in TRPV4 expression increase expression of TNF-α, IL-1β and IL-6

and also showed an increase in expression of TRPV4 is associated with loss of aggrecan and other glycosaminoglycans (GAG's) and decrease in tissue osmolarity with degeneration [106, 137]. Our results suggest the role of TRPV1 and TRPV4 signaling in regulating downstream cascade pathways, which can contribute to the inflammatory environment in IVD and the progression of disc degeneration.

Our results showed a significant positive correlation between NGF and TRP channels in AF and NP. In humans, chronic pain diseases like osteoarthritis and low back pain showed elevated levels of NGF; similarly, elevated levels of NGF are seen in DRG of different inflammatory and pain rat models [154]. Furthermore, nerve fibers that innervates IVD are categorized as nociceptive and believed to be originated from DRG; they express calcitonin gene-related peptide (CGRP), PGP 9.5, substance P (SP), BDNF, TRPV1, neurofilament protein (NFP) [64, 155-158]. Freemont et al. observed a relationship between blood vessels and invading nerve fibers into painful IVDs [86] and suggested that the NGF derived from endothelial cells of blood microvessels is required for neuronal survival and ingrowth into IVD [64, 77]. The exact mechanism by which NGF activates TRP channels is not known, but studies suggest that NGF induced TRPV1 sensitization is a significant contributor [159, 160].

Similarly, studies conducted on rat DRC cells showed NGF increase TRPV1 translocation to the cell surface and possibly mediated through PI3K and PKC signaling events downstream of TrkA [91]. These studies support the possible association of NGF, TRP channels, pro-inflammatory cytokines, and nociception. Furthermore, our study also showed a significant positive correlation between expression of TRPV1 at baseline to one-year follow-up disability scores, suggesting that high TRPV1 expression at baseline can be associated with poor disability, such that TRPV1 may serve as a predictor for surgery. However, any therapeutic implication we suggested in our current study is intriguing but still speculative and more extensive clinical studies are needed to evaluate these findings.

# 6. CONCLUSION AND FUTURE PERSPECTIVES

The present PhD thesis is based on mRNA expression analysis using RT-qPCR to understand better the molecular mechanism of low back pain in disc degeneration patients undergoing lumbar spine fusion surgery. Furthermore, this study illuminates the aspects of inflammation, ECM remodeling and TRP channel expression in Degenerative disc disease (DDD).

Study I confirmed a significant positive correlation between expression of inflammatory cytokines, disability and low back pain intensity in the lumbar disc, which confirms that inflammatory cytokines play a key role in DDD along with pain generation. Furthermore, our results also showed a synergistic interplay between cytokines, which results in upregulation of inflammatory cascades and leads to chronic inflammation suggests that. It is reasonable to assume that targeting a specific cytokine might have limited clinical effects. Instead of targeting cytokine responses like neutrophil and macrophage recruitment responsible for nociception could be a strategy for patients affected by radicular pain associated with nerve root injury. Hence therapies targeting specific actions of different cytokines would be more effective. However, an increase in understanding of the role of different pro-inflammatory cytokines and immune cells in chronic disc degenerative changes and pain could lead to the identification of novel targets for treating degenerative disc disease

Study II confirmed that most MMPs and ADAMTSs express in NP, and their expression is correlated with the degree of disc degeneration on MRI, suggesting the role of these protease in shifting the disc matrix environment towards catabolism results in increased ECM breakdown and progression of degeneration. Current treatment strategies for DDD are restricted mainly to managing the symptoms rather than the underlying cause. Many challenges still exist because these enzymes exist throughout the body and inhibition of MMPs and ADAMTs should be tissue specific and localized only towards degenerated IVD such as intradiscal injections procedures. However, studies have reported that these procedures aggravate disc degeneration [161, 162]. Other potential strategies include gene, protein, and cell-based therapies, which involve the transfer of genetic material into targeted cells leading to replace the loss of disc ECM or to inhibit the catabolic factors. Tissue healing after injury and maintaining structural integrity is a continuous process, even in mature tissues. Hence any attempt to control this activity should be carefully studied. Keeping these challenges in mind and increasing the fundamental knowledge of these

MMPs and ADAMTSs in DDD gives an understanding of how to intervene to improve the treatment for DDD patients.

Study III highlights the association between the expression of TRP channels and patient-related features like pain intensity, disability and degeneration. Furthermore, our study also showed the association between TRP channels expression and inflammatory events in the disc. Our results also showed an association between NGF expression and TRP channels, especially TRPV1. Since NSAIDs have relied heavily on OA and LBP related pain management, new strategies and targets are needed for patients for whom NSAIDs are ineffective or safe. NGF and TRPV1 pathways showed promising alternatives for therapeutic targeting to treat pain associated with moderate to severe OA pain [163]. Tanezumab is the anti-NGF antibody leading in the development and FDA has approved a fast track designation for the treatment of chronic pain for OA and low back patients. Similarly, US FDA has approved a TRPV1 agonist called CNTX-4975, a fast track designation for treating knee OA associated pain [163]. However, more extensive clinical studies are required to evaluate safety and efficacy in treating chronic pain-associated diseases like OA and low back pain.

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# Appendix

# Paper I

**Aripaka, S.S**., Bech-Azeddine, R., Jørgensen, L.M. *et al*. Low back pain scores correlate with the cytokine mRNA level in lumbar disc biopsies: a study of inflammatory markers in patients undergoing lumbar spinal fusion. European Spine Journal, 2021. 30(10): p. 2967-2974.

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ORIGINAL ARTICLE

# Low back pain scores correlate with the cytokine mRNA level in lumbar disc biopsies: a study of inflammatory markers in patients undergoing lumbar spinal fusion

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## Abstract

**Purpose** The molecular mechanism behind pain in degenerative disc disease (DDD) and chronic low back pain (LBP) patients is largely unknown. This present study examines the association of LBP and disability to mediators of the inflammatory cascade, as indexed by mRNA gene expression of pro-inflammatory cytokine markers in the intervertebral disc (IVD).

**Methods** Biopsies of the annulus fibrosus (AF) and the nucleus pulposes (NP) from patients with DDD undergoing 1–2 level fusion surgery at L4/L5 or L5/S1 were obtained from total of 34 patients [9 M, 25 F] with average age of 53 [32–63]. The mRNA expression of TNF-α, IL-1β, and IL-6 in the AF and NP was analyzed using quantitative real-time polymerase chain reaction (RT-qPCR), and the expression level of these markers was correlated to the visual analogue scale (VAS) and Oswestry Disability Index (ODI) scores (0–100) for pain and disability.

**Results** We report a statistically significant positive correlation between pain intensity (VAS score) and the expression of TNF-α in both the AF ($r=0.54$, $p=0.001$) and NP ($r=0.40$, $p=0.02$), similarly with IL-1β in AF ($r=0.37$, $p=0.02$) and IL-6 in NP ($r=0.40$, $p=0.02$). In addition, we found significant positive correlation observed between disability score (ODI) and expression of IL-6 in both AF ($r=0.36$, $p=0.03$) and NP ($r=0.41$, $p=0.01$).

**Conclusion** We conclude that the intensity of LBP and disability is associated with the level of inflammation in the disc.

**Keywords** Intervertebral disc · Low back pain · Degenerative disc · VAS score · TNF-α

## Introduction

Low back pain (LBP) is the foremost cause of disability in the world [1]. It is estimated that about 80% of the population suffers with LBP at some point in life and 10% of these patients are disabled chronically [2]. LBP is also most commonly diagnosed with relation to a degenerative disc disease (DDD), reflected by high correlation between disc degeneration (DD) and LBP [3] although it has been shown that 13 years later this correlation seemed to fade out [4].

The intervertebral disc (IVD) absorbs and distributes applied loads and adds both stability and flexibility to the spine, and serves as a back-movement controller [5]. IVD is composed of the AF, which encircles the NP that is made of a primarily avascular, proteoglycan water-based gel [6], but DD's are invaded by blood vessels. DD is characterized by loss of extracellular matrix, initiated by a shift in balance between expression of catabolic protiens such as matrix metallo proteinases (MMP) and a disintegrin and metalloproteinases with thrombospondin motifs proteins (ADAM-TS) and anabolic protien such as proteoglycans and collagens [7].

Previous studies have shown that the early phase of degeneration is facilitated by an atypical production of pro-inflammatory molecules such as TNF-α, IL-1β, IL-6, IL-8,

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IL-2, IL-4, IL-10, IFN-γ, chemokines and prostaglandins (PGE) 2 secreted by NP and AF cells as well as neutrophils, macrophages and *T* cells [8–10]. Several animal pain models have established the role of several proinflammatory cytokines in peripheral and central nociception. Among the abovementioned pro-inflammatory molecules, TNF-α, IL-1β and IL-6 are of interest as they are implicated in hyperalgesic effects after nerve damage in disc herniation and nerve ingrowth [11]. TNF-α is well known for initiating and orchestrating the response to tissue injury including cell death, proliferation, survival and controlling the release of other cytokines [12]. IL-1β induces decreased matrix production and increased production of degradation enzymes; the matrix metalloproteins [13], which are characteristic for disc degeneration. Along with macrophages and *T* cells, IL-6 is secreted by IVD cells [14] and increased levels of expression are found in herniated discs [15]. Further, IL-6 induces TNF-α expression and apoptosis of neurons in the dorsal root ganglia (DRG) that likely also contributes to allodynia and hyperalgesia [13, 16].

Moreover, it has been shown that increased levels of inflammatory cytokines in the disc, such as TNF-α, IL-1β, IL-6 and IL-8, correlate with disc degeneration [17]. Even serum levels of TNF-α have been showed to be present in about half of fairly severe back pain patients compared to only 15% of healthy controls [18]. All together these findings tempted us to examine the association of LBP and disability experienced by the patients subjectively, and the concentration of several inflammatory mediators in IVD biopsies. We hypothesized that the discal expression of TNF-α, IL-1β and IL-6 in AF and NP of the lumbar IVD correlates with the subjective rating for pain intensity and disability.

## Materials and methods

### Participants

From January 2018 to March 2020, we included 34 patients [9 M, 25 F] undergoing 1, 2 level posterior lumbar instrumented spinal fusion L4/5 and/or L5/S1 with decompression, placement of pedicle screws, discectomy and placement of an intervertebral cage in the disc space.

All participants underwent radiological evaluation with magnetic resonance imaging (MRI) and lumbar X-rays and clinical evaluation in the outpatient clinic by a senior spine surgeon. The radiological-clinical indication for operation was: (a) LBP with or without radicular pain refractory for conservative treatment with analgesics and physiotherapy and concordant 1, 2 levels severe lumbar degenerative changes on MRI of with or without spondylolisthesis, or (b) predominantly leg pain due to severe foraminal stenosis caused by DD. Inclusion criteria were 18–65 of years of age and exclusion criteria were: Symptom duration < 6 months; previous spine surgery; rheumatoid arthritis; ongoing steroid treatment. None of the participants had any significant somatic or psychiatric history on clinical examination other than those related to their lumbar degenerative disorder.

### Rating scales

One to two weeks prior to surgery, all patients noted their pain intensity as assessed by using visual analogue scale (VAS 0–100), where 0 = no pain and 100 = worst pain [19]. Disability due to back pain was assessed by Oswestry Disability Index (ODI 0–100) which ranges from (0 = no disability–100 maximal possible disability) [20]. Demographic data including age, sex, height, and weight were obtained prior to surgery.

### Tissue sampling

Following decompression and exposure of the IVD during surgery, the spine surgeon harvested the AF and NP before discectomy and before preparation of the disc space for placement of the intervertebral cage. An approximately 4 mm × 8 mm sample of the AF was carefully obtained by making a rectangular cut in the exposed disk. If any NP tissue adhered to the underside of the AF sample, judged macroscopically, it was cut off with a new scalpel. Next, several samples of NP were retrieved by gentle use of a forceps in the disc space. During the whole sampling procedure, the surgeon carefully avoided contact of the sampling instruments with the surrounding tissue. The obtained NP and AF tissue samples were immediately frozen separately on dry ice and stored at 80 °C until extraction of RNA.

### RNA extraction cDNA synthesis

Initially, the tissue was digested at 37 °C on a waving shaker set at 40 rpm for 1 h in Dulbecco's modified Eagle medium containing 2 mg/ml proteinase (Pronase; Qiagen). Digestion was terminated by adding 10% fetal bovine serum (FBS) (0.5 ml FBS/5 ml solution) when the tissue appeared nearly digested it is washed with PBS twice. Total RNA extraction from AF and NP was performed using TRIzol (Sigma Aldrich., Denmark), according to manufactures protocol, and the RNA content was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific). Finally, cDNA was synthesized from 0.2 µg of RNA using ImProm-II™ Reverse Transcription System (Promega, USA) which includes Oligo(dT)15 primer. The diluted samples were mixed with 1 µl Oligo(dT)15 primer (0.5 µg/reaction) and incubated for 5 min at 60 °C in an iCycler (Bio-Rad) followed by mixing

with reverse transcription reaction mix (ImProm-IITM 5X Reaction buffer, MgCl2, dNTP mix, recombinant RNasin ribonuclease inhibitor, reverse transcriptase, nuclease-free water) to each sample. Finally, the samples were placed in the iCycler for cDNA synthesis. RT-qPCR was performed using Light Cycler 480 Real-Time PCR System with SYBR Green LightCycler 480 SYBR Green I Master (Roche Diagnostics, IN) for 50 cycles in a fixed sequence at 94 °C for 30 s, 63 °C for 15 s, and 72 °C for 15 s. Amplicons were generated using the primer sets listed in Table 1. We started performing the gene expression analysis using RT-qPCR by validating the housekeeping genes. Reliability, accuracy and reproducibility in RT-qPCR are strongly depended on the choice of suitable housekeeping genes to normalize experimental variations, so we initially analyzed and validated expression of 5 housekeeping genes in IVD using Microsoft Excel-based software programs, geNorm (ver. 3.5), NormFinder (Ver 0.953), and BestKeeper as previously described [21] and selected 3 as stable genes listed in Table 1. These statistical algorithms were used to evaluate the stability of candidate reference genes, and then the overall ranking of the 5 candidate reference genes was determined according to the method described by Chen et al. [22] Expression of all the cytokines was measured relative to the geometric mean of 3 housekeeping genes.

### Statistical analysis

Statistical analysis was performed with the Graphpad Prism 8.2 software. Data are expressed as mean ± SD. Group differences in mRNA expression were evaluated by a Student unpaired *t* test. The correlation of mRNA levels and clinical data was assessed using Pearson correlation coefficient. Similarly, a $p$ value of $< 0.05$ is considered statistically significant for all comparisons.

## Results

### Clinical data

All patients reported pain intensities for back pain and leg pain and average reported scores by VAS were 69 ± 28 and 60 ± 25, respectively. The average ODI scores reported by all patients are 41 ± 15.

### TNF-α, IL-1β, and IL-6 expression in tissue biopsy samples

We successfully extracted RNA from 33 AF to 32 NP biopsies and showed that TNF-α, IL-1β and IL-6 indeed were express in these tissues. Determination of the level of RNA using the RT-qPCR method on these tissues showed that TNF-α and IL-6 expression are more in AF and it is statistically significant when compared to NP (Fig. 1a, b). No differences in the expression of IL-1β (Fig. 1c) were observed between these two tissues.

### Correlation between proinflammatory cytokine levels and pain intensity

Our RT-qPCR analysis showed a significant positive correlation between pain intensity (VAS) and expression of TNF-α (Fig. 2a) and IL-1β (Fig. 2b) in AF, but no correlation observed with expression of IL-6 (Fig. 2c). Similarly, significant positive correlation observed between pain intensity (VAS) and expression of TNF-α (Fig. 2d) and IL-6 (Fig. 2e) in NP, but no correlation observed with expression of IL-1β (Fig. 2f).

### Correlation between proinflammatory cytokine levels and ODI score

Our analysis showed there is significant positive correlation between expression of IL-6 and ODI score in both the AF and NP (Fig. 3a, b). By contrast, TNF-α (Fig. 3c, d) and IL-1β (Fig. 3e, f) expression were not correlated to ODI score.

### Correlation between expression of TNF-α and IL-1β, IL-6 in NP and AF tissues

Because the expression profiles between pain (VAS) and disability (ODI) were partly overlapping, we conducted a post hoc analysis of the intercorrelation between the individual cytokine mRNA's in the two tissues from the patients. We

**Table 1** List of Primers

| Gene | Forward (5′–3′) | Reverse (5′–3′) |
|---|---|---|
| TNF-α | TTC CTG ATC GTG GCA GGC | GCT GAT TAG AGA GAG GTC CCT G |
| IL-1β | ACC AAG TGC CAC AAA GGA AC | CTG CAA TTG AAG CAC TGG AA |
| IL-6 | TTC GCT CTT CCA GTT GGA CT | CAC CAG GGG AAG AAT CTG AG |
| SDHA | CGA GCT GCA TTT GGC CTT TC | TTG ATT CCT CCC TGT GCT GC |
| LDHA | GCC TGT ATG GAG TGG AAT GAA | CCA GGA TGT GTA GCC TTT GAG |
| *β*-Actin | TGG AAC GGT GAA GGT GAC AG | AAC AAC GCA TCT CAT ATT TGG AA |

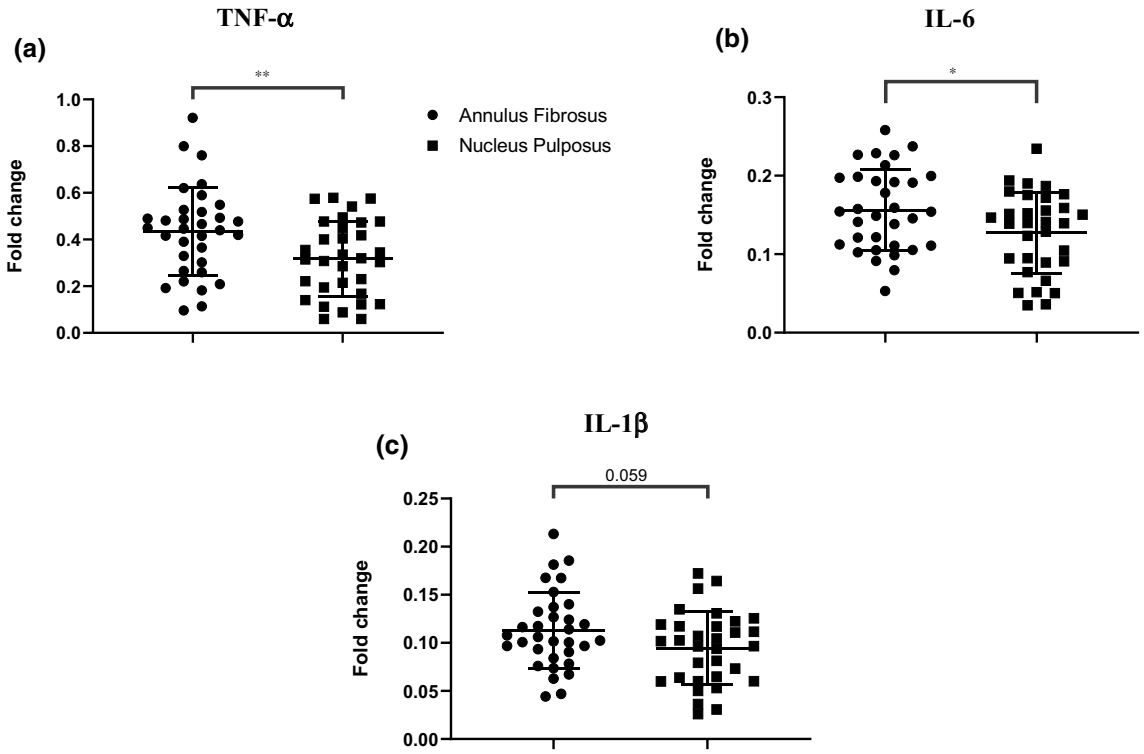


**Fig. 1** The expression of pro-inflammatory cytokines in the annulus fibrosus (AF) and nucleus pulposus (NP): mRNA levels of **a** TNF-$\alpha$ **b** IL-6 **c** IL-1$\beta$ were measured in AF ($n=33$) and NP ($n=32$) from patients undergoing lumbar discectomy as part of fusion surgery. Expression of all the cytokines was measured relative to the geometric mean of 3 housekeeping genes. Values are MEAN ± SD. $p<0.01$, $p<0.05$

observed a significant positive correlation between TNF-α and IL-1β in AF (Fig. 4a) and no correlation observed between TNF-α and IL-6 (Fig. 4b) in AF. We observed no correlation between TNF-α and IL-1β in NP (Fig. 4c) but a significant positive correlation between TNF-α and IL-6 (Fig. 4d).

## Discussion

This study demonstrates statistically significant correlations between the clinical symptoms and expression of cytokines in AF and NP biopsies from LBP patients undergoing lumbar spine fusion. In this investigation, we studied the mRNA expression in human IVD biopsies of pro-inflammatory cytokines. TNF-α was the only cytokine among the 3 cytokines measured which showed significant positive correlation with pain intensity (VAS) and its mRNA expression in both structures supporting previous findings that TNF-α plays an important role in cytokine-mediated neuropathic pain in the peripheray [23]. Similarly, expression of IL-6 and IL-1β showed a significant positive correlation with VAS score. These results suggest that LBP and chronic inflammation in both NP and AF are occurring together. Regarding ODI, only a significant positive correlation with IL-6 was seen (Fig. 3f), but not with other cytokines. Finally, our results showed in agreement with reports published by [24] that both TNF-α and IL-6 are more expressed in the AF than NP.

Many studies have been looking at the role of different cytokines in the pathogenesis of DD with a special focus on TNF-α and IL-6 have been carried out [9]. The expression of TNF-α and its receptors (TNFR1 and TNFR2) are observed in non-degenerated IVD tissues and revealing the importance of tissue repair and remodeling [25], but the degenerative and herniated discs showed higher levels when compared to normal IVD tissues [8]. Hence, it is considered to be one of the initiating factors of the inflammatory cascade in the disc [26]. Inflammatory cascades are believed to be initiated by TNF-α [26] because this cytokine has a major role in regulating the expression of other inflammatory cytokines like IL-1β and IL-6 [27]. The positive correlation between TNF-α and IL-1β in AF and IL-6 in NP in our study may, therfore, be explained by the synergistic

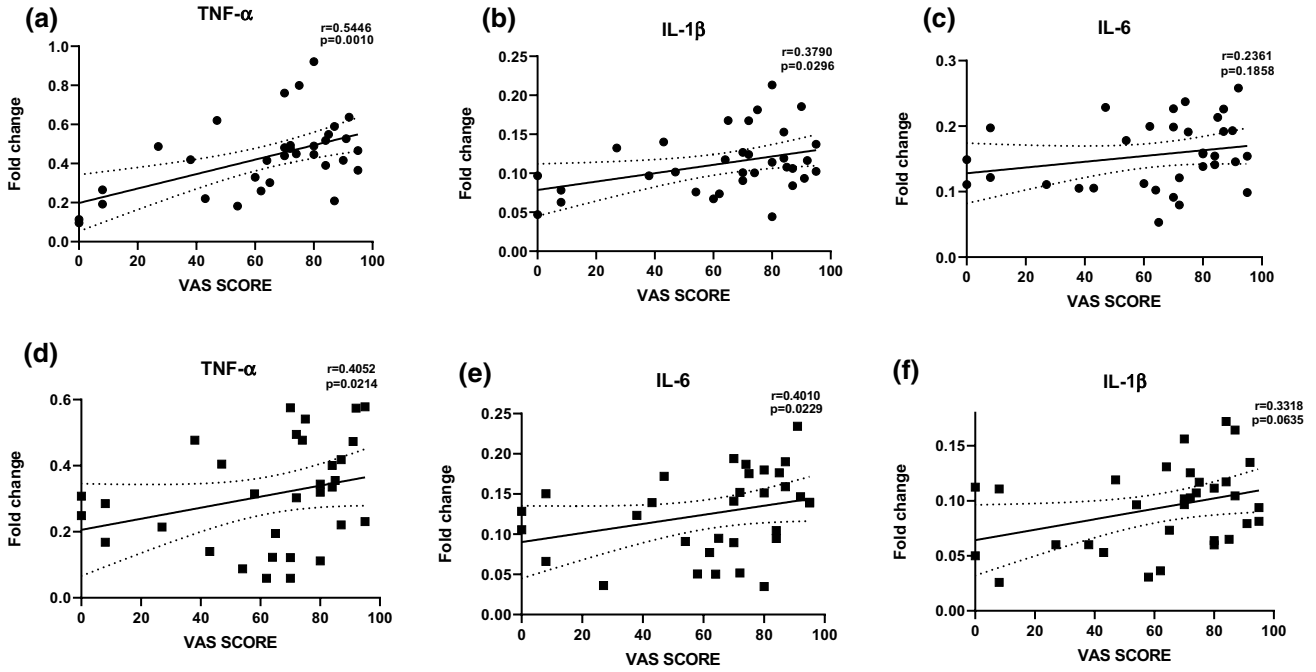


**Fig. 2** Correlation between pro-inflammatory cytokine expression and pain intensity in the AF and NP. **a** In AF, VAS score (1 day before surgery) showed a significantly positive correlation with TNF-$\alpha$ ($r=0.5446$, $p=0.0010$). **b** The positive correlation was demonstrated for IL-1$\beta$ ($r=0.3790$, $p=0.0296$). **c** No correlation was observed with expression of IL-6. **d** Similar trends were observed in NP, significantly positive correlations were observed with TNF-$\alpha$ ($r=0.4052$, $p=0.0214$). **e** There was a postive correlation with IL-6 ($r=0.4010$, $p=0.0220$). **f** No correlation obsereved with expression of IL-1$\beta$. $p<0.05$ is considered statistically significantly for all comparisons. $r$=Pearson correlation coefficient

regulation of IL-1β and IL-6 mRNA expression by TNF-α may be important in DD pathogenesis.

We also found a significant positive correlation between IL-6 and ODI score (Fig. 3f). Because TNF-α is significantly correlated with VAS only, but not with ODI, it seems to suggest that pain and disability are not strongly biochemically linked, corresponding to what is known from a clinical perspective that they are only partially overlapping. Since IL-6 showed a significantly positive correlation to ODI score suggests that IL-6 may play an important role in disability in DD patients.

Along with other immune cells evidence suggests that, intervertebral cells also secrete IL-6 [28]. Our results showed a significant postive correlation between low back pain intensity (VAS score) and IL-6 expression. Similar to our findings, studies performed by Koch et al. showed that chronic pain levels are correlated with circulating IL-6 levels [29] and this is seen associated with neuropathic pain diseases like OA [30], which is supported by a therapeutic study that shows epidural injection of tocilizumab (anti IL-6R) antibody attenuated the leg pain without causing adverse events [31].

Many downstream inflammatory cytokines like ILs are induced by TNF-α and it is considered to be one of the most powerful cytokine among proinflammatory cytokines. Among the effects of different IL's, vasodilation caused by IL-1β can cause the attraction of neutrophils and monocytes to site of injury. Moreover, IL-6 can cause differentiation of monocytes to macrophages initiating maturation of lymphocytes, which will contribute to the hyperalgesia and become more senitive to pain [16]. These cellular downstream cascades caused by inflammatory cytokines can be a connection between inflammation and LBP in DDD patients. The etiology of disc degeneration is still not well understood, and the pathological mechanisms underlying DD and chronic pain have been subject for ongoing debate. Knowledge of these mechanisms is essential for developing a potential therapeutic approach to treat disc degeneration. There is ample evidence to support our findings that pain intensity and disability are associated with inflammatory component. Our results clearly suggest abovementioned cascades, i.e., increase in pro-inflammatory cytokines in disc tissues cause hyperalgesia in newly innervated disc regions. In such cases,

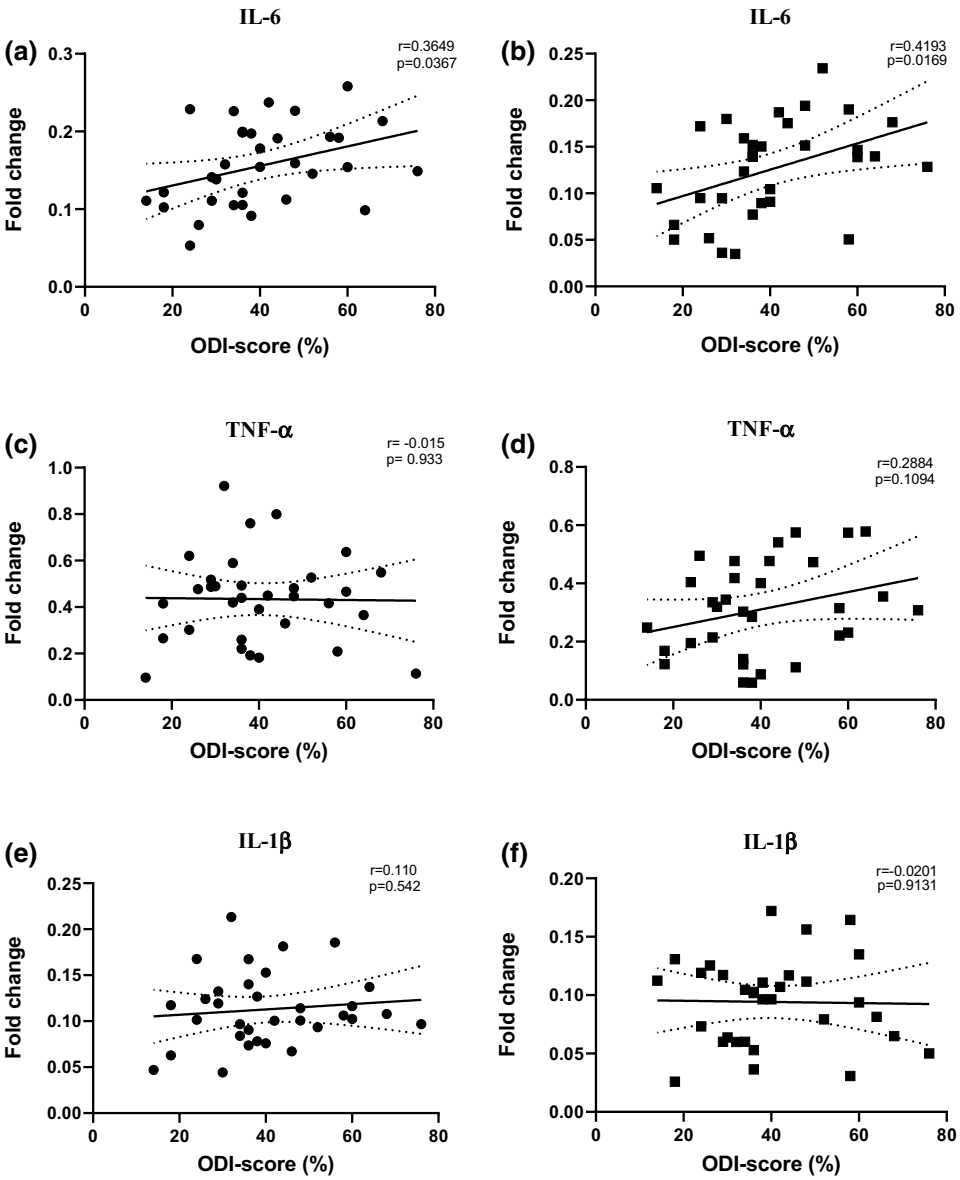


**Fig. 3** Correlation between pro-inflammatory cytokine expression and oswestry disability index (ODI) in AF and NP. **a**, **b** In AF and NP, ODI score (1 day before surgery) showed a significantly positive correlation with IL-6 ($r=0.3649$, $p=0.036$), ($r=0.4193$, $p=0.0169$). **c**, **d** No correlation observed with expression of TNF-$\alpha$ in both AF and NP. **e**, **f** No correlation obsereved with expression of IL-1$\beta$ in both AF and NP. A value of $p<0.05$ is considered statistically significantly for all comparisons. $r$ = Pearson correlation coefficient

the anti-inflammatory therapy can be potential therapeutic method, which may prevent degenerative changes and radicular pain. Since these cytokines have complex networks in regulating the inflammation, targeting a single cytokine might not have expected clinical outcome; hence, targeting multiple cytokine strategies might help in treating the patients [10]. Since the cellular mechanisms of these cytokines where not studied completely, studying the signaling pathways and mechanisms regulating the mediators will provide more understanding on pain mechanisms in low back pain.

## Conclusion

Our study confirmed a significantly positive correlation between the expression of inflammatory cytokines in the lumbar disc and disability and low back pain intensity. The findings support the idea that low back pain may be accompanied by local disc inflammation. We showed a regional difference in the expression of cytokines between AF and NP. Moreover, our results showed a synergistic interplay between the cytokines. Thereby, the inflammatory cascades become upregulated leading to chronic inflammation,

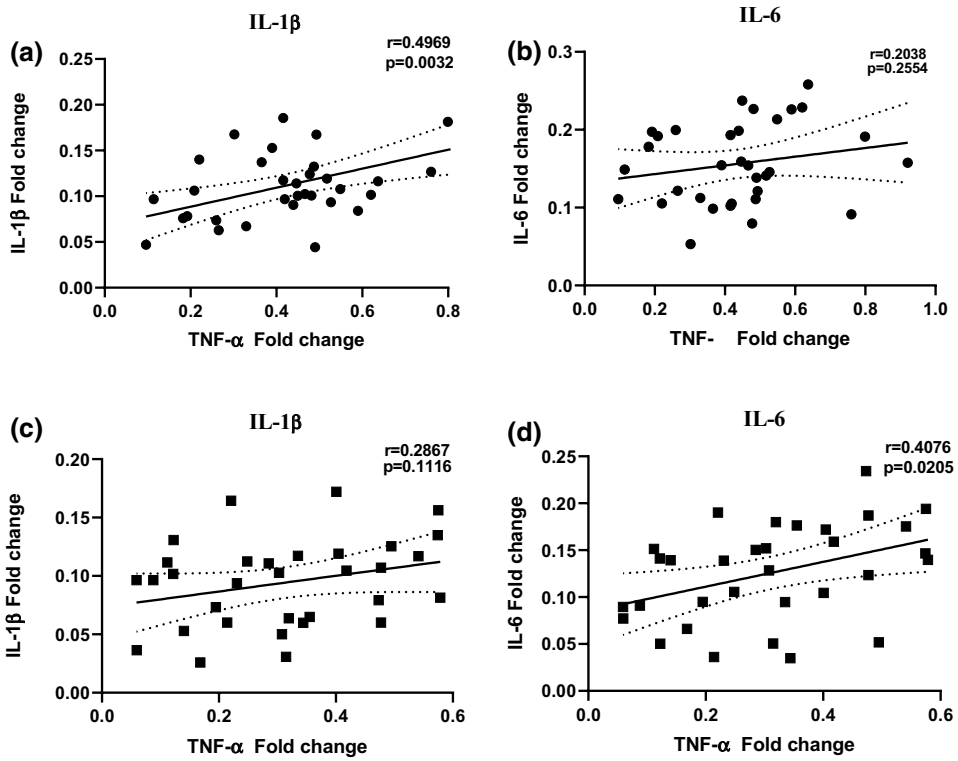


**Fig. 4** Correlation between pro-inflammatory cytokines: **a**. In AF, expression of TNF-$\alpha$ showed a significant positive correlation with IL-1β ($r=0.4969$, $p=0.0032$). **b** No correlation was observed with expression of IL-6. **c** In nucleus pulposes, no correlation observed between expression of TNF-α and IL-1β. **d** There was a significant positive correlation observed between TNF-α and IL-6 ($r=0.4076$, $p=0.0205$). A value of $p<0.05$ is considered statistically significant for all comparisons. $r$ = Pearson correlation coefficient

suggesting that these cytokines are targets for modulation of pain and degeneration.

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## Declarations

**Conflict of interest** The authors have declared no conflict of interest.

**Ethical approval** The study was approved by the Danish ethics committee (H-17026301). All participants provided written informed consent according to the Declaration of Helsinki.

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**DECLARATION OF CO-AUTHORSHIP**

*The declaration is for PhD students and must be completed for each conjointly authored article. Please note that if a manuscript or published paper has ten or less co-authors, all co-authors must sign the declaration of co-authorship. If it has more than ten co-authors, declarations of co-authorship from the corresponding author(s), the senior author and the principal supervisor (if relevant) are a minimum requirement.*

| **1. Declaration by** | |
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| Name of PhD student | Sagar Sanjay Aripaka |
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| Name of principal supervisor | Jens D Mikkelsen |
| Title of the PhD thesis | MOLECULAR BIOLOGY IN THE PAIN GENERATION IN LUMBAR INTERVERTEBRAL DISCS |

| **2. The declaration applies to the following article** | |
|---|---|
| Title of article | Low back pain scores correlate with the cytokine mRNA level in lumbar disc biopsies: a study of inflammatory markers in patients undergoing lumbar spinal fusion |
| **Article status** | |
| Published ☒<br>Date: 23rd May 2021 | Accepted for publication ☐<br>Date: |
| Manuscript submitted ☐<br>Date: | Manuscript not submitted ☐ |
| If the article is published or accepted for publication, please state the name of journal, year, volume, page and DOI (if you have the information). | European Spine Journal, 2021. **30**(10): p. 2967-297, doi.org/10.1007/s00586-021-06868-3 |

| **3. The PhD student's contribution to the article** ***(please use the scale A-F as benchmark)***<br>Benchmark scale of the PhD-student's contribution to the article<br>**A**. Has essentially done all the work (> 90 %) **B**. Has done most of the work (60-90 %) **C**. Has contributed considerably (30-60 %) **D.** Has contributed (10-30 %) **E.** No or little contribution (<10 %) **F.** Not relevant | **A, B, C, D, E, F** |
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| 2. Development of the key methods | B |
| 3. Planning of the experiments and methodology design and development | A |
| 4. Conducting the experimental work/clinical studies/data collection/obtaining access to data | A |
| 5. Conducting the analysis of data | A |
| 6. Interpretation of the results | A |
| 7. Writing of the first draft of the manuscript | A |
| 8. Finalisation of the manuscript and submission | B |
| Provide a short description of the PhD student´s specific contribution to the article.[i]<br>PhD student planned and conducted all the RT-qPCR experiments from RNA extraction protocols, primer designs, housekeeping validations to data analysis. PhD student drafted the manuscript. | |

| **4. Material from another thesis / dissertation**[ii] | |
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**6. Signature of the principal supervisor**

I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge.

Date: 22 Dec2021

Principal supervisor:

| **7. Signature of the PhD student** |
| --- |
| I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge.<br><br>Date: 29 Dec 2021<br><br>PhD student:  |

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[i] This can be supplemented with an additional letter if needed.

[ii] Please see Ministerial Order on the PhD Programme at the Universities and Certain Higher Artistic Educational Institutions (PhD Order) § 12 (4):

*"Any articles included in the thesis may be written in cooperation with others, provided that each of the co-authors submits a written declaration stating the PhD student's or the author's contribution to the work."*

[iii] If more signatures are needed please add an extra sheet.

# Paper II

**Aripaka, S.S.,** Bech-Azeddine R, Jørgensen LM, Mikkelsen JD. The expression of metalloproteinases in the lumbar discs correlates strongly with Pfirrmann MRI grades in lumbar spinal fusion patients. Manuscript submitted to *Brain and Spine Journal* and it is under review.

# APPENDIX

## The expression of metalloproteinases in the lumbar discs correlates strongly with Pfirrmann MRI grades in lumbar spinal fusion patients

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**Abstract:**

**Introduction:** Increased catabolism of the extracellular matrix is observed under degenerative disc disease (DDD). The cleavage of extracellular matrix proteins in the nucleus pulposus (NP) by either matrix metalloproteinases (MMPs) or a disintegrin and metalloproteinases with thrombospondin motifs (ADAMTSs) is believed to be involved in the degeneration, but the mechanisms are not known.

**Research question:** Here, we examine the correlation between expression of several MMPs and ADAMTSs subtypes in lumbar discs from 34 patients with low back pain (LBP) undergoing 1-2 level lumbar fusion surgery (L4/L5 and/or L5/S1) for DDD with or without spondylolisthesis.

**Materials and Methods:** The mRNA levels of MMPs (subtypes 1, 2, 3, 10, and 13) and ADAMTSs (subtypes 1, 4, and 5) were analyzed using quantitative real-time polymerase chain reaction (RT-qPCR) and correlated to the Pfirrmann magnetic resonance imaging classification system (grade I-V) of lumbar DDD.

**Results:** We find a highly significant positive correlation between Pfirrmann grades and the gene expression of MMP1 (r=0.67, p=0.0001), MMP3 (r=0.61, p=0.0002), MMP10 (r=0.6701, p=0.0001), MMP13 (r=0.48, p=0.004), ADAMTS1 (r=0.67, p=0.0001) and ADAMTS5 (r=0.53, p=0.0017). The similar regulation of these transcript suggests their involvement in disc degeneration. Interestingly, a post hoc analysis (uncorrected p-values) also demonstrated a positive correlation between expression of TNF-α, IL-6 and ADAMTSs/MMPs and the Pfirrmann grades.

**Discussion and Conclusion:** These findings show that disc degradation in DDD is strongly associated with the expression of some metalloproteinases.

**Keywords:** ADAMTS**,** Disc Degeneration, MMP, mRNA, Real-time PCR

## 1. Introduction

Low back pain (LBP) represents the top medical expense in western societies and is a leading cause of disability worldwide[1]. LBP is age-related, and several lines of evidence show that the LBP is associated with degenerative disc disease (DDD) [2] even the etiology of DDD is not fully understood. DDD is histologically characterized by cell number reduction, extracellular matrix (ECM) loss and inflammation [3, 4]. The intervertebral disc (IVD) is an essential component of the spine and consists of an outer fibrous ring, the annulus fibrosus (AF), rich in collagen type I providing strength [5] and the centrally located nucleus pulposus (NP), which is rich in collagen type II and proteoglycans, mainly aggrecan. In healthy discs, a careful regulation between growth factors and catabolic enzymes entails that the rate of synthesis and breakdown of the ECM is in equilibrium [6, 7]. Therefore, any loss of collagen and proteoglycans is key in developing DDD[8]. Matrix metalloproteinases (MMPs) and a disintegrin and metalloproteases with thrombospondin motifs (ADAMTSs) are families of enzymes that cleave collagens and proteoglycans, respectively, and we consider these molecules to be important in LBP and DDD.

MMPs are a large family of zinc-dependent proteolytic enzymes associated with extracellular matrix protein turnover and degradation[8-12]. Currently, 24 MMP human subtypes have been identified and classified into six groups based on their substrate specificity[13]. MMPs are usually secreted in inactive forms and require a regulatory activator protein to be active[14]. High expression of MMP-1,-3 are seen in recurrent disc herniation [8], but whether expression of any MMP subtypes relates to the progression of the DDD is unknown.

The other major component of the NP along with collagens is proteoglycans, mainly aggrecan. Aggrecan is the most common proteoglycan that makes 50% of the dry weight of NP, which plays an important role in water absorbing capacity and also contributes in the diffusion of nutrients from periphery and in the maintenance of disc height and ability to withstand compressions [7, 15]. Specific change associated with disc degeneration is the loss of proteoglycans which decreases the disc's water holding capacity[16, 17]. ADAMTSs are synthesized as pre-pro-enzymes later cleaved by furin or furin-like proteases to activated forms which are secreted and associated with proteolytic events of ECM components and leading to degradation [18]. Even though MMPs lyses aggrecan and collagens [19, 20], there is increasing

evidence for the role of ADAMTSs in the degradation of aggrecan as well [21, 22]. Studies focused on osteoarthritis with disc degeneration showed upregulation in expression of ADAMTS in degenerated articular cartilage [23, 24]. ADAMTS 4 and 5 have received increased attention because they can specifically cleave cartilage proteoglycans. Loss of aggrecan occurs during IVD tissue degeneration [25, 26]. ADAMTS subtype-1, -4, -5, degrade aggrecan and the expression of the same subtypes is found to be increased in degenerated IVD tissue. Previous studies have reported the expression of MMPs and ADAMTSs in IVD tissues and their implication in catabolism and ECM loss [25].

In our previous study, we showed increased expression of inflammatory cytokines correlates with pain intensity and disability in lumbar discs from patients undergoing lumbar spine fusion [27]. The present study was aimed to gain better insight into the role of different MMP and ADAMTS expression in IVD's from same patients and also study the relevance to inflammation, We therefore determined levels of MMP transcript subtypes (1, 2, 3, 10, and 13) and ADAMTS subtypes s (-1, -4, and -5) as previously described [8] in the NP and the association to lumbar disc degeneration in patients with chronic lumbar pain, as measured by the Pfirrmann magnetic resonance imaging classification system (grade I-V) of lumbar DDD [28]. Our hypothesis is that upregulation of MMP and ADAMTS expression implicated in disc ECM destruction is modulated by inflammation and upregulated under in chronic LBP. Considering therapeutic intervention, we were interested to correlate such expression to other clinical parameters and biomarkers.

## 2.Materials and Methods:

### 2.1 Participants

We included 34 patients [9M, 25F] undergoing 1-2 level posterior lumbar instrumented spinal fusion L4/5 and/or L5/S1 with the placement of pedicle screws and an intervertebral cage in the disc space. All participants were radiologically evaluated with magnetic resonance imaging (MRI) and lumbar X-rays and clinical evaluation in the outpatient clinic by a senior spine surgeon. Inclusion criteria were: 18-65 of years of age; predominantly LBP with DDD and/or spondylolisthesis or predominantly radiculopathy with severe foraminal stenosis with or without spondylolisthesis; scheduled 1-2 level fusion with pedicle screws and placement of a transforaminal lumbar interbody cage; and pain (either predominantly radiculopathy or LBP with

or without radiculopathy). Exclusion criteria were: symptom duration < 6 months; previous spine surgery; rheumatoid arthritis; or ongoing steroid treatment. None of the participants had any significant somatic or psychiatric history on clinical examination other than those related to their lumbar degenerative disorder. The study was approved by the Danish ethics committee (H-17026301). All participants provided written informed consent according to the Declaration of Helsinki.

**2.2 Informed Consent Statement:**

Informed consent was obtained from all subjects involved in the study.

**2.3 Pfirrmann MRI Grading System**

The degree of disc degeneration was assessed for each patient using the 5-level Pfirrmann MRI grading system on T-2 weighted sagittal MRI sequences. A Pfirrmann grades I and II indicates a non-degenerative disc, whereas grade III, IV, and V signify an increasingly degenerative disc [28].

**2.4 Tissue Sampling**

Following decompression and exposure of the IVD, the surgeon harvested the AF and the NP before preparation of the disc space for placement of the intervertebral cage. A window of the AF was cut open with a scalpel, and any adherent NP to the underside of the AF sample was cut off using a new scalpel. Next, several samples of NP were retrieved by gentle use of forceps in the disc space. The samples were immediately frozen on dry ice and stored at -80 °C until further processing. Only NP tissues were used for this study. During the whole sampling procedure, the surgeon carefully avoided contact of the sampling instruments with the surrounding tissue.

**2.5 RNA extraction and cDNA synthesis:**

RNA extraction from NP was performed using TRIzol (Sigma Aldrich., Denmark), according to manufactures protocol, and the RNA content was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific). Initially, the tissue was digested at 37°C on a waving shaker set at 40rpm for 1 hour in Dulbecco's Modified Eagle Medium containing 2mg/ml proteinase (Pronase; Qiagen). Then, digestion was terminated by adding 10% fetal bovine serum

(FBS) (0.5ml FBS/5ml solution) when the tissue appeared nearly digested, it is washed twice with PBS. Finally, cDNA was synthesized from 0.2µg of RNA using ImProm-II™ Reverse Transcription System (Promega, USA).

RT-qPCR was performed using a Light Cycler 480 Real-Time PCR System (Roche Diagnostics, IN) with SYBR Green I Master Mix for 40 cycles in a fixed sequence at 94 °C for 30 seconds, 60 °C for 15 seconds, and 72 °C for 15 seconds. Amplicons were generated using the primer sets listed in Table 1. We started performing the gene expression analysis using RT-qPCR by validating the housekeeping genes, so we initially analyzed and validated the expression of 5 housekeeping genes as previously described [29] in IVD using microsoft excel-based software programs, geNorm (ver. 3.5), NormFinder (Ver 0.953), and BestKeeper. From this analysis in tissues, three genes listed in Table 1 were found to be stably expressed in the validation and accordingly used as housekeeping genes. Statistical algorithms were used to evaluate the stability of candidate reference genes, and then the overall ranking of the 5 candidate reference genes was determined according to the method described by Chen et al [30]. Expression of all the MMPs andADAMTSs were measured relative to the geometric mean of 3 housekeeping genes.

| Gene | Forward (5`- 3`) | Reverse (5`- 3`) |
|---|---|---|
| **TNF- α** | TTC CTG ATC GTG GCA GGC | GCT GAT TAG AGA GAG GTC CCT G |
| **IL-6** | TTC GCT CTT CCA GTT GGA CT | CAC CAG GGG AAG AAT CTG AG |
| **SDHA** | CGA GCT GCA TTT GGC CTT TC | TTG ATT CCT CCC TGT GCT GC |
| **LDHA** | GCC TGT ATG GAG TGG AAT GAA | CCA GGA TGT GTA GCC TTT GAG |
| **β-Actin** | TGG AAC GGT GAA GGT GAC AG | AAC AAC GCA TCT CAT ATT TGG AA |
| **Aggrecan** | GTG CCT ATC AGG ACA AGG TCT | GAT GCC TTT CAC CAC GAC TTC |
| **MMP- 1** | GCC ATC ACT TAC CTT GCA CT | AGA CAC CAC ACC CCA GAA CA |
| **MMP-3** | TCC TAC TGT TGC TGT GCG TG | AGG TTC ATG CTG GTG TCC TC |
| **MMP-2** | TAC AGG ATC ATT GGC TAC ACA CC | GGT CAC ATC GCT CCA GAC T |
| **MMP-10** | TGA GTT TGA CCC CAA TGC CA | GTC TTC CCC CTA TCT CGC CT |
| **MMP-13** | GGC TTA GAG GTG ACT GGC AA | ATC AGG AAC CCC GCA TCT TG |
| **ADAMTS-1** | AGG ATG AAA CGC CGG AAC AA | CCC CAC CAC AAG ACA AGT GA |

| | | |
|---|---|---|
| **ADAMTS-4** | ACT GGT GGT GGC AGA TGA CA | TCA CTG TTA GCA GGT AGC GCT TT |
| **ADAMTS-5** | TCG GGA GGA TTT ATG TGG GC | TGG AAT CGT CAT GGG AGA GG |

**Table:1** ***List of primers used in this study***

### 2.6 Statistical Analysis:

Statistical analysis was performed with the Graphpad Prism 8.2 software. The correlation of mRNA levels and clinical data was assessed using spearman's correlation coefficient. Similarly, A p-value of $<0.05$ is considered statistically significant for all comparisons.

## 3. Results

### 3.1 Expression of specific MMPs and ADAMTSs correlate strongly with Pfirrmann MRI grades

After surgical collection of the IVD specimens, mRNA expression analysis was performed using RT-qPCR on NP tissue. We found significant positive correlations between Pfirrmann grade and expression of MMP-1 ($\rho=0.67$, $p<0.0001$) (Figure 1A), MMP-3($\rho=0.61$, $p=0.0002$) (Figure 1B), MMP-10($\rho=0.671$, $p<0.0001$) (Figure 1C), MMP-13 ($\rho=0.48$, $p=0047$) (Figure 1D), ADAMTS-1 ($\rho=0.679$, $p<0.0001$) (Figure 1E), ADAMTS-5 ($\rho=0.53$, $p=0.001$) (Figure 1F). Performing a post-hoc analysis with the expression of pro-inflammatory cytokines, we found a significant positive correlation with Pfirrmann grade and expression of TNF-α ($\rho=0.37$, $p<0.03$) (Figure 1G) and IL-6 ($\rho=0.35$, $p=0.04$) (Figure 1H). We did not find any statistical correlation between Pfirrmann grade and expression of MMP2, ADAMTS-4, IL-1β, NGF or BDNF (data not shown).

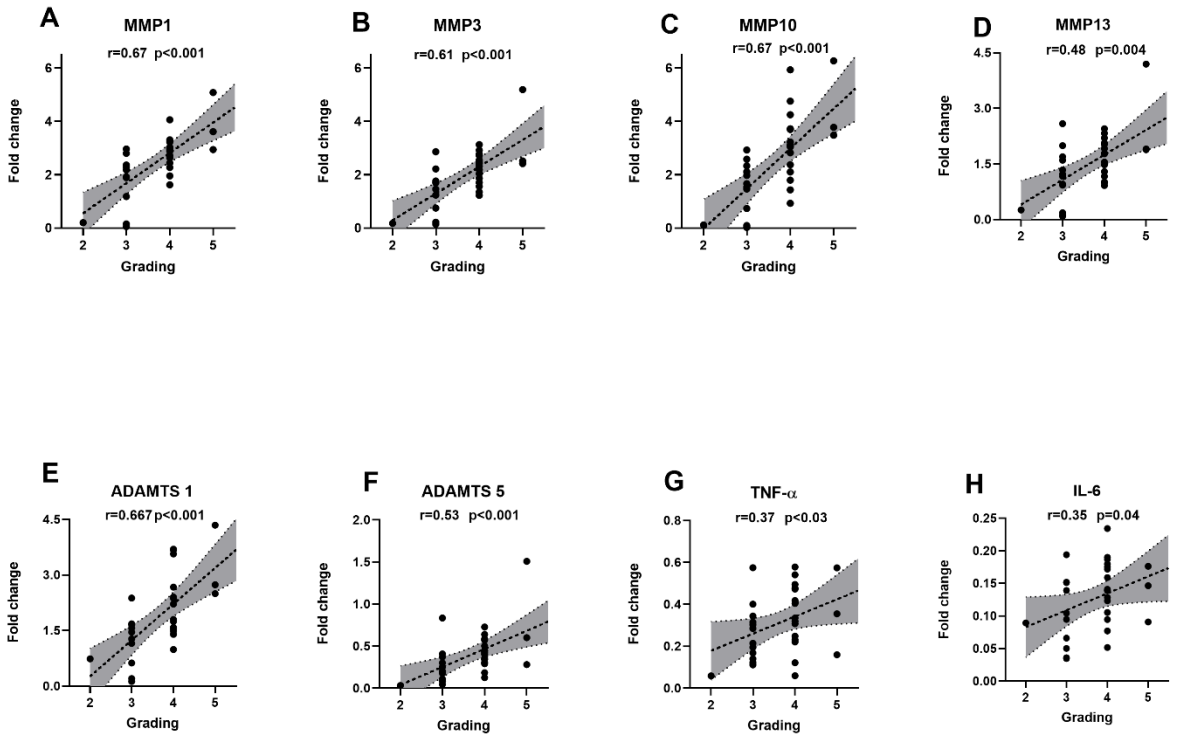


***Figure 1.*** *Expression of MMPs and ADAMTSs correlate with Pfirrmann MRI grades: Significant positive correlation observed between Pfirrmann MRI grades and expression of* ***(A)*** *MMP-1 (ρ=0.67, p<0.0001) ,* ***(B)*** *MMP-3(ρ=0.61, p=0.0002),* ***(C)*** *MMP-10(ρ=0.671, p<0.0001),* ***(D)*** *MMP-13 (ρ=0.48, p=0047),* ***(E)*** *ADAMTS-1(ρ =0.679, p<0.0001) ,* ***(F)*** *ADAMTS-5 (ρ=0.53, p=0.001),* ***(G)****TNF-α (ρ=0.37, p<0.03) and (H) IL-6 (ρ=0.35, p=0.04) . p<0.05 (two tailed) are considered statistically significantly for all comparisons. ρ=spearmen's correlation coefficient.*

### 3.2 Correlation between mRNA expression of MMPs and ADAMTSs

Since the transcripts are involved in proteolytic activity, we performed a post hoc pair-wise correlation analysis between expression of MMPs and ADAMTSs levels and demonstrated a strongly significant positive correlations between the expressions of these genes (Figure 2A&B).

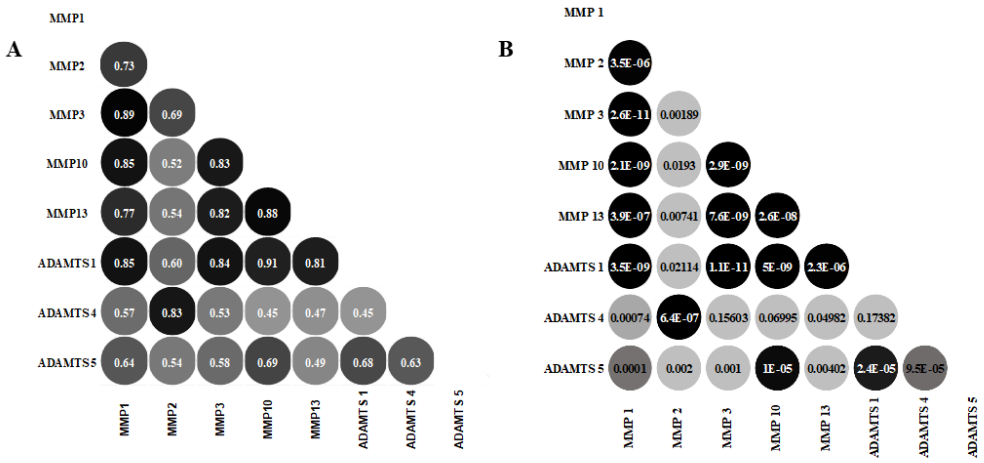


***Figure 2.*** *Correlation between mRNA expression of MMPs and ADAMTSs: Multiple significant positive correlations were found concerning the mRNA expression status of almost all of the MMPs and ADAMTS studied.* ***(A)*** *shows the correlations(ρ) between MMPS and ADAMTSs, and* ***(B)*** *shows corresponding significance values (p). $p<0.05$ (two-tailed) are considered statistically significant for all comparisons. ρ=spearmen's correlation coefficient.*

### 3.3 Correlation between transcript levels of aggrecan with age

We observed a relation between expression level of aggrecan and the age of the patients (r=-0.42, ρ=0.012) (Figure 3), Similarly, aggrecan showed significant positive correlations with expression of MMP (-2, -10, -13), ADAMTS (-1, -4, -5), but none with pro-inflammatory cytokines. Similarly, we observed negative correlations between expression of MMP2, ADAMTS4 and age of the patients (data not shown).

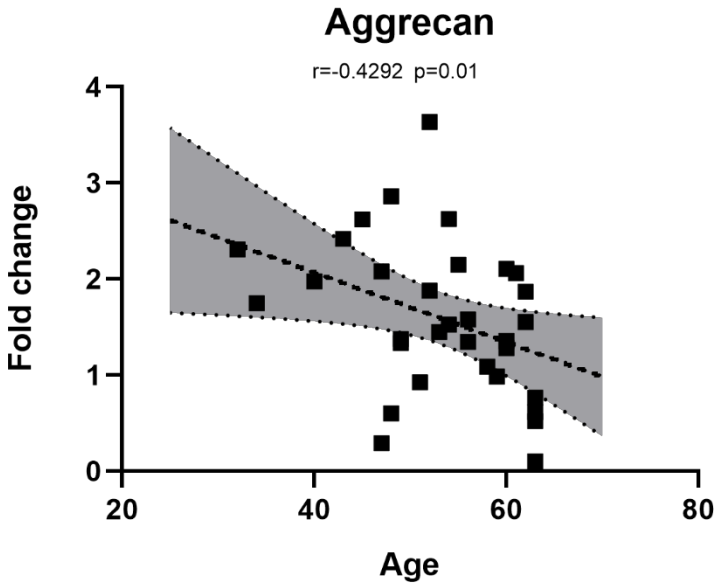


***Figure 3.*** *Correlation between expression of ECM gene aggrecan with age: Significant negative correlation observed between the expression of aggrecan with the age of the patients undergoing lumbar spine fusion (r=-0.42, ρ=0.012). p<0.05 (two-tailed) are considered statistically significant for all comparisons. ρ=spearmen's correlation coefficient.*

### 3.4 Correlation of transcript levels of MMPs and ADAMTSs with pro-inflammatory cytokines.

To elucidate the mechanisms behind disc degeneration, we conducted a post hoc analyses of the intercorrelation between the individual cytokine mRNA's expression to the expression of different MMPs and ADAMTSs. Interestingly, a relatively strong and significant positive correlations between TNF-α and MMP2 (r=0.36, p=0.039) (Figure 4A), ADAMTS 4 (r=0.46, p=0.007) (Figure 4B), and ADAMTS5 (r=0.33, p=0.03) (Figure 4C), but not to the other proteases. Similarly, we found significant positive correlations between IL-6 and MMP1 (r=0.36, p=0.04) (Figure 5A), MMP10 (r=0.36, p=0.037) (Figure 5B), MMP13 (r=0.34, p=0.05) (Figure 5c), ADAMTS 1 (r=0.35, p=0.049) (Figure 5D), ADAMTS4 (r=0.42, p=0.016) (Figure 5E), ADAMTS5 (r=0.39, p=0.024) (Figure 5F), but no correlation with MMP (-2, -3, -13).

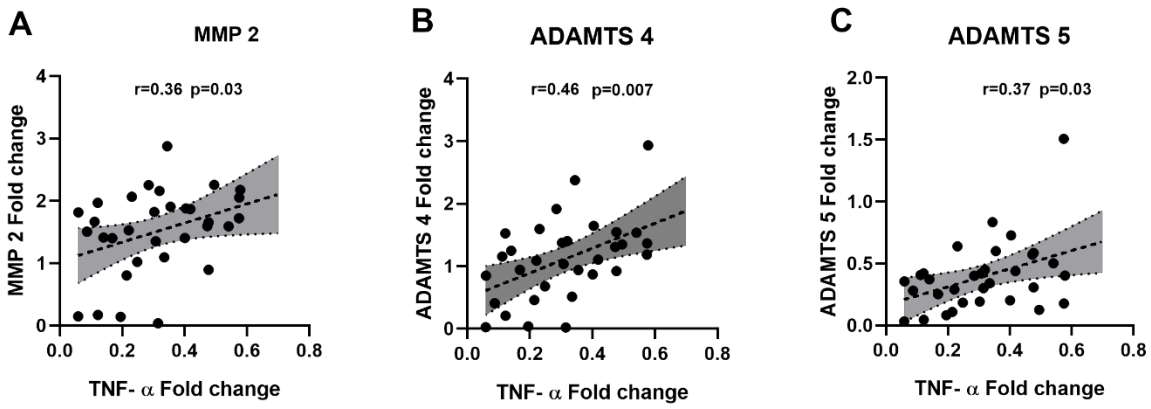


***Figure 4.*** *Correlation between pro-inflammatory cytokine TNF-α expression and MMP and ADAMTS**:** A post hoc analysis of the intercorrelation between TNF-α mRNA expression to the expression of different MMPs and ADAMTSs and we found significant positive correlations between* ***(A)*** *TNF-α and MMP2(r=0.36, p=0.039),* ***(B)*** *ADAMTS 4(r=0.46, p=0.007), and* ***(C)*** *ADAMTS5 (r=0.33, p=0.03). p<0.05 (two-tailed) are considered statistically significant for all comparisons. ρ=spearmen's correlation coefficient.*

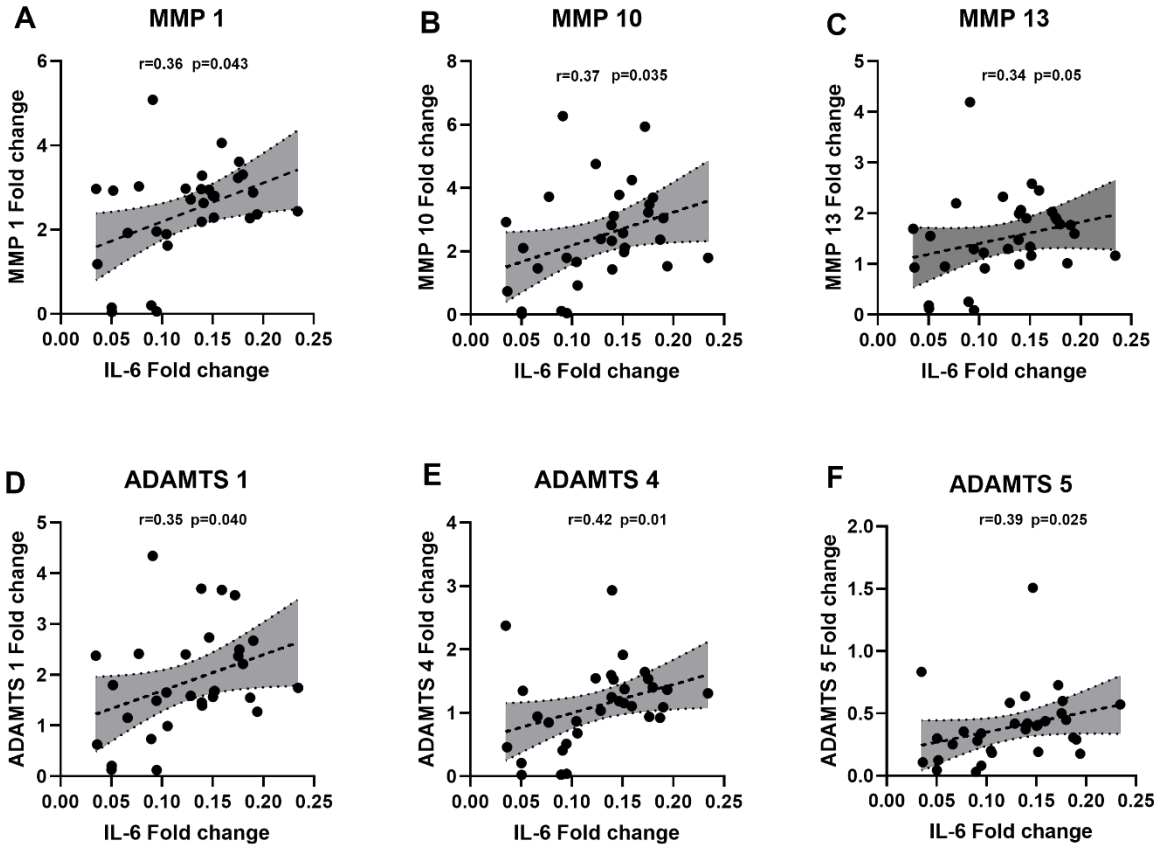


***Figure 5.*** *Correlation between pro-inflammatory cytokine IL-6 expression and MMP and ADAMTS: A post hoc analyses of the intercorrelation between IL-6 mRNA expression to the expression of different MMPs and ADAMTSs and we found significant positive correlations between* ***(A)*** *IL-6 and MMP1(r=0.36, p=0.04) ,* ***(B)*** *MMP10(r=0.36, p=0.037),* ***(C)*** *MMP13 (r=0.34, p=0.05),* ***(D)*** *ADAMTS 1 (r=0.35, p=0.049),* ***(E)*** *ADAMTS4 (r=0.42, p=0.016),* ***(F)*** *ADAMTS5 (r=0.39, p=0.024). p<0.05 (two tailed) are considered statistically significantly for all comparisons. ρ=spearmen's correlation coefficient.*

## 4. Discussion

In the present study, we have demonstrated significant positive correlations between Pfirrmann MRI grades of disc degeneration and the mRNA expression profile of MMP (-1, -3, -10, -13) and

ADAMTS (-1, -5) in NP biopsies from LBP patients undergoing lumbar spine fusion surgery. We also analyzed the expression of these genes in AF, it became clear that no correlations with degeneration grade were observed, and further no differences were found in the post-hoc analysis, likely reason for these results we believe is that the loss of collagen type II and aggrecan, which are essential components of the NP extracellular matrix and molecular targets for the majority of MMP's and ADAMTS's results in ECM breakdown and progression of disc degeneration

***Regulation of gene expression of MMPs and ADAMTSs with degenerative changes in IVD:***

Non-degenerated IVD expresses MMPs, although their expression is minimal and even devoid in young IVDs [31]. Previous studies have shown increased expression of different MMPs in degenerated IVD tissues [31-35]. These studies indicate that ECM loss by proteolytic cleavage is key in degeneration, shifting the disc dynamics towards increased catabolism leading to loss of function and pain generation. MMP-1 belongs to a subclass of collagenases that break down the fibrillar collagens type I, II and III dominantly present in NP and inner annulus fibrosus [36]. The present study showed positive correlation between MMP-1 and degeneration grade. These findings are supported by previous studies [6] which showed that with increasing degeneration an increase in the proportion of NP cells displaying MMP-1-immunoreactivity. Our results on MMP-3 are also in agreement with previous histopathological observations reporting positive correlation between expression of MMP-3 and MRI grading in degenerated disc [37]. MMP-13 is a member of one of the three collagenases and more potent in cleaving collagen type II than MMP-1 [38]. Previous studies demonstrated more MMP-13 immunoreactive cells within the NP of degenerated discs where collagen II is most abundant [32, 33]. Together with the present data a role of MMP-13 in degeneration, probably via collagen II loss from the NP ECM is proposed, further suggesting that ECM loss due to collagen breakdown is driven by MMPs.

Several studies have shown ADAMTS in normal IVD, which signifies their role in normal tissue remolding and homeostasis [32, 39]. Our study showed a significant positive correlation between the NP expression of ADAMTS-1 and 5, but not 4, to the Pfirrmann MRI grading system supporting a previous study conducted on human degenerated endplates. Here a marked upregulation of ADAMTS-5 but not ADAMTS-4 is reported, and it is concluded that ADAMTS-5 and TNF-α plays important role in degenerative endplates-induced low back pain [40]. Similarly,

work on NP cell cultures showed that expression of ADAMTS-5 increased in under senescence induced by continuous passage of cultures [41]. Patel et. al [42] found a higher level of ADAMTS-4 in advanced level degeneration whereas the content of ADAMTS-5 protein levels does not change in contrast to our mRNA data.

Our post-hoc analysis on the pair-wise correlations showed co-expression of MMP-10 with other MMPs and ADAMTSs. Along with the proteolytic activity of MMP-10, it has been shown that MMP-10 is a potent activator of MMP pro-enzymes [43, 44]. These pair-wise correlations may explain that several MMP family members are activated simultaneously. Previous studies describe this activation of pro-MMPs by MMP10 as "super activation" [43, 45]. Such potent and convergent activation of these enzymes might dynamically shift the ECM metabolism to more catabolism by favoring MMP activity over inhibitors.

***Expression of aggrecan in relation to age of the patients:***

Interestingly, we found a significant negative correlation between the expression of aggrecan and age, and this is independent of the disc degradation, Aged disc mainly contains aggrecan in a non-aggregated form and decreased glycosaminoglycan chain length which might be derived from proteolytic damage [46]. This non-aggregated aggrecan might not have the same functional ability as the intact aggregates because their sizes and matrix interactions are diminished [47, 48]. Hence age-related ECM molecular alterations cause structural integrity and biomechanical function loss in the disc is independent of the degeneration [49].

***Regulation of inflammatory pathways on the expression of MMPs and ADAMTSs:***

Cytokines also up-regulate a wide variety of catabolic mediators like ADAMTS-4/5, MMP-1, -2, -13, -14, which suppress the expression of essential ECM genes [50-53]. The link between cytokine expression and MMPs could be important because Seguin et al. [54] showed that TNF-α induced MMP-2 activity post-translationally by controlling MMP-14 expression mainly through the extra cellular signal-regulated kinase pathway. Similarly, we also found significant positive correlations between the expression of TNF-α and ADAMTS- 4/5, which in line with a report demonstrating that TNF-α and IL-1β regulate expression of ADAMTS-4/5 through Mitogen-activated protein kinase and nuclear factor kappa light chain enhancer of activated B cells signalling pathways [54, 55]. Furthermore, IL-6 also synergistically potentiates the catabolic

actions of IL-1β and TNF-α [56], and when treated with a cocktail of cytokines, a significant decrease in proteoglycan synthesis and an increase in PGE-2 and MMP-13 occur [56]. Together, our findings suggest the critical role of cytokines in ECM metabolism and enable us to propose the role of intracellular pathways like MAPK, NF-κB signalling pathways in regulating the expression of these cytokines, proteases, and therapeutic blockage one of these would be expected to prevent loss of ECM. Similarly, it would be interesting to study these associations with Modic type changes because a recent study by Chen, Y. et al. showed that disc degeneration is one factor leading to different types of Modic changes and suggested severe degeneration means severe endplate damage and hence type II changes often occur. However, it is very intriguing to implicate these therapeutic or preventive strategies from our study, but still, it is speculative, further studies are needed to confirm their therapeutic potential.

## 5. Conclusion:

Our results showed an imbalance between catabolism and anabolism of IVD matrix components. We showed that most MMPs and ADAMTSs express in NP, and their expression levels increase with degeneration grade, suggesting the role of these proteases in ECM breakdown and progression of degeneration. Our results also propose that stimuli coming from the release of inflammatory cytokines participate in the regulation of MMPs and ADAMTSs, and dysregulation of their activity can increase the disc's structural loss. Our results contribute to understanding the role of different MMPs and other aggrecanases in disc degeneration etiology with the potential to integrate novel biomarkers in diagnosis, therapy effects, and the prognosis for patients with DDD.

**Disclosure:** The authors have declared no conflict of interest

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**Author's Contribution**: JDM, RBA, and LMJ performed study design, and SSA conducted experiments. SSA and JDM did the data analysis, contributed both to data interpretation; SSA wrote the first draft manuscript, and all authors approved the final manuscript.

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**DECLARATION OF CO-AUTHORSHIP**

*The declaration is for PhD students and must be completed for each conjointly authored article. Please note that if a manuscript or published paper has ten or less co-authors, all co-authors must sign the declaration of co-authorship. If it has more than ten co-authors, declarations of co-authorship from the corresponding author(s), the senior author and the principal supervisor (if relevant) are a minimum requirement.*

| **1. Declaration by** | |
|---|---|
| Name of PhD student | Sagar Sanjay Aripaka |
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| Name of principal supervisor | Jens D Mikkelsen |
| Title of the PhD thesis | MOLECULAR BIOLOGY IN THE PAIN GENERATION IN LUMBAR INTERVERTEBRAL DISCS |

| **2. The declaration applies to the following article** | |
|---|---|
| Title of article | The expression of metalloproteinases in the lumbar discs correlates strongly with Pfirrmann MRI grades in lumbar spinal fusion patients |
| **Article status** | |
| Published ☐<br>Date: | Accepted for publication ☐<br>Date: |
| Manuscript submitted ☒<br>Date: 25th Oct 2021 | Manuscript not submitted ☐ |
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| 2. Development of the key methods | B |
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| 4. Conducting the experimental work/clinical studies/data collection/obtaining access to data | A |
| 5. Conducting the analysis of data | A |
| 6. Interpretation of the results | A |
| 7. Writing of the first draft of the manuscript | A |
| 8. Finalisation of the manuscript and submission | B |
| Provide a short description of the PhD student´s specific contribution to the article.[i] <br>PhD student planned and conducted all the RT-qPCR experiments from RNA extraction protocols, primer designs, housekeeping validations to data analysis. PhD student drafted the manuscript. | |

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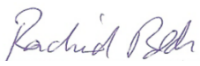
| 1. | 211221 | Rachid Bech-Azeddine | Associate professor |  |
| 2. | 211221 | L. M. Jørgensen | Associate professor |  |
| 3. | 221221 | Jens D Mikkelsen | Professor |  |

**6. Signature of the principal supervisor**

I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge.

Date: 22 Dec2021

Principal supervisor:

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[iii] If more signatures are needed please add an extra sheet.

# Paper III

**Aripaka S.S.,** Bech-Azeddine R, Jørgensen LM, Mikkelsen JD. Transient Receptor Potential (TRP) Channels mRNA Transcripts in the Lumbar Intervertebral Discs: Biomarkers for inflammation, pain, disability, and clinical outcome. Manuscript submitted *European Spine Journal.*

# Transient Receptor Potential (TRP) Channels mRNA Transcripts in the Lumbar Intervertebral Discs: Biomarkers for inflammation, pain, disability, and clinical outcome

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# Transient Receptor Potential (TRP) Channels mRNA Transcripts in the Lumbar Intervertebral Discs: Biomarkers for inflammation, pain, disability, and clinical outcome


## Abstract

**Purpose:** Transient receptor potential (TRP) channels are widely expressed cation channels that play an essential role in mediating Ca2+ homeostasis and are considered potential regulators of inflammatory pain. This study investigates the expression of the TRP channel subtypes TRPV1, TRPV4, TRPC6, TRPM2, TRPM8 in lumbar intervertebral disc (IVD) biopsies from patients with chronic low back pain (LBP).

**Methods:** We determined the expression of these TRP channel subtypes in the annulus fibrosus (AF) and the nucleus pulposus (NP) from 46 patients with LBP undergoing 1-2 level lumbar fusion surgery for degenerative disc disease. The mRNA transcripts were analyzed using quantitative real-time polymerase chain reaction (RT-qPCR), and the expression levels were correlated to Visual Analog Scale (VAS) and Oswestry Disability Index (ODI) scores (0-100) for pain and disability.

**Results:** A significant positive correlation between VAS score and the mRNA transcripts for TRPV1, TRPC6, TRPM2, TRPM8 was demonstrated in the AF. We also showed a significant positive correlation between ODI scores and expression of TRPV1, TRPM8. Further, there is a significant positive correlation between TNF-α in the AF to TRPV1, TRPM2 and TRPM8, and IL-6 to TRPV1 in the NP. Interestingly, a 12-month postoperative follow-up of treatment response to surgery demonstrated a significant correlation between only TRPV1 expression at baseline and the follow-up ODI scores, which indicate this marker to be a potential predictor of the effect of surgery.

**Conclusion:** These results strongly suggest the association between inflammatory mediators, TRP channels expression in lumbar disc biopsies in patients with chronic LBP.


**Keywords**: low back pain; TRP channels; lumbar fusion surgery; Degenerative Disc

# APPENDIX

## INTRODUCTION:

Low back pain (LBP) is considered a symptom rather than a disease [1]. Nevertheless, LBP is the main cause of years lived with disability, and an estimated 80% of the worldwide population suffer from LBP at least once in their lifetime [2]. Furthermore, LBP is commonly associated with degeneration of the intervertebral disc (IVD), and conservative therapy such as analgesics is often the only treatment for these patients.

The IVD is a mechanosensitive structure that lies between the spinal vertebrae [3]. The biochemical composition of this tissue will define the mechanical properties by absorbing and distributing the applied loads generated by muscle activity and body weight. IVD primarily contains the highly hydrated, avascular and proteoglycan-rich central nucleus pulposus (NP), surrounded by the annulus fibrosus (AF), richer in collagen I. The cellular content of NP is low (5000 cells/$mm^3$), and it is poorly vascularized. Therefore, the IVD is prone to degeneration with loss of extracellular matrix (ECM) [4]. ECM is mainly composed of proteoglycans, which has a water holding capacity. Thus, tissue dehydration and weakening, such as tears and clefts, occur if the ECM is low. These changes influence the mechanical properties of IVD and hence alter mechanobiology [5].

The onset and regulatory mechanisms for degenerative disc disease (DDD) are not completely understood. However, patients affected by DDD are characterized by secretion of inflammatory molecules like TNF-α, IL-1β, IL-6, IL-8, and neutrophils, macrophages, and T cells [6, 7] originating from cells in both NP and AF. These inflammatory mediators in the blood can affect the release of other molecules, i.e. ion channels like transient receptor potential (TRP) channels, sodium channels, and mechanosensitive ion channels in nociceptors, which lead to sensitization and pain hypersensitivity [8]. Interestingly, TRP channels have emerged as receptors for inflammation-associated molecules and conduct inflammation-mediated pain [9-11]. TRP channels are cation-selective transmembrane receptors with diverse physiological functions and are regulated by diverse stimuli. Stimulation of TRP channels causes channel opening and influx of calcium ions. Six families of TRP channels have been identified and classified according to their sequence homology and topological differences: TRPA (ankyrin), TRPC (canonical), TRPM (melastatin), TRPV (vanillin), TRPP (polycystin), and TRPML (mucolipin). Apart from

TRPA, every subfamily has several members [12]. TRP channels are expressed in almost all excitable and non-excitable tissues [13], are primarily present on the cell surface, and are involved in sensing chemical and physical stimuli such as temperature, pH, mechanical stress, and osmolarity [12]. Any dysregulation in TRP channels is considered to influence the physiological immune response [14].

Some lines of evidence suggest that TRP channels in IVD cells may contribute to degeneration and pain [10]. Altered functions and regulation of TRP channels affect the inflammatory, osmotic and mechanical sensitivity, which mediate a variety of sensations, including pain [3]. It has been shown that the expression and activity of TRP channels are altered in IVD and painful joints [15]. More recent studies showed dysregulation of TRPV4 and TRPC6 channels by several stimuli like inflammation, mechanical and osmotic sensitivity [3]. During pathological conditions, many TRP channels are sensitized, and their activation threshold is reduced, leading to an increase in perception of hyperalgesia and allodynia [8].

The association between gene expression and pain intensity, disability, and degeneration is not established completely. To better understand the role of different TRP channels in IVD, this study was designed to determine the expression of TRP channels like TRPV1, TRPV4, TRPC6, TRPM2, and TRPM8 in degenerated human IVD. These particular channels among the TRPs were selected because of their involvement as molecular nociceptors in peripheral sensory neurons and musculoskeletal tissues and actively transducing thermal, chemical, and mechanical stimuli, and showed altered expression in other pathological conditions [3, 8, 14]. Furthermore, as a post-hoc analysis, we also wanted to understand the role of inflammation on the expression of TRP channels because we have shown in a previous study that there is a positive correlation between the expression of inflammatory cytokines to pain intensity and disability [7].

**Materials and Methods:**

**Participants**

A total of 48 patients (36F, 12M) of 18-65 years of age with LBP and radiculopathy scheduled to undergo fusion surgery with pedicle screws and placement of a transforaminal lumbar interbody

cage were included. The preoperative magnetic resonance image (MRI) and lumbar X-rays were used to evaluate spondylolisthesis, instability, foraminal stenosis and Pfirrmann grade (I-V).

**Rating Scales:**

Before surgery, patients were assessed for disability by Oswestry Disability Index (ODI 0 –100), which ranges from 0 (no disability) to 100 (highest disability) [16], and for pain intensity in the lower back by a visual analog scale (VAS), which ranges from 0 (no pain) to 100 (worst pain) [17].

**Pfirrmann MRI Grading System**

The degree of disc degeneration was assessed for each patient using the 5-level Pfirrmann MRI grading system on T-2 weighted sagittal MRI sequences. A Pfirrmann grade I and II indicates a non-degenerative disc, whereas grade III, IV, and V signify an increasingly degenerative disc [18].

**Tissue Sampling**

Tissue sampling was performed as previously detailed [7]. Briefly, after the decompression procedure, the surgeon isolated and collected the AF and NP. First, a window of the AF was cut open with a scalpel, and any adherent NP tissue was removed. Next, several NP samples were retrieved from the disc space using forceps. Both NP and AF tissues were investigated in this study.

**RNA extraction, cDNA synthesis, and RT-qPCR:**

RNA extraction procedure and subsequent use of RT-qPCR were performed as previously detailed [7]. Briefly, total RNA was extracted from frozen AF and NP tissue using the Phenol–chloroform extraction method using TRIzol (Sigma Aldrich., Denmark), according to manufacturer protocol. Next, RNA concentration and purity were determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific). Finally, cDNA was synthesized from 0.2µg of RNA using ImProm-II™ Reverse Transcription System (Promega, USA). RT-qPCR performed with iQ SYBR® Green Supermix (BioRad, California, USA) for 40 cycles in a fixed sequence at 94 °C for 30 seconds, 60 °C for 15 seconds, and 72 °C for 15 seconds. Amplicons were

generated using the primer sets listed in Table 1. Succinate Dehydrogenase Complex Flavoprotein Subunit A (*SDHA*), Lactate dehydrogenase A (*LDHA)* and Beta-Actin (*ACTB*) were used as housekeeping genes, these genes are selected based on validation assay previously detailed [7], and all TRP channels expression was measured relative to the geometric mean of the three housekeeping genes.

| Gene | Forward (5`- 3`) | Reverse (5`- 3`) |
|---|---|---|
| **TNF- α** | TTC CTG ATC GTG GCA GGC | GCT GAT TAG AGA GAG GTC CCT G |
| **IL-6** | TTC GCT CTT CCA GTT GGA CT | CAC CAG GGG AAG AAT CTG AG |
| **IL-1β** | ACC AAG TGC CAC AAA GGA AC | CTG CAA TTG AAG CAC TGG AA |
| **SDHA** | CGA GCT GCA TTT GGC CTT TC | TTG ATT CCT CCC TGT GCT GC |
| **LDHA** | GCC TGT ATG GAG TGG AAT GAA | CCA GGA TGT GTA GCC TTT GAG |
| **β-Actin** | TGG AAC GGT GAA GGT GAC AG | AAC AAC GCA TCT CAT ATT TGG AA |
| **TRPV1** | CTG CCC GAC CAT CAC AGT C | CTG CGA TCA TAGAGC CTG AGG |
| **TRPV4** | CTA CGG CAC CTA TCG TCA CC | TTA GGC GTTT TCT TGT GGG TCA |
| **TRPC6** | ATC TTG ACG GAG AGT GCG G | CTG GTG GTA GCA AAG CGT A |
| **TRPM2** | ACC CTT TTA CAC GGC AGA GA | TAC TGG ATC GTG GAC AGT GG |
| **TRPM8** | GCA AGT GTG GCT ATG CCC A | CCA AAG GCG TCG GTA GGA A |

**Table 1:** ***List of primers used in this study***

**Statistical analysis**

Statistical analysis was performed with the GraphPad Prism 8.2 software. Data are expressed as Mean ± SD. An unpaired student t-test evaluated group differences in mRNA expression. The correlation of mRNA levels and clinical data was assessed using the Pearson correlation coefficient. Similarly, a p-value of $< 0.05$ is considered statistically significant for all comparisons and for post-hoc analyses the p-value is uncorrected for multiple comparisons.

**Results:**

The average age of the included patients was 52 years ± 9 years [18-65]. The VAS score for back pain was 65 ± 26, and the ODI score was 42 ± 17. We found an expression of all five TRP channels in both AF and NP biopsies. The levels of TRPV1 and TRPV4 was higher in AF when

compared to NP, whereas the expression of TRPC6, TRPM2, TRPM8 did not differ between AF and NP (Fig. 1).

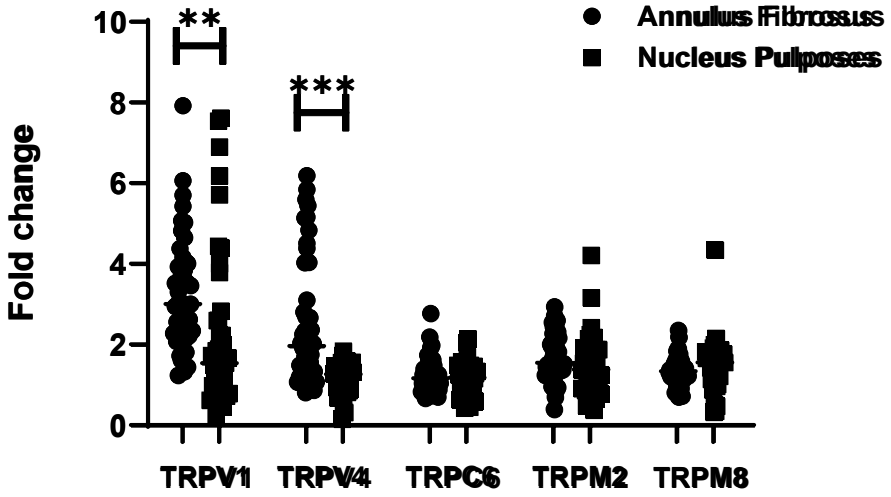



***Fig.1 The expression of TRP channels in the annulus fibrosus (AF) and nucleus pulposus (NP):*** *mRNA level of five different TRP channels (TRPV1, TRPV4, TRPC6, TRPM2, and TRPM8) were measured in AF (n=46) and NP (n=44). A significant difference was observed in the expression of TRPV1 and TRPV4 between AF and NP. Expression of all TRP channels was measured relative to the geometric mean of 3 housekeeping genes. Values are Mean ± SD. **p<0.01, ***p<0.001*

**Correlation between the expression of TRP channels and clinical rating scores for pain and disability**

In the AF and illustrated in Fig. 2, there was a strong and highly significant positive correlation between the VAS pain score and the expression of TRPV1 (Fig. 2a); TRPC6 (Fig. 2c); TRPM2 (Fig. 2d); and TRPM8 (Fig. 2e). In particular very high significance was found for TRPV1 and TRPM8. On the other hand, no correlation was found for TRPV4 (Fig. 2b).

In contrast to the expression in the AF, the VAS scores for pain intensity did not correlate with the expression level of any of the TRP channels in the NP (data not shown).

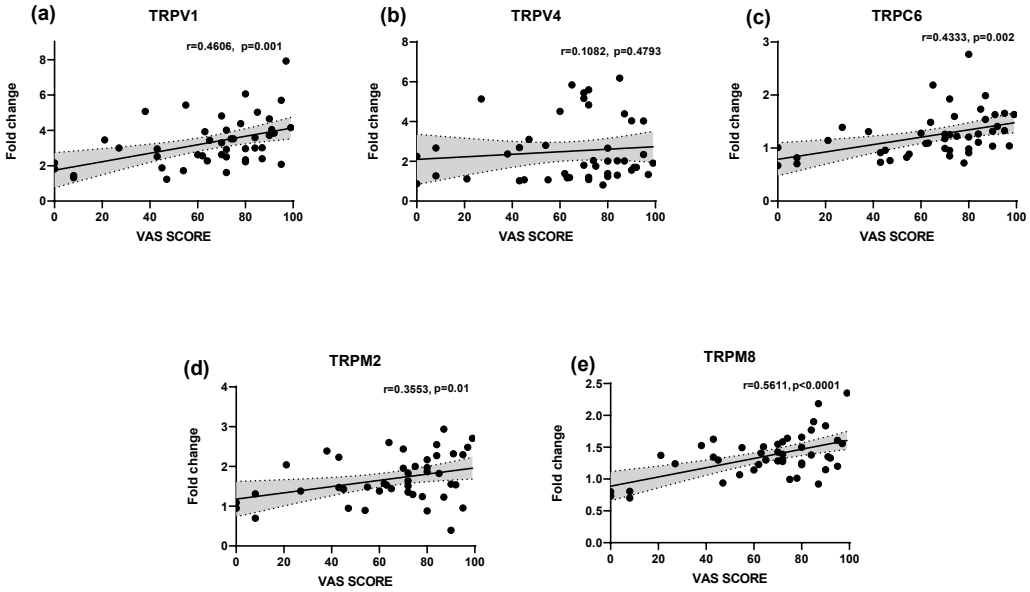



***Fig.2 Correlation between TRP channel expression in AF and VAS score for pain intensity:*** *(a) VAS score showed a significantly positive correlation with TRPV1(r=0.46, p=0.001). (b) No correlation observed with TRPV4. (c), (d), (e) Positive correlations demonstrated with TRPC6(r=0.43, p=0.002), TRPM2(r=0.35, p=0.01), TRPM8 (r=0.56, p<0.0001). No correlations were observed in NP. $p < 0.05$ is considered statistically significant for all comparisons. r = Pearson correlation coefficient*

Moreover, in the AF we also observed a strong and highly significant positive correlation between the ODI score for disability and expression of TRPV1 (Fig. 3a) and TRPM8 (Fig. 3b) was observed. By contrast, expression of TRPV4, TRPC6, and TRPM2 was not found to be correlated, and ODI scores did not correlate with expression of any TRP channels in the NP (data not shown).

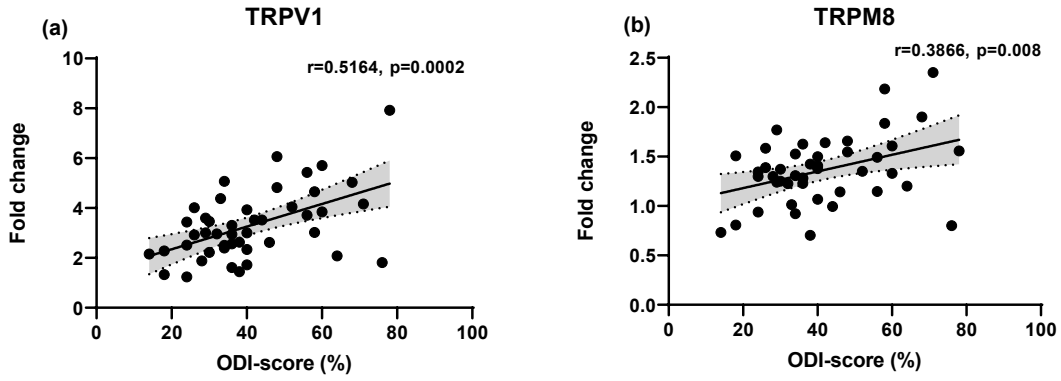



***Fig.3 Correlation between TRP channels expression Oswestry disability index (ODI) in AF:*** *(a) ODI score showed a significantly positive correlation with TRPV1 (r=0.51, p=0.0002). (b) TRPM8 showed positive correlation (r=0.386, p=0.008). TRPV4, TRPM2, and TRPC6 did not show any correlations in AF. No correlations were observed in NP. $p < 0.05$ is considered statistically significant for all comparisons. r = Pearson correlation coefficient*

**Correlation between the expression of TRP channels and clinical rating scores for disability one year after surgery**

Interestingly, when assessed for disability one year after the surgery, which was undertaken in a subpopulation of 34 patients, we here demonstrate that the expression of TRPV1 level at baseline was significantly correlated with the ODI score for disability at the 1-year follow-up (Fig 4). The expression of the other examined TRP channels in AF and NP were not associated with the outcome scores for pain and disability at the one-year follow-up.

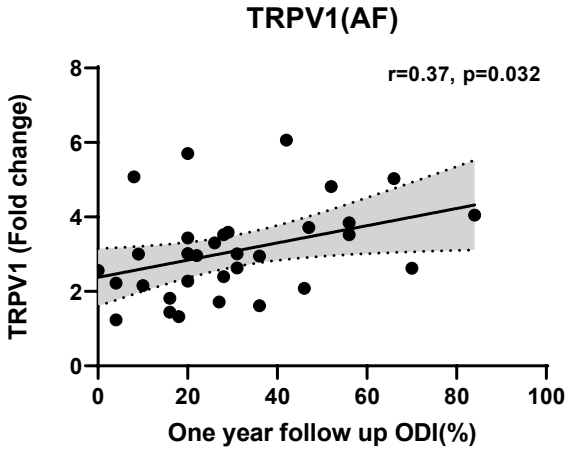



***Fig.4*** *Significant positive correlation between TRPV1 channel and one-year follow-up ODI scores (r=0.37, p=0.03). p < 0.05 (uncorrected) is considered statistically significant for all comparisons. r = Pearson correlation coefficient*

**Post hoc analyses**

In order to further elaborate the mechanisms of expression of TRP channels in patients with low back pain, we conducted a post-hoc analyses correlating TRP expression to other biomarkers of lower back pain and disease, namely Pfirrmann MRI classification for disc degeneration (grade I-V), pro-inflammatory cytokine gene expressions (TNF-α, IL-1β, IL-6) and Nerve Growth Factor (NGF) mRNA.

A significant positive correlation between Pfirrmann grade (I-V) for disc degeneration and expression levels of TRPV1 in the NP was found (Fig 5). No other markers in the AF nor the NF displayed such correlation.

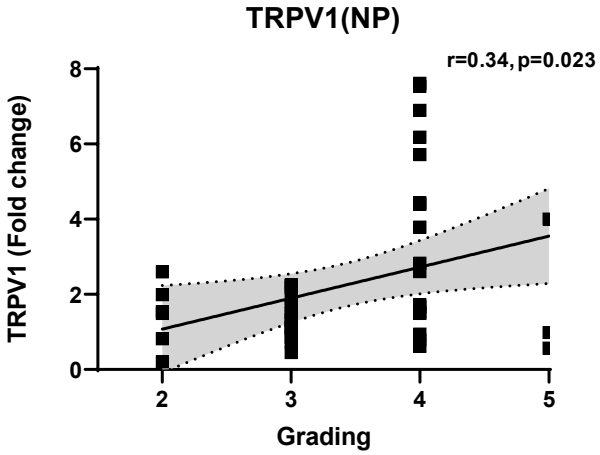
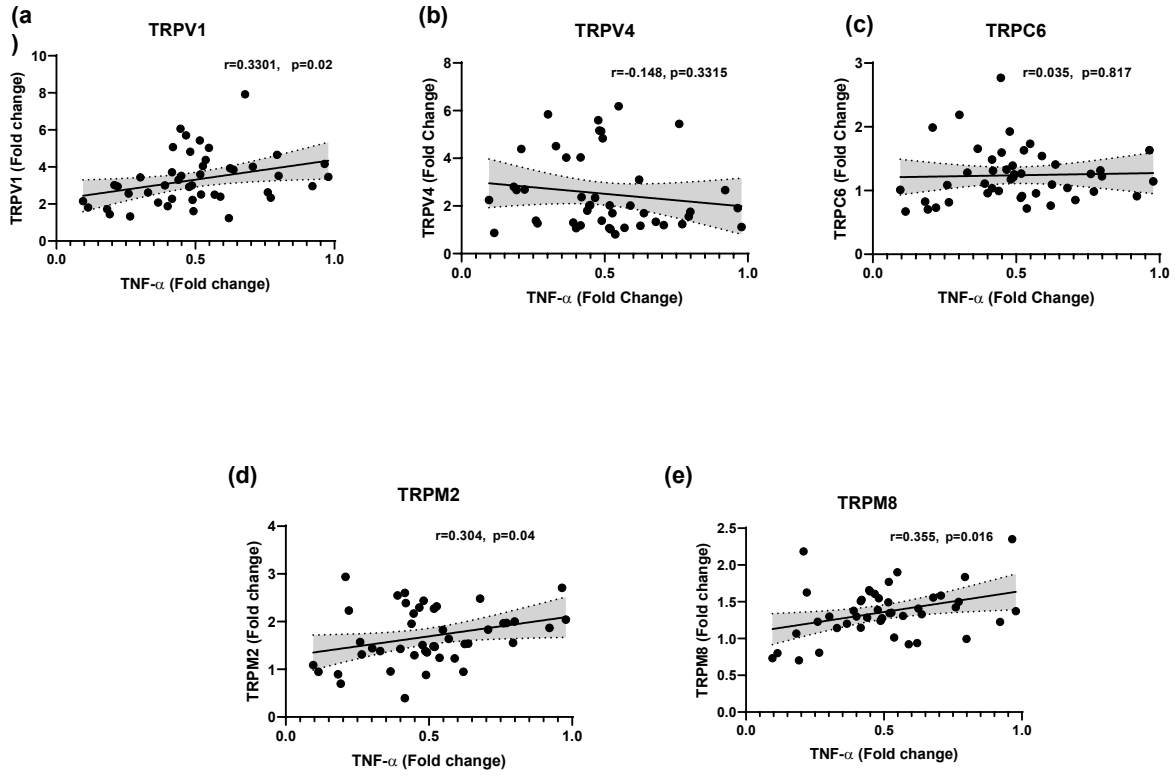



**Fig.5 Expression of TRP channels in NP correlate with Pfirrmann MRI grades:** Significant positive correlation observed between Pfirrmann MRI grades and expression of TRPV1 (r=0.34, p=0.023). No other channels showed any correlation with Pfirrmann MRI grades. $p < 0.05$ is considered statistically significant for all comparisons. r = Pearson correlation coefficient.

Next, to elucidate the mechanisms of TRP channels in pain generation, we correlated the expression of TRP levels in AF and NP with the most dominant pro-inflammatory cytokines (Fig. 6-7). With regard to TNF-α in the AF (Fig 6a-e), a significant positive correlation to TRPV1 (Fig 6a), TRPM2 (Fig 6d), TRPM8 (Fig 6e), but not for TRPV2 (Fig 6b), and TRPC6 (Fig. 6C). Furthermore, we also analysed the expression in the NP and found that TRPV4 was correlated to TNF-α expression in this tissue, but not any of the other TRP mRNA transcripts (data not shown). Interestingly TRPV4 is also strongly correlated to IL-1β in the NP (Fig. 7a). However, with regard to IL-6, only TRPV1 in NP was found to be correlated (Fig 7b).

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2195 ***Fig.6 Correlation between TNF-α expression and TRP channel expression in AF (a-e)***. *In the*
2196 *AF, TNF-α showed significant positive correlation with TRPV1 (r=0.33, p=0.02) (a), TRPM2*
2197 *(r=0.30, p=0.04) (d), and TRPM8 (r=0.35, p=0.016) (e), and no correlations observed with*
2198 *TRPV4 and TRPC6 (b, c). p < 0.05 (un-corrected) is considered statistically significant for all*
2199 *comparisons. r = Pearson correlation coefficient*.

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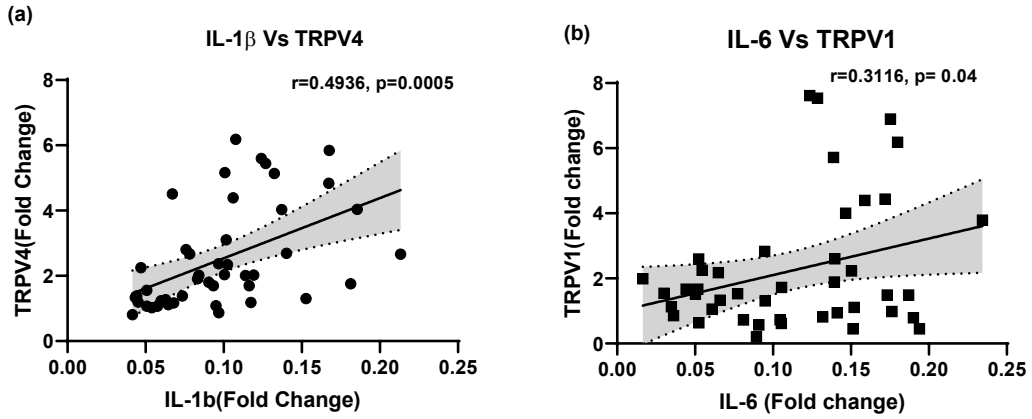


***Fig.7 Correlation between pro-inflammatory cytokine (IL-1β, IL-6) and TRP channel expression (AF and NP).*** *In AF IL-1β showed a correlation with TRPV4(r=0.49, p=0.0005) (a), no other channels showed any correlation with IL-1β in both AF and NP. Further, TRPV1 showed correlation with IL-6 (r=0.31, p=0.04) (b) in NP, and no other channels showed correlation with IL-6 in both AF and NP. p < 0.05 (un-corrected) is considered statistically significant for all comparisons. r = Pearson correlation coefficient.*

Finally, the expression of most of the TRP channels correlates significantly (uncorrected) with the expression of NGF in both AF (TRPV1 and TRPM2) and NP (TRPV1, TRPV4, TRPC6, TRPM2 and TRPM8) (Fig 8a-g).

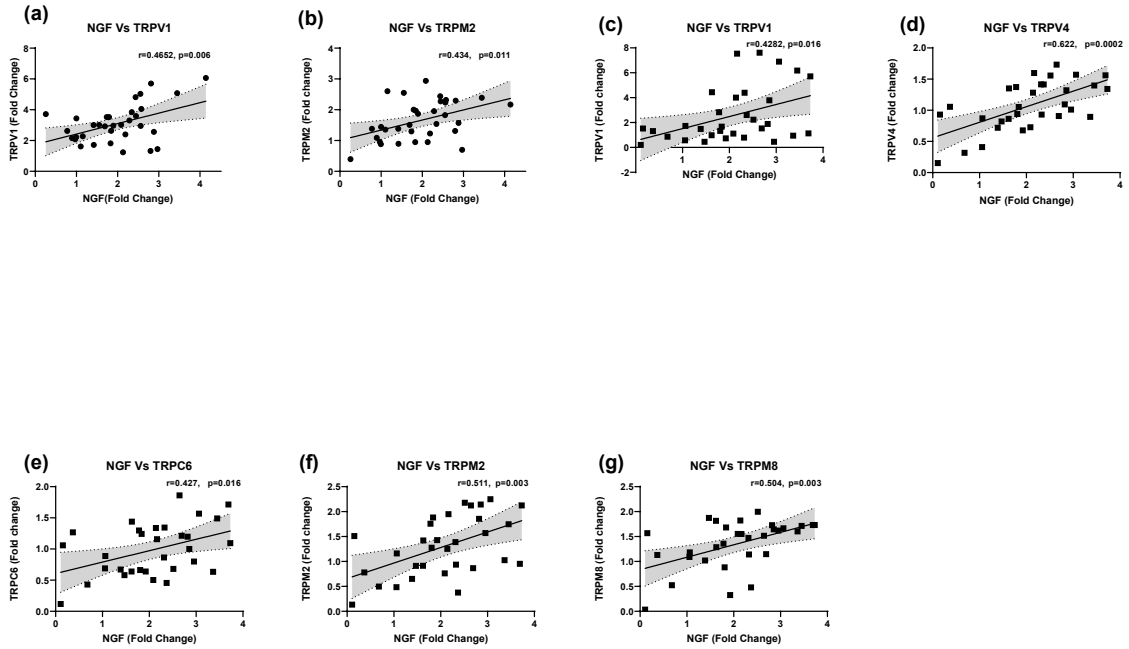


***Fig.8 Correlation between expression of TRP channels and NGF:*** *We made a post-hoc analysis on 34 IVD samples. In AF (a, b) Expression of NGF showed significant positive correlation with TRPV1 (r=0.46, p=0.006) and TRPM2 (r=0.43, p=0.01), but not with the other channels. In NP (c-h), expression of NGF showed significant positive correlation with all TRP channels i.e., TRPV1 (r=0.42, p=0.01), TRPV4 (r=0.62, p=0.0002), TRPC6(r=0.42, p=0.016), TRPM2 (r=0.51, p=0.003) and TRPM8 (r=0.50, p=0.003). $p < 0.05$ (uncorrected) is considered statistically significant for all comparisons. r = Pearson correlation coefficient.*

**Discussion:**

This study shows that the expression of selected TRP channels in the lumbar disc correlates significantly with the patient's rating scores (VAS) for lumbar pain and disability (ODI). Further, the expression also correlates with the Pfirrmann MRI classification for disc degeneration, the expression of pro-inflammatory markers (TNF-α, IL1-beta, IL-6), as well as nerve growth factor (NGF). This strongly indicates that TRP channels, known to be centrally implicated in pain perception, are also regulated in the disc under the progression of degeneration and

inflammation. Interestingly, we showed that for TRPV1, the expression of this particular channel was significantly correlated with the clinical outcome of the operation at one year follow up.

We believe this is one of the first studies showing a significant positive correlation between a transcript (here TRPV1 expression) at the time of operation and the treatment response to lumbar fusion surgery (here ODI score) at baseline and one-year follow-up. As such, TRPV1 may serve as a predictor for the effect of surgery: a high TRPV1 expression at baseline may predict a poor disability at follow-up. A previous study, as well as our findings reports a significant positive correlation between the expression of TRPV1 and ODI scores at baseline [19], but here extended to a more sustained marker. To what extent TRPV1 is a biomarker for more general clinical outcome measures require further investigations.

Pro-inflammatory cytokines are essential regulators of pain, and their increased synthesis and release from the IVD produce inflammatory pain [5, 20]. We have recently reported that TNF-α and IL-6 in the disc are linked to higher pain intensity and disability [7]. In the present study, conducted on partly the same patients, expression of TRPV1 also displays a positive correlation with the expression of TNF-α and IL-6. It is considered that TRPV1 channels, as a member of the TRPV (vanillin) in humans [21], are located on nerve terminals of dorsal root ganglion neurons that innervate IVDs and plays a role in nociception and pain management [13]. Previous studies showed that TNF-α causes an increase in the probability of channel opening to heat, capsaicin and protons [22]. Furthermore, Yu et al. [23] showed that an inflammatory stimulus increased the expression of TRPV1 [23]. These data indicate that TRPV1 is an interplay with cytokines is important in inflammation-mediated pain. Interestingly, TRPV1 is also expressed in non-neuronal cells like chondrocytes [9] and IVD cells [15], but their role in these tissues with respect to inflammation and pain is unknown. One possibility is their role in mediating the acidic microenvironment [24]. Change in IVD homeostasis by the accumulation of lactic acid due to poor fluid transport in degenerative IVD can increase the acidity and thus enhance TRPV1 functionality [24, 25]. We propose that activation of these channels causes a transient increase in intracellular calcium in chondrocytes and IVD cells with subsequent exocytosis of neuropeptides and also the regulation of several inflammatory genes that encodes cytokines, growth factors, prostaglandins (PG), and matrix metalloproteinases (MMPs) which are also involved in cell

hemostasis as well as inflammation [26]. The correlation between expression of TRPV1, disabling pain, and pro-inflammatory cytokines is in line with these observations.

Studies conducted by Walter et al. on bovine IVD [27] demonstrated an increase in TRPV4 expression with a decrease in tissue osmolarity due to loss of aggrecan and other glycosaminoglycans with advancing degeneration [28]. Furthermore, in the same study, increased TRPV4 expression increased calcium release and increased the expression of TNF-α, IL-1β and IL-6, which supports our findings and implicates a broader role of TRPV4 signalling in contribution to inflammatory response and potential involvement in the progression of DDD.

We show here that the expression of TRPM2 and TRPM8 showed correlations with TNF-α expression and pain intensity, which is in line with Sadowska et al., demonstrating an influence of TRPM2 expression on pain intensity and chronicity [3]. It has been shown that expression of TRPM8 is increased in the rat chronic constriction injury models [29] [30]. TRPM2 channels are suggested to have a role in the various cellular process like cell survival and sensing cellular stress.TRPM2 may be activated by reactive oxygen species mainly by $H_2O_2$ and functions as a sensor for cellular stress [31]. Similarly, TRPM8, a sensor of cold temperatures and animal models of neuropathic pain studies, showed TRPM8 channels expression increased in DRG in response to sciatic or spinal nerve damage, which correlated to the development of neuropathic pain [30,40]. TRPM8 is a drug target for treating cold allodynia, a characteristic feature of neuropathic pain, and blockage of TRPM8 showed a decrease in cold hypersensitivity in nerve injury models. Association of TRPM8 expression with pain intensity suggest the involvement of TRPM8 in sensory nerve functions in the context of low back pain.

One striking observation was the significant correlation between NGF and most of the examined TRP channels in both AF and NP. NGF plays a vital role in the growth and differentiation of sensory and sympathetic neurons during development [32]. The growth factor also plays a significant role in pain mediation and nociception via binding to its receptor tropomyosin kinase A (TrkA) in primary afferent sensory nerve fibres [33]. Several studies have reported elevated levels of NGF in the DRG in several inflammatory and pain models in rats [see 34] for review. Elevated levels of NGF is found in the synovial fluid and IVDs from patients with chronic pain diseases like osteoarthritis and low back pain, respectively [35]. Furthermore, Freemont et al.

[36] showed that the growth of nociceptive nerves into painful degenerated IVD is linked with NGF production by endothelial cells of blood microvessels growing into IVD [36]. This demonstrates that the NGF can induce neural growth into poorly innervated IVD [37] and contribute to the painful degenerated discs. The exact mechanisms of pain generation are unknown, but previous studies suggested that NGF-induced sensitization of TRPV1 can be an important contributor [38] and TRPV1 is known to play a vital role in nociception modulated by NGF activity [39]. NGF was also shown to increase translocation of TRPV1 to the cell surface in cultured DRG neurons possibly mediated through PI3K and/or PKC signaling events downstream of TrkA [39]. These results support our findings that there is a link between NGF and TRP channels, especially TRPV1 channels, in pain associated disc degeneration.

Altogether, our results provide evidence that TRP channels are expressed in AF and NP of degenerated lumbar discs and highlight a significant relationship between expression of TRP channels with patient-associated features like lumbar pain intensity, disability, and MRI based degeneration scores. Furthermore, the association between cytokines and TRP channels suggests the role of TRP channels in the regulation of several inflammatory events. While treatment strategies for low back pain rely mainly on NSAIDs, this precipitates new options for providing patients with adequate relief. Our results provide further insights into TRPV1 as a diagnostic assessment and therapeutic target.

**Conflict of interest**: The authors have declared no conflict of interest.

**Ethical approval**: The study was approved by the Danish ethics committee (H-17026301). All participants provided written informed consent according to the Declaration of Helsinki.

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**DECLARATION OF CO-AUTHORSHIP**

*The declaration is for PhD students and must be completed for each conjointly authored article. Please note that if a manuscript or published paper has ten or less co-authors, all co-authors must sign the declaration of co-authorship. If it has more than ten co-authors, declarations of co-authorship from the corresponding author(s), the senior author and the principal supervisor (if relevant) are a minimum requirement.*

| **1. Declaration by** | |
|---|---|
| Name of PhD student | Sagar Sanjay Aripaka |
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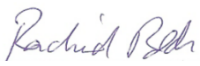
| Name of principal supervisor | Jens D Mikkelsen |
| Title of the PhD thesis | MOLECULAR BIOLOGY IN THE PAIN GENERATION IN LUMBAR INTERVERTEBRAL DISCS |

| **2. The declaration applies to the following article** | |
|---|---|
| Title of article | Transient Receptor Potential (TRP) Channels mRNA Transcripts in the Lumbar Intervertebral Discs: Biomarkers for inflammation, pain, disability, and clinical outcome. |
| **Article status** | |
| Published ☐<br>Date: | Accepted for publication ☐<br>Date: |
| Manuscript submitted ☒<br>Date: 21st Dec 2021 | Manuscript not submitted ☐ |
| If the article is published or accepted for publication, please state the name of journal, year, volume, page and DOI (if you have the information). | |

| **3. The PhD student's contribution to the article (*please use the scale A-F as benchmark)*** <br>Benchmark scale of the PhD-student's contribution to the article <br>**A**. Has essentially done all the work (> 90 %) **B**. Has done most of the work (60-90 %) **C**. Has contributed considerably (30-60 %) D. Has contributed (10-30 %) **E.** No or little contribution (<10 %) **F.** Not relevant | **A, B, C, D, E, F** |
|---|---|
| 1. Formulation/identification of the scientific problem | D |
| 2. Development of the key methods | B |
| 3. Planning of the experiments and methodology design and development | A |
| 4. Conducting the experimental work/clinical studies/data collection/obtaining access to data | A |
| 5. Conducting the analysis of data | A |
| 6. Interpretation of the results | A |
| 7. Writing of the first draft of the manuscript | A |
| 8. Finalisation of the manuscript and submission | B |
| Provide a short description of the PhD student´s specific contribution to the article.[i] <br>PhD student planned and conducted all the RT-qPCR experiments from RNA extraction protocols, primer designs, housekeeping validations to data analysis. PhD student drafted the manuscript. | |

| **4. Material from another thesis / dissertation**[ii] | |
|---|---|
| Does the article contain work which has also formed part of another thesis, e.g. master's thesis, PhD thesis or doctoral dissertation (the PhD student's or another person's)? | Yes: ☐ No: ☒ |
| If yes, please state name of the author and title of thesis / dissertation. | |
| If the article is part of another author's academic degree, please describe the PhD student´s and the author´s contributions to the article so that the individual contributions are clearly distinguishable from one another. | |

**5. Signatures of the co-authors**[iii]

| | Date | Name | Title | Signature |
|---|---|---|---|---|
| 1. | 211221 | Rachid Bech-Azeddine | Associate professor |  |
| 2. | 211221 | L. M. Jørgensen | Associate professor |  |
| 3. | 221221 | Jens D Mikkelsen | Professor |  |

**6. Signature of the principal supervisor**

I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge.

Date: 22 Dec2021

Principal supervisor: 

**7. Signature of the PhD student**

I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge.

Date: 22 Dec 2021

PhD student: 

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[i] This can be supplemented with an additional letter if needed.

[ii] Please see Ministerial Order on the PhD Programme at the Universities and Certain Higher Artistic Educational Institutions (PhD Order) § 12 (4):

*"Any articles included in the thesis may be written in cooperation with others, provided that each of the co-authors submits a written declaration stating the PhD student's or the author's contribution to the work."*

[iii] If more signatures are needed please add an extra sheet.

# Annexure I

# Selection of stable House-keeping genes for RT-qPCR Normalization.

**Aim:**

To explore the expression stability of potential reference genes in Human Lumbar intervertebral disc Biopsy specimens for Normalization of quantitative Real-Time Polymerase chain reaction.

**Materials and Methods.**

Lumbar disc tissue obtained from the patients undergoing a lumbar spinal fusion procedure including a discectomy for low back pain and presenting a disc degeneration on MRI at the surgical level at Center of Rheumatology and Spine Diseases, Rigshospitalet – Glostrup. Samples from 12 patients have been selected for this study specimens are collected from L4/L5 and L5/S1 regions of spine showing spondylosis and radiculopathy. Immediately after surgery the samples where stored in dry ice for transport and after receiving they are stored at -80°C until further analysis.

**RNA extraction:**

Total RNA extraction was performed using TRI reagent (sigma Aldrich., Denmark), according to manufactures protocol. The RNA content was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific). Throughout the RNA extraction the samples were kept on ice.

**cDNA synthesis**

The ImProm-IITM Reverse Transcription System (Promega, USA) was used to reverse transcribe the RNA. The concentration of each RNA sample was measured with a spectrophotometer (NanoDrop, USA) and the samples were diluted to the same final concentration determined by the sample with the lowest RNA yield. The diluted samples were mixed with 1 µl Oligo(dT)15 primer (0.5 µg/reaction) and incubated for 5 minutes at 60 °C in an iCycler (Bio-Rad), in order to amplify mRNA specifically, since the primers recognize mRNA's poly-A tale. Then reverse transcription reaction mix (ImProm-IITM 5X Reaction buffer, MgCl2, dNTP mix, recombinant RNasin ribonuclease inhibitor, ImProm IITM Reverse transcriptase, nuclease-free water) was added to

each sample. Finally, the samples were placed in the iCycler for cDNA synthesis. The cycles used for the reverse transcription were: 1. 5 min at 25 °C 2. 60 min at 42 °C 3. 15 min at 70 °C the cDNA samples were placed one ice for 2 minutes, then added 80 µl 0.5xTris-EDTA buffer (Fluka 93302) and stored at -20 °C.

**Selection of reference Genes:**

Five candidate reference genes were chosen on their common use as reference genes and insights from previous study (Zhou ZJ, et al. (2014)) which showed the importance of suitable housekeeping genes to study for normalization in Human cartilage endplate of the lumbar spine. According their study LDHA and SDHA where stable expressing genes and GAPDH, ACTB and 18S where least stable expressing genes. So, we have selected these 5 genes to study their stability in our biopsy's. The primers sequences were shown below, and they have been adopted from (Zhou et al., 2014). The primers were synthesized by LGC, Biosearch Technologies., Ltd (Denmark).

| Gene Symbol | mRNA gene name | Accession no. | Primer Sequence | Amplicon size(bp) |
|---|---|---|---|---|
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase | NM_002046.3 | 5′-AGAAGGCTGGGGCTCATTG-3′<br>3′-AGGGGCCATCCACAGTCTTC-5′ | 258 |
| ACTB | Actin,beta | NM_001101.3 | 5′- AGCGAGCATCCCCCAAGTT- 3′<br>3′-GGGCACGAAGGCTCATCATT-5′ | 285 |
| 18S | 18S ribosomal RNA | NM_003286.2 | 5′-CAGCCACCCGAGATTGAGCA-3′<br>3′-TAGTAGCGACGGGCGGTGTG-5′ | 253 |
| LDHA | Lactate dehydrogenase A | NM_005566.3 | 5′-GCCTGTATGGAGTGGAATGAA-3′<br>3′-CCAGGATGTGTAGCCTTTGAG-5′ | 157 |
| SDHA | Succinate dehydrogenase complex, subunit A | NM_004168.2 | 5′-AGACCTCCCGCACCTGAAGACG-3′<br>3′-ATCAATCCGCACCTTGAAGTTG-5′ | 175 |

***Table 1. Primer sequences used for this analysis***

**qPCR:**

qPCR is a powerful tool to quantify gene expression by exponentially amplifying small amounts of nucleic acid, obtained from RNA extraction and cDNA synthesis by reverse transcription. In qPCR, specific forward and reverse primers are used for the gene of interest, and a fluorescent DNA-binding dye, which emits light upon binding to dsDNA. This enables increasing fluorescence with increasing amounts of dsDNA produced, providing the ability to quantify the

concentration of mRNA expressed in the sample. The qPCR reaction is run in a thermal cycler which illuminates the samples at a specific wavelength and records the emitted fluorescence (Livak & Schmittgen, 2001). The qPCR Master Mix was composed of RNase free water, 15 pmol/µl of each primer mixed with 2x iQ™ SYBR® Green Supermix (BioRad), as a fluorescent dye. The samples were loaded in a 96-well plate, followed by loading of the Master Mix. The plate was centrifuged at 1000g for 30 seconds and finally placed in the thermal LightCycler 480II (Roche). The cycles used are 40 to 50 cycles in a fixed sequence at 94 °C for 30 seconds, 60 °C for 15 seconds, and 72 °C for 15 seconds followed by a melting curve programme. A reverse transcriptase negative control was included to ensure the absence of genomic DNA contamination, and the no-template control was also run to exclude contamination or dimer formation for each primer pair. The amplification specificity was confirmed by melting curve analysis and agarose gel electrophoresis of the products. For each primer pair, a series of 10-fold of three dilutions of cDNA (10- to 1,000-fold dilution) were made to generate a standard curve. The PCR amplification efficiency (E) was determined by the slope of the standard curve: $E\ (\%)=(10[-1/\text{slope}]\ -1)\ \times 100\%$.

**Statistical Analysis**

The Ct values for each sample were compiled and run through the Microsoft Excel-based software programs, geNorm (ver. 3.5) (Vandesompele et al., 2002), NormFinder (Ver 0.953) (Andersen, Jensen, & Ørntoft, 2004), and BestKeeper (Pfaffl, Tichopad, Prgomet, & Neuvians, 2004). These statistical algorithms were used to evaluate the stability of candidate reference genes, and then the overall ranking of the 12 candidate reference genes was determined according to the method described by (Chen, Pan, Xiao, Farwell, & Zhang, 2011). For geNorm and NormFinder, data were analyzed by transforming raw Ct values into relative quantities using the ΔCt method. The lowest Ct value was subtracted from the raw Ct values of qRT-PCR for each gene to give the ΔCt value. The equation $E^{-\Delta Ct}$ was applied to each data point. Therefore, all data were expressed relative to the expression of the most highly-expressed gene. BestKeeper analysis was based on the raw Ct values.

## RESULTS

### Specificity of amplification products and Expression levels of selected genes:

To determine the specificity of amplification products multicurve analysis were performed and the results shows a single product of expected length for each selected gene. Primer dimers and non-specific amplicons were not detected, because the no template control samples showed no signal. Absence of signal in the reverse transcriptase negative control suggested no genomic DNA contamination. All PCR assays showed efficiency values between 96.7% and 102.1%.

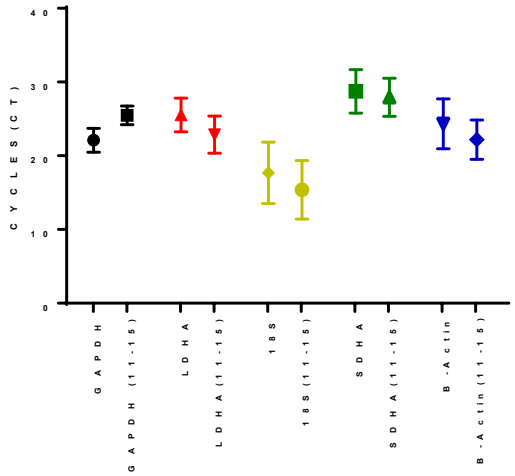



**Fig 1.** ***The expression of levels of reference genes were represented separately like (1-8 patients has one group and 11-15 patients has one group), since for first 10 patients the actual biopsy region from IVD is not clearly know, so they were not pooled along with other patient groups. The expression of selected genes varied widely with Ct value ranging from 11.5 (18sRNA) to 33.4 cycles (SDHA). Average Ct values were between 22 to 30 cycles.***

### NormFinder analysis:

NormFinder is a model-based algorithm, which identifies the most stable reference genes based on combing samples into groups. The main goal of this approach is to calculate the inter- and intra-group variation of the candidate reference genes and then combine both results into a stability value M. Genes with the lowest value are considered to be the most stable (Andersen et al., 2004). Initially patients 1-8 were analyzed to identify the stable reference gene. According to normfinder, the most stable reference gene in these samples were found to be SDHA, with M value of 0.904, followed by Beta-actin, LDHA, GAPDH and18S. Next patient samples (11-15) were analyzed,

and according to normfinder, LDHA was found to be stable expressing gene, with M value 0.171, followed by SDHA, beta-actin (ACTB) ,18S, and GAPDH. Finally, when all the samples were analyzed together, SDHA is found to be stably expressing gene, with M value 0.603, followed by ACTB, LDHA, GAPDH, 18S. Finally, GAPDH and 18S was found to most unstable genes in 3 subsets.

| RANK | ALL PATIENTS (M) | Patients (1-8) (M) | Patients (11-15) (M) |
|---|---|---|---|
| 1 | SDHA (0.603) | SDHA (0.904) | LDHA (0.171) |
| 2 | Beta-actin (0.801) | Beta-actin (0.959) | SDHA (0.171) |
| 3 | LDHA (0.920) | LDHA (1.007) | Beta-actin (0.571) |
| 4 | GAPDH (1.447) | GAPDH (1.219) | 18S (1.430) |
| 5 | 18S (1.726) | 18S (1.966) | GAPDH (1.860) |

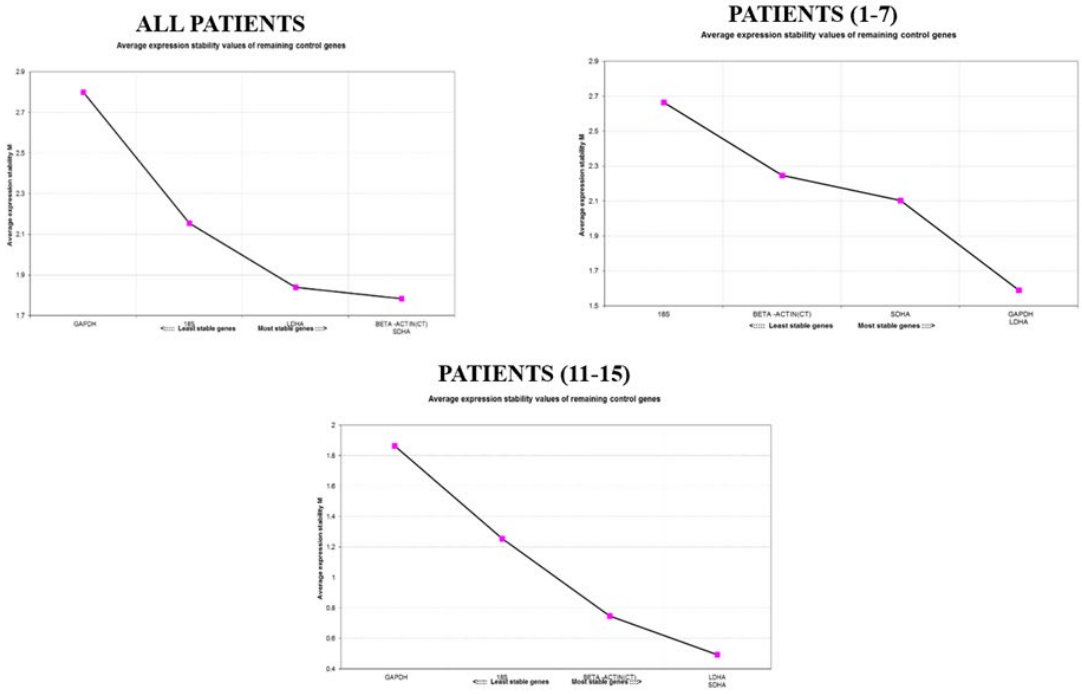
**BestKeeper analysis:**

BestKeeper analyzes the expression stability of reference genes using raw Ct values. Gene expression variation is determined by the calculated standard deviation (SD) and coefficient of variance (CV) for all candidate reference genes based on the whole data set of their Ct values (Pfaffl et al., 2004). Those with the lowest CV±SD were identified as the most stable genes. The analysis showed that SDHA, LDHA, ACTB found to stably expressing genes with low CV±SD value.

| RANK | ALL PATIENTS (CV±SD) | Patients (1-8) (CV±SD) | Patients (11-15) (CV±SD) |
|---|---|---|---|
| 1 | SDHA (7.76±2.21) | ACTB (5.34±1.18) | SDHA (4.11±1.05) |
| 2 | LDHA (7.92±1.94) | LDHA (6.27±1.60) | LDHA (7.62±2.13) |
| 3 | ACTB (7.95±1.87) | SDHA (7.47±2.15) | ACTB (8.49±1.94) |
| 4 | GAPDH (11.20±2.62) | GAPDH (10.91±2.65) | GAPDH (9.76±2.17) |
| 5 | 18S (19.48±3.26) | 18S (15.98±2.83) | 18S (22.02±3.39) |

**GeNorm:**

GeNorm is a program designed to analyze the expression stability of candidate reference genes on the assumption that the ratio of the expression level of two ideal reference genes is constant in all samples. And the average expression-stability M value for each investigated gene is calculated with the average of pairwise variations, according to which the expression stability of all reference genes is ranked. Genes with the lowest M value are the most stable. In our results when all the samples were analyzed together, the genes with smallest M value was SDHA followed by beta-actin and LDHA. Therefore, these are more stable genes. In sub group analysis, LDHA, SDHA and GAPDH were found to stable expressing genes for patient group (1-8). SDHA, LDHA, Beta-actin were found to be stable expressing genes for patient group (11-15). 18S uniformly unstable in all the groups.



**Final ranking of candidate reference genes.**

Since the discrepancies in expression stability of candidate reference genes among the algorithms, a method taking into account all the three sets of results were applied to calculate the final ranking. Specifically, the geometric means of the three ranking numbers produced by geNorm, NormFinder, and BestKeeper were calculated for each candidate reference gene; those with the smallest geometric means were considered to be the most stable (Chen et al., 2011).From the analysis SDHA,LDHA and Beta-actin were found more stable across all the 3 algorithm's used.

**Discussion**

The purpose of this study was to choose appropriate internal controls to ensure credible evaluation of gene expression levels in human lumbar disc specimens. All the 5 candidates were selected from previous investigations, and they were reported to have relatively stable expression levels (Andersen et al., 2004; Pfaffl et al., 2004). Our studies showed that the most stable gene in all samples was SDHA, which is among the less commonly used. And a combination of three reference genes was recommended, i.e., SDHA, LDHA and ACTB, based on a comprehensive consideration of the results of all algorithms. The application of multiple references is beneficial for normalization (Andersen et al., 2004), (Pombo-Suarez, Calaza, Gomez-Reino, & Gonzalez, 2008) (Ren et al., 2010). We compared our results with (Zhou et al., 2014) study and our results were quite similar with their study i.e., SDHA was found to be stably expressing gene in there study also along with LDHA and ACTB. Surprisingly GAPDH and 18S which are most commonly used housekeeping genes in most of studies has performed poorly in our study. Our results, however, further confirmed the necessity of assessing the reference genes in each tissue and experimental condition.

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# Annexure II

## One Year follow-up analysis

A total of 34 patients 12-month follow-up data available for analysis. At 12 months postoperative follow-up, there were a statistically significant improvements in VAS back pain and ODI scores. The VAS back pain scores were reduced from 63 to 46 and ODI scores reduced from 42 to 32. The ratio patients reaching minimal clinically important difference (MCID) was 36% for ODI (MCID≥10), and 44% for VAS back pain (MCID≥18)

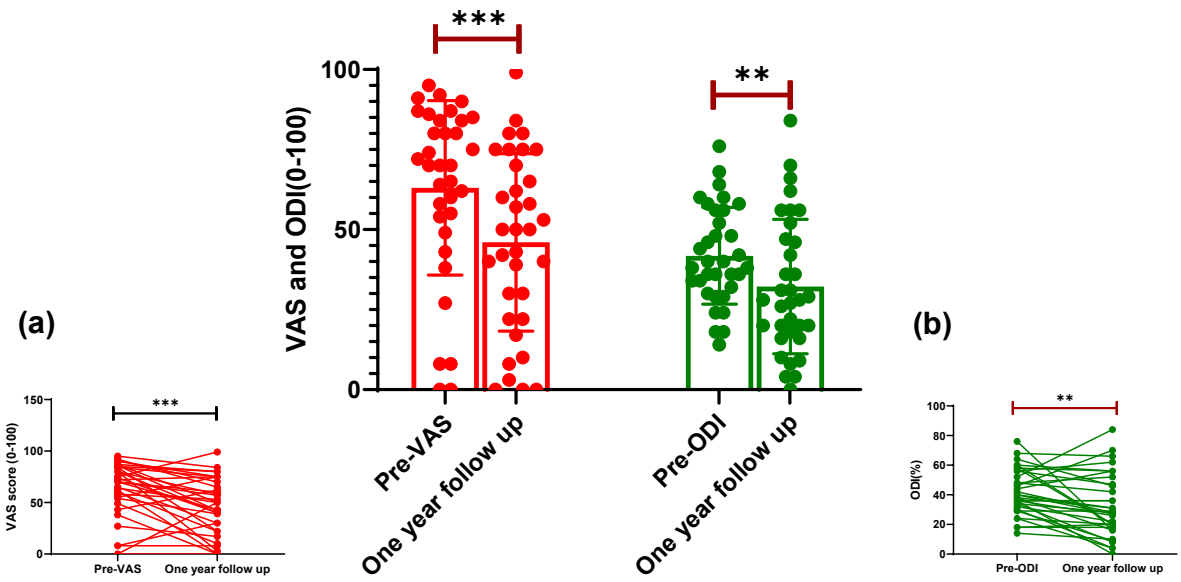


***Fig 1. Decrease in VAS and ODI scores at 12 months postoperative follow-up. (a) Significant difference in VAS back pain scores observed after 12-month follow-up analysis. (b) Significant improvement in the surgery outcome as there is reduction in disability scores observed after 12 months. Values are MEAN ± SD. Group difference were evaluated by Paired t test ***p < 0.001, **p < 0.01***

We further studied to identify any potential biomarker to predict the effect of surgery and correlated the baseline expression of proinflammatory markers we have studied to one year follow

up clinical scores. We found the baseline expression of IL-1β in AF showed significant positive correlation with 12 month follow up VAS scores. Similarly, the expression of IL-1β showed a significant positive correlation to 12 month follow up VAS scores in NP. We also find significant positive correlation with 12-month follow-up ODI scores with baseline expression of TNF- α and IL-6 in NP. The expression of other MMPs and ADAMTSs did not correlate to 12 month follow up scores.

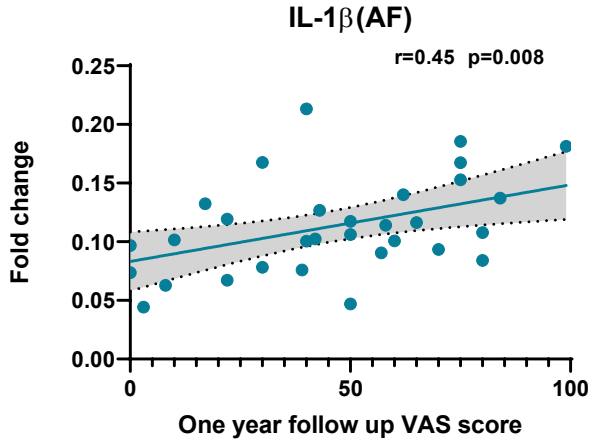


***Fig 2. Expression of baseline IL-1β correlates with 12 months follow VAS scores (r=0.45, p=0.0008). p < 0.05 is considered statistically significant for all comparisons. r = Pearson correlation coefficient.***

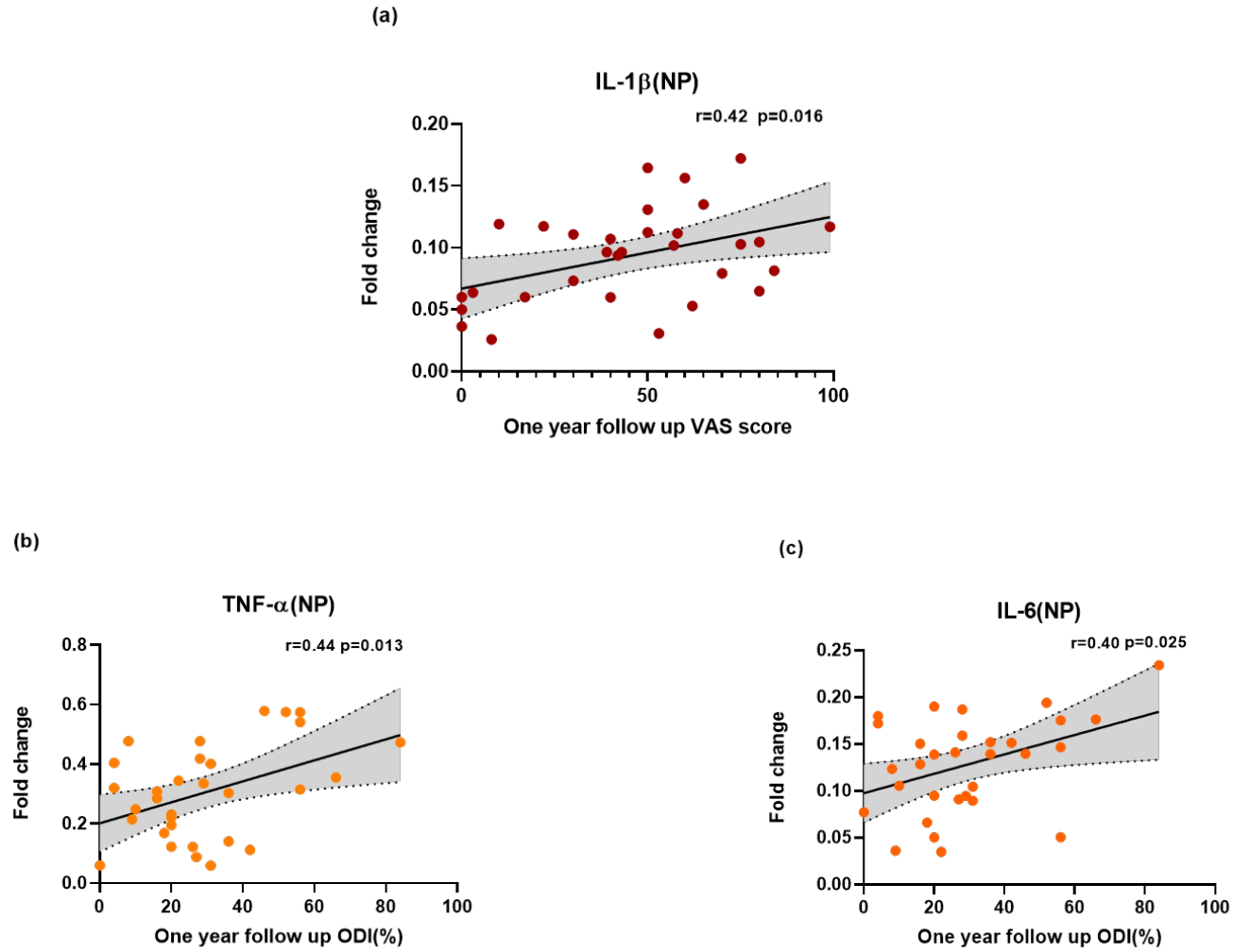


***Fig 3. Association between expression of pro-inflammatory cytokines at baseline to follow up clinical scores in NP (a) Expression of IL-1β at baseline showed significant positive correlation to 12 month follow up VAS scores (r=0.42, p=0.016). (b)&(c) Expression of TNF-α (r=0.44, p=0.013) and IL-6 (r=0.40, p=0.025) at baseline showed significant positive correlation with follow up disability scores. p < 0.05 is considered statistically significant for all comparisons. r = Pearson correlation coefficient.***

We are still in process of collecting the follow up information, but our results showed that subjects a reduction in post-surgical pain, but baseline expression of several cytokines showed association with treatment response one-year follow-up. These results suggest the expression of the pro-inflammatory cytokines may serve as a predictor for the surgery outcomes, because higher

expression at baseline may predict a poor outcome at follow-up. Further investigation has to be done to confirm at what extents these cytokine markers can be a predictor for surgical outcomes.