Diss. ETH No. 26777

Identification and characterization of the transient receptor potential (TRP) channels in the intervertebral disc

A thesis submitted to attain the degree of
DOCTOR OF SCIENCES of ETH ZURICH
(Dr. sc. ETH Zurich)

presented by
Aleksandra M. Sadowska
M.Sc. Biomedical Engineering, ETH Zurich
Born on 31.01.1990
Citizen of Poland

accepted on the recommendation of
Prof. Dr. Karin Kozak-Würtz, examiner
Prof. Dr. Stephen J. Ferguson, co-examiner
Prof. Dr. med. Oliver N. Hausmann, co-examiner

2020

In dedication to my family and William

For their advice, patience and faith

"The beginning of knowledge is the discovery of something we do not understand."
– Frank Herbert

"Things are only impossible until they are not."
– Jean-Luc Picard

Table of Contents

Acknowledgements

The work presented in this thesis is the result of the great collaborative efforts of several researchers from many different backgrounds and institutions. Without their generous support and expertise, many of the findings presented in this thesis would have not been possible.

First and foremost, I would like to thank my supervisor Prof. Karin Wuertz-Kozak for her mentorship, friendship and constant support during my PhD journey. I am particularly grateful for the numerous opportunities she gave me to expand my knowledge and gain new experiences. Our research involved many unknowns, but she always encouraged her group to take risks and change directions when necessary, which motivated me to push harder and explore new perspectives. I have many fond memories of our interactions over the last 3 and a half years, and I hope to be a similar role model to someone one day.

Dr. Oliver Hausmann is a dear collaborator who was involved in nearly all the projects presented in this thesis. Oliver gave me invaluable insights into life in the clinics and the struggles experienced by patients suffering from lower back pain. Oliver also made this research possible by providing us with the test samples themselves. I would like to thank Oliver for all his efforts, time and translational ideas, which greatly enriched our research.

The research presented here was conducted in collaboration with the Laboratory of Orthopedic Technology led by Prof. Stephen J. Ferguson at the Institute for Biomechanics at ETH Zurich. I would like to thank Prof. Stephen J. Ferguson for letting me use the lab space, as well as for creating a friendly work atmosphere. Stephen always made me feel that I am a part of his team. I remember many cheerful coffee breaks with Stephen and I often found myself seeking his valuable input into my research dilemmas. I feel very grateful for his support and help throughout our research.

I had a great time working in HPP and sharing these days with many talented and intelligent individuals among them, but not limited to, my past and present colleges: Elena Cambria, Petra Cazzanelli, Dr. Olga Krupkova and Dr. Matthew Randall. I would also like to give special thanks to our lab manager Helen Greutert for all her efforts into making our lab run smoothly. I would also like to acknowledge a number of students, who have helped me on different projects, among them Birsen Altinay, Georgios Makris, Dominique Michel

and Jemima Schadow. Discovering science together with them was fun and I appreciate the opportunity to have lead their projects. I would especially like to express my gratitude to Birsen Altinay, who worked alongside me during the last months of my PhD. Birsen was a great support during those months and she helped me finish the last research project in a timely fashion.

My time at ETH would not have been the same without the friendship of the other past and present members of the Institute for Biomechanics, especially Pascal Behm, Dr. Enrico De Pieri, Dr. Benedikt Helgason, Graeme Paul and Dr. Duncan Tourolle. We shared many great scientific chats and fun outdoor adventures together that enriched my time while working on my doctoral projects.

I would also like to show my gratitude to all our project collaborators and especially to Dr. Wolfgang Hitzl, Prof. Lisbet Haglund and the Orthopedic Research Laboratory at the McGill University for all their time and effort put into this research. Successful execution of a research project does not only rely on the scientific contribution alone, therefore I would also like to recognize the invaluable assistance of our administrative and technical staff, including Anina Eglin, Sofia Delamanis, Ingrid Okanta and Catherine Palmer, who all contributed to the smooth workflow at the Institute for Biomechanics. Finally, I would like to acknowledge the Swiss National Foundation and Eurospine for their financial support, which made this research possible.

My journey leading to this thesis started long before I joined the ETH thanks to the support of my loving family and friends. I wish to show special gratitude to my mum, Magdalena, who supported my education, encouraged me to be brave, to take risks and to never give up. It is with her help that I had the courage to pursue my interests and to join the ETH. Lastly, I am forever grateful to Dr. William Enns-Bray for his unending support, inspiration and everyday encouragement. William has a talent for asking the most difficult questions, which motivate me to give better answers, to write stronger texts and to aim higher. His adventurous mindset inspires me to live my life to the fullest and to boldly venture into the future.

May 2020
Aleksandra Sadowska

Summary

Low back pain (LBP) has a high prevalence and is a major public health problem globally. Intervertebral disc degeneration (IVD), which is the central cause of LBP, is a natural process with an early onset. Due to the rapidly aging population in the western society, it is predicted that occurrences of LBP as well as its related costs will further increase, causing a high economic burden on the individual and the society. Biomechanical, biological and genetic factors have an impact on the development of painful IVD degeneration (also called degenerative disc disease). However, treatment options are limited. Therefore, there is a clear need for advancing our understanding of IVD degeneration and designing novel treatment strategies. Transient receptor potential (TRP) channels constitute a family of Ca^{2+} permeable cell membrane receptors and have recently emerged as potential contributors to tissue homeostasis, inflammation and pain. TRP channels were investigated as biomarkers and potential therapeutic targets in many other tissues, but so far, their role remains largely unknown in the IVD. This thesis attempts to address this knowledge gap. The objective of this thesis was to identify and characterize TRP channels and pro-inflammatory factors in the IVD, with the goal to evaluate their therapeutic potential.

In the first study, the occurrences of inflammatory processes and the expression of TRPC6 and TRPV4 in human degenerated tissue from the lumbar and cervical IVDs were measured and compared. Aside from genes with known implication in degenerative disc disease (DDD) or disc herniation (DH), four previously not recorded genes from the interferon and TRP families (IFNα1, IFNα8, IFNβ1, TRPC6) could be detected in the human degenerated IVD tissue. A correlation between gene expression and age (IL-15) and IVD degeneration grade (IFNα1, IL-6, IL-15, TRPC6), but not Modic grade, was identified. Significant differences were found between cervical and lumbar IVDs (IL-15), nucleus and annulus (IL-6, TNF-α, TRPC6), single-level and multi-level surgery (IL-6, IL-8) as well as DDD and DH (IL-8), while sex had no effect. Multiple gene-gene pair correlations, either between different cytokines (e.g. IL-6 and IL-8) or between cytokines and TRP channels (e.g. TRPC6 and IL-15/IL-8/IL-6), exist in the disc. Presented findings support the importance of IL-6 and IL-8 during IVD degeneration, but additionally demonstrate a potential pro-inflammatory role of IL-15 and interferon type I as well as a mechanistic role of TRPC6 in the disease. This study concluded that despite existing tissue- or patient-specific differences, novel anti-

inflammatory or TRP-modulatory strategies for the treatment of disc pathologies may be applicable independently of the spinal region.

In the second study, the gene and protein expression pattern of currently known TRP channels in non-degenerated and degenerated human IVD tissue was analyzed. Patient- and tissue- specific characteristics, such as age, IVD degeneration grade, pain intensity and chronicity and others were included in the analysis. For the first time we could show that 26 out of 28 currently known TRP channels are expressed in the IVD on the gene level, thereby revealing novel therapeutic candidates from the TRPC, TRPM and TRPML subfamilies. TRPC6, TRPM2 and TRPML1 displayed enhanced gene and protein expression in degenerated IVDs as compared with non-degenerated IVDs. Furthermore, the gene expression of TRPC6 and TRPML1 was influenced by the IVD degeneration grade. Pain intensity and/or chronicity affected the gene and/or protein expression of TRPC6, TRPM2 and TRML1, therefore suggesting their therapeutic potential. Interestingly, decreased gene expression of TRPM2 was observed in patients treated with steroids. This is the first study to present the link between TRP channels and pain in the IVD. Thus, these results represent the current benchmark for the TRP channel expression pattern in the IVD. Our findings support the importance of TRP channels in IVD homeostasis and pathology, and their possible application as pharmacological targets for the treatment of IVD degeneration and LBP.

The objective of the third study was to investigate the effect of hypo-osmotic stress, which is one of the main hallmarks of IVD degeneration, on the gene expression pattern and calcium signaling of TRPV4 and TRPM7 channels in bovine IVD cells. As the first in the field, we presented a complete screening of currently known TRP channel in bovine IVD cells. In line with the results obtained in human IVD tissue, TRPC1, TRPM7 and TRPV4 were overall the most highly expressed TRP channels in bovine IVD cells. We determined that TRPV4 gene expression was downregulated in hypo-osmotic condition, whereas its Ca^{2+} flux increased. RNA sequencing identified over 3000 up- or down-regulated targets, from which we selected aggrecan, ADAMTS9 and IL-6 and investigated whether their altered gene expression is mediated through either the TRPV4 or TRPM7 channel, using specific activators and inhibitors (GSK1016790A/ GSK2193874 for TRPV4 and Naltriben/ NS8593 for TRPM7). Application of TRPV4 selective activator (GSK1016790A) induced the expression of IL-6 under iso-osmotic condition, proportionally to hypo-osmotic

stimulation alone, indicating that this effect might be TRPV4-mediated. However, the application of TRPV4 selective blocker (GSK2193874) failed to inhibit the increase of IL-6 under hypo-osmotic condition. A treatment with TRPM7-activator did not cause significant changes in the gene expression of tested targets. We concluded that although TRPV4 and TRPM7 are likely involved in osmosensing in the IVD, neither of them mediates hypo-osmotically-induced gene expression changes of aggrecan, ADAMTS9 and IL-6.

This thesis has made several significant contributions to the field of spine research. Through a detailed identification and characterization of TRP channels in the IVD, it revealed their important role in IVD degeneration. It demonstrated that the expression levels of TRP channels and pro-inflammatory factors may depend on the specific characteristics of the IVD tissue (e.g. lumbar *versus* cervical, degeneration grade) or patients (e.g. pain intensity/chronicity). Importantly, results of these experiments highlighted novel therapeutic targets from the TRPC, TRPM and TRPML family, which are not only linked to low back pain intensity or chronicity, but also to the level of inflammation. These findings may contribute to the design of new biomarker-based tests and help develop novel pharmacological treatments for painful IVD degeneration and LBP. Moreover, RNA sequencing data presented in this thesis can serve spine scientist as a source for further research.

In conclusion, IVD tissue- and/or patient-dependent expression pattern of TRP channels presented over the course of this study suggests that TRP channels have a potential to become novel therapeutic targets in painful IVD degeneration. Their pharmacological modulation may prove beneficial in treating pain and inflammation, could contribute to the regulation of the IVD tissue homeostasis and might ultimately change clinical practice related to discogenic back pain in the future.

Zusammenfassung

Schmerzen im unteren Rückenbereich (Low Back Pain, LBP) haben eine hohe Prävalenz und stellen weltweit ein großes Problem für das Gesundheitswesen dar. Die Degeneration der Bandscheibe BS) ist ein natürlicher Prozess der bereits in jungen Jahren startet und eine zentrale Ursache von LBP darstellt. Aufgrund der rasch alternden Bevölkerung in der westlichen Gesellschaft wird prognostiziert, dass das Auftreten von LBP sowie die damit verbundenen Kosten weiter zunehmen werden, was zu einer hohen wirtschaftlichen Belastung für den Einzelnen und die Gesellschaft führen wird. Biomechanische, biologische und genetische Faktoren haben einen Einfluss auf die Entwicklung der schmerzhaften BS-Degeneration (auch degenerative disc disease = DDD genannt). Die Behandlungsmöglichkeiten für DDD sind jedoch begrenzt. Es besteht daher ein klarer Bedarf, unser Verständnis der BS-Degeneration zu vertiefen und neue Behandlungsstrategien zu entwickeln. TRP-Kanäle (Transient Receptor Potential) bilden eine Familie von $Ca2^{+}$-durchlässigen Zellmembranrezeptoren und haben in jüngster Zeit vermehrt Beachtung als potenzielle Mitverursacher von Gewebehomöostase, Entzündungen und Schmerzen gewonnen. TRP-Kanäle wurden als Biomarker und potenzielle therapeutische Zielstrukturen in vielen anderen Geweben untersucht, aber ihre Rolle in der BS ist bisher weitgehend unbekannt. Die vorliegende Arbeit versucht, diese Wissenslücke zu schließen. Ziel dieser Arbeit war die Identifizierung und Charakterisierung von TRP-Kanälen und pro-inflammatorischen Faktoren in der BS mit dem Ziel, ihr therapeutisches Potenzial zu bewerten.

In der ersten Studie wurde das Auftreten von Entzündungsprozessen und die Expression von TRPC6 und TRPV4 in degenerierten BS der Lenden- und Halswirbelsäule untersucht und verglichen. Neben Genen deren Relevanz in der DDD oder dem Bandscheibenvorfall (BSV) bekannt ist, konnten vier bisher nicht erkannte Gene aus den Interferon- und TRP-Familien (IFNα1, IFNα8, IFNβ1, TRPC6) im menschlichen degenerierten BS-Gewebe nachgewiesen werden. Es wurde eine Korrelation zwischen der Genexpression und dem Alter (IL-15) und dem BS-Degenerationsgrad (IFNα1, IL-6, IL-15, TRPC6), aber nicht dem Modic-Grad, festgestellt. Signifikante Unterschiede wurden zwischen zervikalen und lumbalen Bandscheiben (IL-15), Nukleus und Annulus (IL-6, TNF-α, TRPC6), ein- und mehrstufiger Chirurgie (IL-6, IL-8) sowie DDD und BSV (IL-8) festgestellt, während das

Geschlecht keinen Einfluss hatte. Mehrere Gen-Genpaar-Korrelationen, entweder zwischen verschiedenen Zytokinen (z.B. IL-6 und IL-8) oder zwischen Zytokinen und TRP-Kanälen (z.B. (TRPC6 und IL-15/IL-8/IL-6), konnten in den untersuchten Bandscheiben nachgewiesen werden. Die vorgestellten Befunde unterstreichen die Bedeutung von IL-6 und IL-8 in der BS-Degeneration, zeigen aber zusätzlich eine mögliche pro-inflammatorische Rolle von IL-15 und Interferon Typ I sowie eine mechanistische Rolle von TRPC6 bei der Erkrankung. Diese Studie kam zu dem Schluss, dass trotz bestehender gewebe- oder patientenspezifischer Unterschiede neuartige anti-inflammatorische oder TRP-modulatorische Strategien zur Behandlung von Bandscheibenpathologien unabhängig von der Wirbelsäulenregion anwendbar sein könnten.

In der zweiten Studie wurde das Gen- und Proteinexpressionsmuster von TRP-Kanälen in nicht-degeneriertem und degeneriertem, menschlichen BS-Gewebe untersucht. Patienten- und gewebespezifische Merkmale wie Alter, BS-Degenerationsgrad, Schmerzintensität und Chronizität und andere wurden in die Analyse einbezogen. Wir konnten zum ersten Mal zeigen, dass 26 von 28 derzeit bekannten TRP-Kanälen in der BS auf Genebene exprimiert werden, wodurch neue therapeutische Kandidaten aus den Unterfamilien TRPC, TRPM und TRPML aufgedeckt wurden. TRPC6, TRPM2 und TRPML1 zeigten in degenerierten BS im Vergleich zu nicht-degenerierten BS eine erhöhte Gen- und Proteinexpression. Darüber hinaus wurde die Genexpression von TRPC6 und TRPML1 durch den BS-Degenerationsgrad beeinflusst. Schmerzintensität und/oder Chronizität beeinflussten die Gen- und/oder Proteinexpression von TRPC6, TRPM2 und TRML1, was auf ihr therapeutisches Potenzial hindeutet. Interessanterweise wurde bei Patienten, die mit Steroiden behandelt wurden, eine verminderte Genexpression von TRPM2 beobachtet. Dies ist die erste Studie, die den Zusammenhang zwischen TRP-Kanälen und Schmerzen in derBS darstellt. Somit repräsentieren diese Ergebnisse den aktuellen Maßstab für das Expressionsmuster der TRP-Kanäle in der BS. Unsere Ergebnisse unterstützen die Bedeutung der TRP-Kanäle in der BS-Homöostase und -Pathologie und ihre mögliche Anwendung als pharmakologische Zielstrukturen für die Behandlung von BS-Degeneration und LBP.

Das Ziel der dritten Studie war es, die Wirkung von hypo-osmotischem Stress, der eines der Hauptmerkmale der BS-Degeneration ist, auf das Genexpressionsmuster und die Kalzium-Signalisierung der TRPV4- und TRPM7-Kanäle in bovinen BS-Zellen zu untersuchen. Es

handelt sich hierbei um das erste vollständige TRP-Kanal-Screening in bovinen BS-Zellen. In Übereinstimmung mit den Ergebnissen die immenschlichen BS-Gewebe erzielt wurde, waren TRPC1, TRPM7 und TRPV4 insgesamt die am höchsten exprimierten TRP-Kanäle in bovinen BS-Zellen. Wir stellten fest, dass die Expression des TRPV4-Gens im hypo-osmotischen Zustand herunterreguliert war, während sein Ca^{2+}-Fluss zunahm. Die RNA-Sequenzierung identifizierte über 3000 hoch- oder herunterregulierte Ziele, aus denen wir Aggrecan, ADAMTS9 und IL-6 auswählten und untersuchten, ob ihre veränderte Genexpression entweder durch den TRPV4- oder den TRPM7-Kanal vermittelt wird. Hierzu wurden spezifische Aktivatoren und Inhibitoren (GSK1016790A/ GSK2193874 für TRPV4 und Naltriben/ NS8593 für TRPM7) verwendet. Die Anwendung von GSK1016790A (TRPV4-Aktivator) induzierte die Expression von IL-6 unter iso-osmotischen Bedingungen, proportional zur alleinigen hypo-osmotischen Stimulation. Dies deutete darauf hin, dass dieser Effekt TRPV4-vermittelt sein könnte. Die Anwendung von GSK2193874 (TRPV4-Blocker) konnte jedoch den Anstieg von IL-6 unter hypo-osmotischen Bedingungen nicht hemmen. Wir kamen zu dem Schluss, dass TRPV4 und TRPM7 zwar wohl an der Osmoseregulierung in der IVD beteiligt sind, keiner von beiden TRP Kanälen jedoch die hypo-osmotisch induzierte Genexpressionsänderungen von Aggrecan, ADAMTS9 und IL-6 vermittelt.

Diese Arbeit hat mehrere bedeutende Beiträge auf dem Gebiet der Wirbelsäulenforschung geleistet. Durch eine detaillierte Identifizierung und Charakterisierung von TRP-Kanälen in der BS hat sie deren wichtige Rolle bei der BS-Degeneration aufgezeigt. Sie hat gezeigt, dass die Expression von TRP-Kanälen und pro-inflammatorischen Faktoren von gewebe-relevanten Charakteristika (z.B. dem Ort oder dem Stadium der Degeneration) oder von patientenspezifischen Merkmalen (z.B. Schmerzintensität/Chronizität) abhängen kann. Wichtig ist, dass die Ergebnisse dieser Experimente neue therapeutische Zielstrukturen aus der TRPC-, TRPM- und TRPML-Familie hervorheben konnten, die nicht nur mit der Schmerzintensität oder Chronizität, sondern auch mit dem Grad der Entzündung in Zusammenhang stehen. Diese Erkenntnisse könnten zur Entwicklung neuer biomarker-basierter Tests beitragen und die Entwicklung neuer pharmakologischer Behandlungen für schmerzhafte BS-Degeneration und LBP unterstützen. Darüber hinaus können die in dieser Arbeit vorgestellten Daten aus der RNA-Sequenzierung den Wirbelsäulenwissenschaftlern als Quelle für weitere Forschungen dienen.

Zusammenfassend lässt sich sagen, dass das in dieser Arbeit aufgedeckte gewebe- und/oder patientenabhängige Expressionsmuster von TRP-Kanälen darauf schließen lässt, dass sich TRP-Kanäle als neue therapeutische Zielstrukturen bei schmerzhafter IVD-Degeneration eignen können. Ihre pharmakologische Modulation könnte sich bei der Behandlung von Schmerzen und Entzündungen als vorteilhaft erweisen, zur Regulierung der BS-Gewebehomöostase beitragen und letztlich die klinische Praxis im Zusammenhang mit BS-bedingten Rückenschmerzen in der Zukunft verändern.

Chapter 1

1 Introduction

1.1 Thesis motivation

Low back pain: causes and impact on the society

Low back pain (LBP) is a common cause of activity limitation, disability and lost productivity worldwide [1]. It is estimated that up to 80% of adults in the western society will develop LBP at least once during their lives [1, 2]. In 2013, LBP was determined to be the leading cause of the years lived with disability (YLDs) metric in 45 of 50 developed countries and 94 of 138 developing countries [3]. This affects not only patients' life quality, but also results in a high economic burden on a society. The total costs depend on a multitude of factors and are generally divided into direct (related to healthcare e.g. in- and outpatient treatment, medication, use of utility devices etc.) and indirect costs (related to paid productivity losses e.g. absence from work). Several studies have been performed to estimate costs related to LBP in Europe. Although, total (direct and indirect) numbers differ between the studies, depending on the study design and methodology (e.g. ~€10 billion in Switzerland in 2005 [4] or ~€740 million in Sweden in in 2008–2011 [5]), the common factor is that costs and prevalence of LBP are predicted to rise due to the population growth and aging [3, 4, 6, 7].

Intervertebral disc (IVD) degeneration is considered the major underlying cause of the LBP [8]. Although IVD degeneration is a natural process observed with aging that will remain asymptomatic for a large percentage of individuals throughout their lifetime, it may progress into painful degenerative disc disease (DDD). In the symptomatic IVD degeneration, biomechanical, inflammatory and environmental factors contribute to the development of painful degenerative changes [9]. Despite immense therapeutic potential, the link between degeneration, inflammation, pain and mechanical signals is not well understood. Consequently, it is still unclear how IVD cells sense and convert mechanical signals into biochemical signals as well as whether that response depends on the IVD's physiological condition. Due to the fact that answering the existing knowledge gap could create an opportunity for the development of new treatment strategies for DDD, these questions remain of high scientific and clinical relevance. The solution may lie in the identification of new therapeutic targets and biomarkers that may act as cellular sensors and transduce local environmental changes in the IVD cells. Biomarkers can be defined as biological molecules, which can be objectively measured and can be used to indicate health or disease [10].

Identification of new LBP-specific biomarkers could not only help to improve the identification and characterization of the medical condition, but could also provide scientists with new targets for a pharmaceutical treatment, help optimize drug dosing or contribute to mechanistic understanding of the observed differences between individuals in clinical trials [10, 11]. In 2013, the World Health Organization (WHO) outlined search for biomarkers as one of the key future research directions necessary for improved care and prevention of LBP [12].

Transient receptor potential (TRP) channels and inflammation

Transient receptor potential (TRP) channels represent a superfamily of cation selective transmembrane receptors with diverse structure, physiological function and activation mechanism [13]. TRP channels function as sensors for chemical and physical stimuli, such as mechanical stress, osmolarity, temperature, pH or oxidative stress [13, 14]. Hence, TRP channels are important for signal transduction, cell adaptation to local environmental changes as well as cell survival. The IVD is biomechanically an active tissue: its primary function is the transmission of loads arising from muscle activity and body weight as well as it experiences diurnal osmotic changes [14, 15]. Thus, as mechano- and osmosensors, TRP channels may constitute an interesting target for the investigation of spinal diseases. So far, TRP channels have been implicated in the development of pathologies in a multitude of other tissues such as liver, brain, endothelium, muscle or cartilage [16]. As a result, TRP channels have been highly researched as therapeutic targets in other tissues, with many completed and ongoing clinical trials [17]. Recent evidence suggests the involvement of TRP channels in joint diseases such as osteoarthritis and DDD, but their biological function and potential therapeutic applicability is still unclear [14, 15]. Thus, a scrutinized identification of the presence and function of TRP channels in the IVD is required in order to assess their suitability as a therapeutic targets for the treatment of DDD and LBP.

1.2 Thesis aims

The overall objective of this thesis was to identify and characterize TRP channels in the IVD, investigate their role in inflammation and osmosensing in the IVD as well as assess their potential as therapeutic targets. The specific aims of this thesis and the challenges that they address are as follows:

Aim 1: Investigate the occurrence of inflammatory processes and the expression of TRPC6 and TRPV4 channels in human tissue samples from degenerated lumbar and cervical IVDs. The first aim was to measure and compare the gene expression levels of selected known as well as novel pro-inflammatory molecules, and TRPC6 and TRPV4 channels in IVD degeneration in both the lumbar and cervical IVDs. As the majority of research on IVD degeneration has been conducted on the lower back, only limited data exists on the mechanisms of painful cervical degeneration. In this study, the first goal was to seek whether the molecular differences in the inflammation between the degenerated lumbar and cervical IVDs exist. Secondly, the aim was to measure the expression levels of TRPC6 and TRPV4 channels and to test whether their expression levels correlate with the expression of selected pro-inflammatory targets. Differences in the tissue and patient characteristics such as spinal region (cervical *versus* lumbar), IVD zone (nucleus pulposus *versus* annulus fibrosus), IVD degeneration grade, Modic changes, pathology (DDD *versus* disc herniation), the extent of a surgery, age and sex were accounted in the analysis. We hypothesized that significant differences exist between expression levels of pro-inflammatory targets based on specific patient and IVD characteristics, and that TRPV4 and TRPC6 are expressed on the gene level in the degenerated human IVD tissue. Furthermore, the identification of differently expressed targets could support the development of more individualized and targeted treatments for IVD degeneration and discogenic back pain.

Aim 2: Characterize the TRP channels expression pattern on the gene and protein level in human IVDs. Firstly, the second aim was to measure the gene expression levels of all known TRP channels in human degenerated and non-degenerated IVD tissue. Secondly, the goal was to test whether the TRP channels gene and protein expression levels are influenced by specific patient or tissue characteristics, such as age, the degree of degeneration, prior steroid treatment, body mass index (BMI), type of spinal

disease or zonal differences (nucleus pulposus or annulus fibrosus) as well as pain intensity and chronicity. The overall goal of this study was to determine members of the TRP channel family that could constitute potential therapeutic targets for the treatment of inflammation and pain in the human IVD. We hypothesized that apart from previously investigated TRPV4 and TRPC6, other members of TRP channel family are as well expressed in the degenerated human IVD tissue. Moreover, we hypothesized that the differences in the TRP channels gene expression levels may be influenced by specific tissue characteristics such as the IVD degeneration grade as well as pain intensity and chronicity.

Aim 3: Determine whether IVD cells sense and transduce osmotic stress through selected TRP channels and identify molecular targets affected by hypo-osmotic stress. The third aim was to measure gene expression levels of all currently known TRP channel in bovine non-degenerated IVD cells in order to select the most prominently expressed TRP channels. Next, with the application of osmotic treatment, the goal was to identify the main TRP osmosensor(s) using gene expression analysis and calcium flux measurement. As a decreased tissue osmolarity is a major hallmark of IVD degeneration, this study focused on hypo-osmotic stress. In parallel, RNA sequencing was performed to identify molecular targets with differential expression patterns caused by hypo-osmotic treatment, which were subsequently validated by qPCR. The goal of this study was to advance the current understanding of the molecular changes observed during IVD degeneration and we hypothesized that TRP channels may sense osmotic-stress and mediate ECM molecules and pro-inflammatory cytokine gene expression under hypo-osmotic condition.

1.3 Thesis outline

The thesis has been organized into eight chapters. The first chapter presents the motivation, aims and outline of the thesis. The second chapter specify the necessary background information concerning the intervertebral disc, IVD degeneration and degenerative disc disease as well as current treatment strategies and TRP channels. The following chapters present the studies conducted to answer the above-described thesis aims. The final chapter concludes this thesis in a synthesis of the scientific findings in terms of the novelty and contribution to the scientific field. The content of each chapter is described in detail below:

Chapter 1 describes the clinical, societal and economical motivation of this thesis and presents the thesis aims and the thesis outline.

Chapter 2 provides an overview of scientific publications on the intervertebral disc and TRP channels. The anatomy of the spine and intervertebral disc, the biology of intervertebral disc degeneration, background information on TRP channels as well as the current state-of-the-art in diagnostic and treatment strategies are discussed in this chapter.

Chapter 3 presents a review paper on inflammation in the IVD, introduces a concept of inflammaging and may serve as an introduction to the first and second aims of this thesis. Factors related to the IVD degeneration such as aging, local and systemic inflammation and inducers of inflammation (senescence, mechanical loading, matrix degradation, bacterial infection and obesity) as well as a clinical perspective are discussed in detail in this chapter.

Chapter 4 presents a study in which the first aim of this thesis was addressed and where the inflammation in the lumbar and cervical spine was investigated. In this study, we reported four novel targets (IFNα1, IFNα8, IFNβ1, TRPC6) in addition to genes with known implication in DDD and DH such as IL-6, IL-8 or TNF-α. TRPV4 and IL-8 were the two most highly expressed genes in the analyzed dataset. A correlation between gene expression and age (IL-15) and IVD degeneration grade (IFNα1, IL-6, IL-15, TRPC6) was identified. Significant differences were detected between cervical and lumbar IVDs (IL-15), nucleus and annulus (IL-6, TNF-α, TRPC6), single-level and multi-level surgery (IL-6, IL-8) as well as DDD and DH (IL-8), while Modic changes and sex had no effect. Moreover, a positive correlation between the interleukins and the TRP channels (TRPC6 and IL-15/IL-8/IL-6) was found and a negative correlation between the interferons and TRP channels (TRPC6/V4 and IFNα1/β1 or TRPC6 and IFNα8) was further determined.

Chapter 5 addresses the second aim of this thesis and describes a study in which human non-degenerated and degenerated tissue obtained from 37 individuals was screened for TRP channels expression on the gene and/or protein level. We could show that 26 out of 28 currently known TRP channels are expressed in the human IVD tissue on the mRNA level. We could demonstrate an increased gene and protein expression of TRPC6, TRPM2 and TRML1 in degenerated *versus* non-degenerated IVDs. Additionally, the gene and/or protein expression of these TRP channels was influenced by pain intensity and/or chronicity.

Interestingly, decreased TRPM2 gene expression was detected in patients treated with steroids. This study supports the relevance of TRP channels in the IVD degeneration and introduces novel therapeutic candidates from the TRPC, TRPM and TRPML subfamilies.

Chapter 6 presents a literature review on osmosening, osomosignalling and inflammation in relation to the osmotic changes in the IVD, therefore providing the background information for the third aim. This review summarizes the current knowledge on how IVD cells sense osmotic changes in their local environment and how do they translate these signals into physiological or pathophysiological responses. Among others, presumed IVD cell membrane osmosensors and osmosignaling mediators as well as how this knowledge could be translated to clinics is discussed.

Chapter 7 describes the final study, which addresses the third aim of this thesis, on TRP channels as osmosensors and hypo-osmolarity-induced genome-wide changes in IVD cells. We could demonstrate that TRPC1, TRPV4 and TRPM7 are the most highly expressed TRP channels in bovine nucleus pulposus and annulus fibrosus cells, and that the expression of TRPM7 and TRPV4 decreases with passaging. Moreover, we could show that TRPV4 exhibits increased calcium flux under hypo-osmotic conditions. Additionally, we identified over 3000 differently up- or down-regulated targets by hypo-osmolarity. By the application of hypo-osmotic treatment and pharmacological modulation, we investigated whether the altered gene expression of aggrecan, ADAMTS9 and IL-6 is mediated through TRPV4 or TRPM7 channels. Out of the three targets, only IL-6 showed a similar response pattern between the hypo-osmotic treatment and the TRPV4 agonist treatment. However, a treatment with a TRPV4 antagonist did not reverse IL-6 increase. While treatment with TRPM7 agonist had no significant effect on the calcium response and the tested targets. These findings indicate that although TRPV4 and TRPM7 may sense hypo-osmotic stimuli, it is unlikely that either of them mediates IL-6 expression under hypo-osmotic loading.

Chapter 8 concludes the thesis by underlining the main findings and contribution to the spine research field. This chapter also discusses the limitations related to methods and conclusions described in the earlier chapters, and provides the outlook on possible future of spine research aimed at targeting low back pain.

References

1. Freburger, J.K., et al., *The rising prevalence of chronic low back pain.* Arch Intern Med, 2009. **169**(3): p. 251-8.
2. Walker, B.F., *The prevalence of low back pain: a systematic review of the literature from 1966 to 1998.* J Spinal Disord, 2000. **13**(3): p. 205-17.
3. Global Burden of Disease Study, C., *Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013.* Lancet, 2015. **386**(9995): p. 743-800.
4. Wieser, S., et al., *Cost of low back pain in Switzerland in 2005.* Eur J Health Econ, 2011. **12**(5): p. 455-67.
5. Olafsson, G., et al., *Cost of low back pain: results from a national register study in Sweden.* Eur Spine J, 2018. **27**(11): p. 2875-2881.
6. Ravindra, V.M., et al., *Degenerative Lumbar Spine Disease: Estimating Global Incidence and Worldwide Volume.* Global Spine J, 2018. **8**(8): p. 784-794.
7. Geurts, J.W., et al., *The Impact of Chronic Discogenic Low Back Pain: Costs and Patients' Burden.* Pain Res Manag, 2018. **2018**: p. 4696180.
8. Hughes, S.P., et al., *The pathogenesis of degeneration of the intervertebral disc and emerging therapies in the management of back pain.* J Bone Joint Surg Br, 2012. **94**(10): p. 1298-304.
9. Anderson, D.G. and C. Tannoury, *Molecular pathogenic factors in symptomatic disc degeneration.* Spine J, 2005. **5**(6 Suppl): p. 260S-266S.
10. Biomarkers Definitions Working, G., *Biomarkers and surrogate endpoints: preferred definitions and conceptual framework.* Clin Pharmacol Ther, 2001. **69**(3): p. 89-95.
11. Khan, A.N., et al., *Inflammatory biomarkers of low back pain and disc degeneration: a review.* Ann N Y Acad Sci, 2017. **1410**(1): p. 68-84.
12. Duthey, B.J.P.m.f.E. and the world. Global Burden of Disease *Background paper 6.24 low back pain.* 2013: p. 1-29.
13. Ramsey, I.S., M. Delling, and D.E. Clapham, *An introduction to TRP channels.* Annu Rev Physiol, 2006. **68**: p. 619-47.
14. Krupkova, O., J. Zvick, and K. Wuertz-Kozak, *The role of transient receptor potential channels in joint diseases.* Eur Cell Mater, 2017. **34**: p. 180-201.
15. Walter, B.A., et al., *Reduced tissue osmolarity increases TRPV4 expression and pro-inflammatory cytokines in intervertebral disc cells.* European cells & materials, 2016. **32**(7): p. 123-36.

16. Nilius, B., *TRP channels in disease.* Biochim Biophys Acta, 2007. **1772**(8): p. 805-12.
17. Moran, M.M., *TRP Channels as Potential Drug Targets.* Annu Rev Pharmacol Toxicol, 2018. **58**: p. 309-330.

Chapter 2

2 Background

2.1 Spine and intervertebral disc

Spine functional anatomy

The vertebral column (also known as spine) consists of 33 bony vertebra, out of which 24 are movable and nine are fused vertebrae - five of those fused form the sacrum and the remaining four form the coccyx (commonly term as tailbone) [1]. The vertebral column can be divided into five main regions: cervical (neck) numbered C1 to C7, thoracic (mid back) numbered T1 to T12, lumbar (lower back) numbered L1 to L5, sacrum and coccyx region (**Figure 1**) [1]. Each region has a distinctive function. The cervical spine for example supports the weight of the head and provides it with a range of motion; the thoracic spine has much less mobility than the cervical spine, but it protects the nearby organs such as the heart or lungs and supports arm movement and bending over motion; the lumbar spine bears the body weight. Concurrently, the spine has three main functions: 1) To provide the support for the upright posture, 2) to allow movement and mobility, 3) to protect the spinal cord and to absorb shock [1].

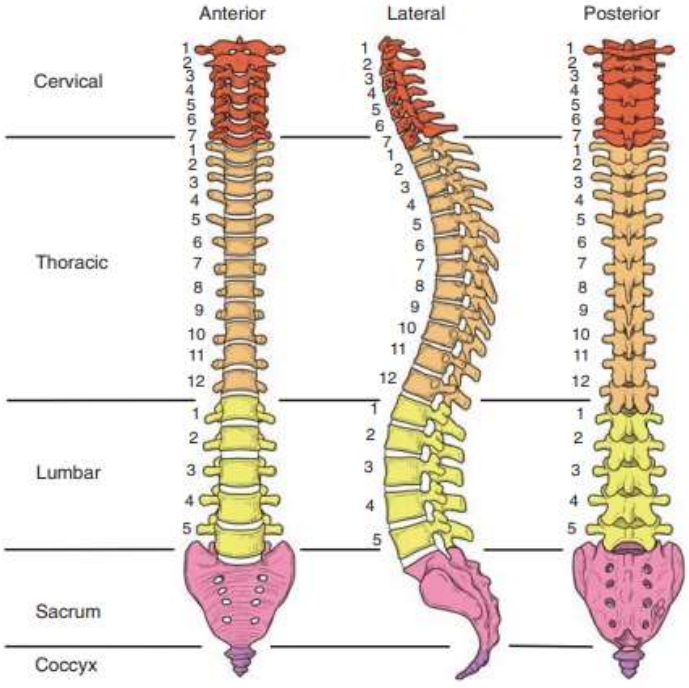


Figure 1. Anatomy of a spine. Reprinted from [2] with permission from Springer.

Intervertebral disc (IVD) functional anatomy

The intervertebral disc (IVD) is an avascular, cartilaginous joint-like structure located between adjacent vertebrae, starting from the second segment of the cervical spine (C2) until the sacrum. An IVD consists of two main parts: the outer annulus fibrosus (AF) and the inner nucleus pulposus (NP) and is separated from the adjacent vertebra by thin endplates (EP) consisting of hyaline cartilage and fibrocartilage (**Figure 2**) [3].

- **Annulus Fibrosus (AF):** The AF is a tough tissue consisting of layers of highly organized lamellae of collagen type I fibers, which are aligned parallel to each other at approximately 30–42° to the vertical axis and run in opposite directions. The AF is populated by fibrochondrocyte-like cells of mesenchymal origin. The main functions of the AF include restraining the NP, providing stability against shear and torsion and providing the ability for angular movements (flexion-extension/lateral flexion) [3].
- **Nucleus pulposus (NP):** The NP is a well-hydrated, gel-like structure located in the center of the IVD (surrounded by the AF) and consists of proteoglycans (PG), predominantly aggrecan, that are entrapped in a collagen (COL) type II network. Due to the chemical characteristics of PGs and COL II, the NP possesses osmotic properties necessary to resist and transmit compressive loads and provides disc height. The NP is sparsely populated by a mix of chondrocyte-like mesenchymal cells and notochordal-derived cells, which become fibrochondrocyte-like with disc maturation and aging [3].
- **Vertebral Endplate (also known as cartilaginous endplate (EP or CEP)):** The EP is a vascularized, porous and permeable barrier located at the interface between the AF and the vertebral bone. It consist of hyaline cartilage and fibrocartilage and possesses two main roles: 1) it allows nutrition and water exchange between the IVD and vertebrae and 2) it prevents the NP from bulging into a vertebra [3]. With ageing, EPs experience various types of lesions and calcification, which may play a pathological role in the development of the IVD degeneration due to e.g. decreased nutrient supply to the IVD [4, 5].

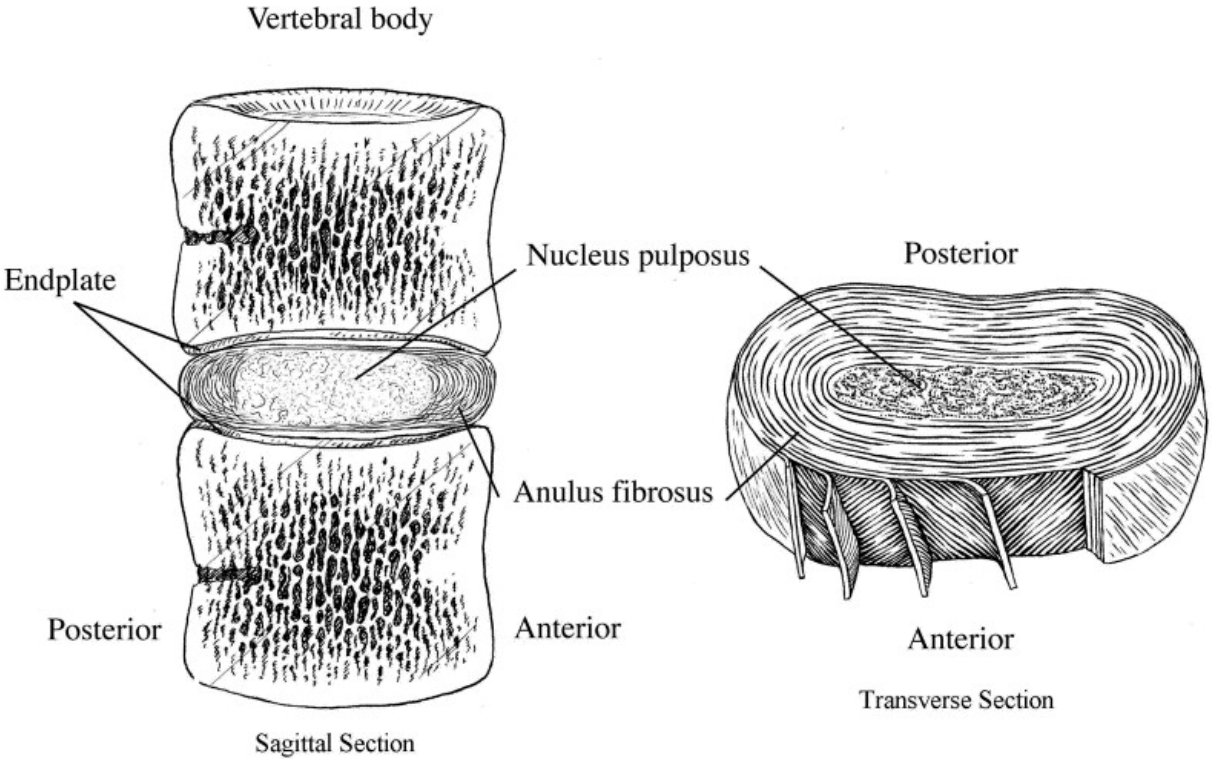


Figure 2. The sagittal and transverse sections of the lumbar disc. Reprinted from [6] with permission from Elsevier.

2.2 Pathologies of intervertebral disc

IVD degeneration and degenerative disc disease

IVD degeneration is a natural age-related process beginning early in life and is characterized by exaggerated destruction of the extracellular matrix (ECM), decreased ECM production and increased cell death resulting in compromised tissue integrity, which consequently affects the IVD's ability to fulfill its mechanical function [7-9]. Aging-induced IVD degeneration is however often asymptomatic, meaning that many individuals with degenerated IVDs will not experience common symptoms linked to the IVD degeneration, such as pain or disability. However, in certain individuals, IVD degeneration becomes painful. Symptomatic IVD degeneration (often referred to as degenerative disc disease (DDD) or painful IVD degeneration) is characterized by low-grade, chronic inflammation, neovascularization and neoinnervation in addition to factors such as an imbalance of anabolic (e.g. collagen type II (COL-2), proteoglycans (PG)) and catabolic (e.g. metalloproteinases (MMPs), interleukin (IL), fibronectin fragments) factors, reduced

hydration of the NP, clefts and tears in AF, which are also commonly observed in the asymptomatic IVD degeneration [7]. Symptomatic IVD degeneration is thought to be the leading cause of low back pain (LBP); however, the underlying mechanisms of IVD degeneration are still unclear [10]. Factors such as un-physiological loading, trauma, inflammation, genetics, life style and age are all believed to contribute to the development of painful IVD degeneration [11-14] and are further discussed in the chapter 3.

Disc herniation

Disc herniation (DH) describes a condition when a ruptured AF causes the NP to protrude and push either on the outer rings of AF or into the spinal canal (**Figure 3**) [15]. Moreover, injured IVD tissue releases and/or attracts cytokines (e.g. IL-6, IL-8, and CCL2), further contributing to pro-inflammatory environment and leading to pain sensation [16-18]. In addition to low back pain, irritated nerve roots may cause a feeling of weakness, sharp pain or numbness in the limbs – a condition referred to as sciatica and a common symptom of DH [19]. IVD degeneration, genetics and trauma are common causes for disc herniation [20]. Despite the fact that DH is treatable by e.g. lumbar microdiscectomy surgery, the post-operative reoccurrence rate is estimated to be up to 15% and is usually caused by insufficient sealing of the injured IVD, leading to continued exposure of the protruding tissue [21] or insufficient removal of disc tissue [22]. Moreover, it was shown that factors such as smoking, heavy lifting, weight, height and gender (male) might contribute to the reoccurrence of DH [21, 23].

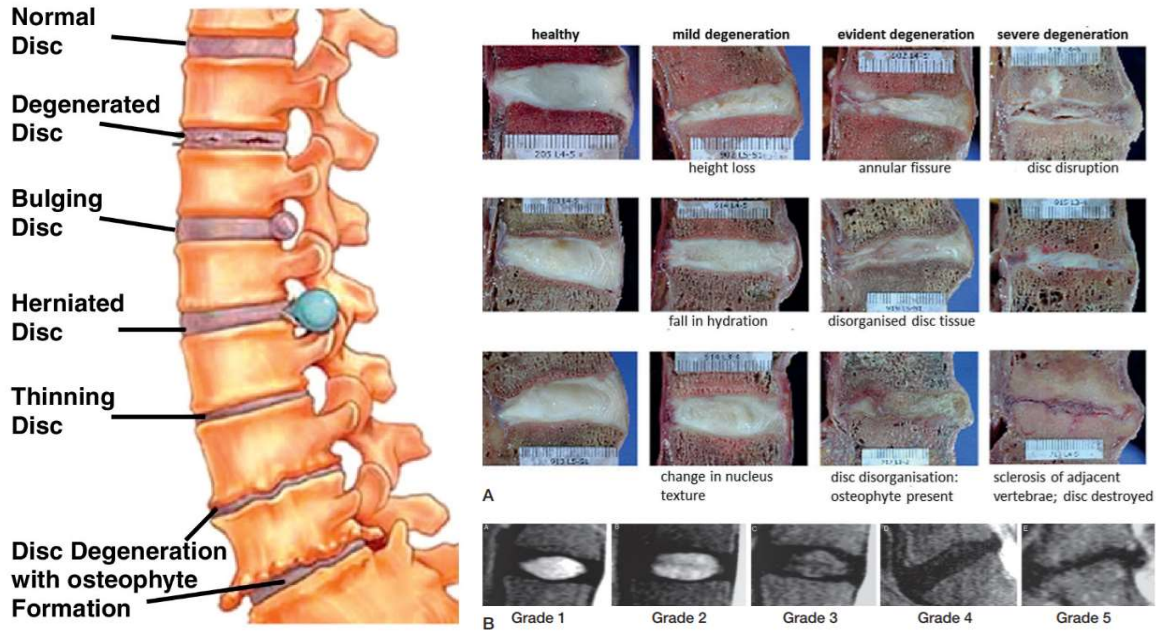


Figure 3. Left: Schematic representation of IVD pathologies. Adapted from [24] with a permission from Elsevier. **Right**: (**A**) Human IVD at various stages of degeneration from healthy (left) to severely degenerated (right) and (**B**) representative MRI images showing degenerated IVDs graded with the Pfirrmann degeneration grade. Reprinted from [25] with permission under the terms of the Creative Commons Attribution-Non-Commercial License from Taylor & Francis.

Low back pain

Discogenic pain can be classified into three subtypes: chronic (lasting longer than 3 months), subacute (lasting between 1 to 2 months) and acute (lasting up to 1 month) [26, 27]. Most people will experience low back pain at some point of their lifetime, whereas the prevalence of chronic back pain was estimated to be approximately 23 % [27]. It was reported that around one out of 10 patients experiencing acute low back pain might develop chronic back pain [27]. IVD degeneration and DH are one of the most common sources of LBP, but other conditions such as muscle/ligament strain, osteoporosis and arthritis are listed among other contributing factors [28, 29]. However, low back pain is very often non-specific and as such, by the definition, has no clear pathoanatomical cause [27]. As prevention strategies are limited to the avoidance of risk factors, non-specific LBP is often treated with

pharmacological (e.g. paracetamol, opioids) and manual (e.g. physiotherapy, massage, acupuncture, exercise) therapies [30].

2.3 State-of-the-art in diagnosis and treatment

Diagnosis

An accurate diagnosis is a prerequisite for a successful treatment. Physical examination, patient self-assessment (e.g. via pain questionnaires), occupational and psychosocial factors as well as imaging are common tools used to determine the sources of the LBP [31]. Magnetic Resonance Imaging (MRI) is a sensitive imaging technique allowing to evaluate structural changes in the IVD such as tears, narrowing and loss of IVD height, reduction in IVD hydration and changes to endplates (called Modic changes (MC)) [32]. Using MRI, Pfirrmann *et al.* [33] classified the degree of IVD degenerative changes in a scale ranging from grade I (regular IVD height, a clear distinction between AF and NP and a white IVD appearance on the MRI, **Figure 2**) to grade V (collapsed IVD space with inhomogeneous structure and dark appearance on the MRI, **Figure 3**) [32]. Modic *et al.* [34] created a grading scale for the changes to the endplates including type 1 changes (bone marrow edema), type 2 (infiltration of fatty marrow) and type 3 (bone sclerosis) [32]. Clinical evidence suggests that Modic changes may be a source of pain and between 19 to 69 % of DDD patients were reported to have changes to endplates, whereas in asymptomatic IVD degeneration, Modic changes are rather uncommon [35, 36]. Despite the fact that MRI has been proved useful in identifying structural changes to IVD (such as disc herniation), the obtained findings has been so far not associated with low back pain severity, which hinders the diagnosis of the painful disc degeneration [37, 38]. In the recent years, advanced imaging techniques, focused on the correlation of imaging findings with molecular markers e.g. pH-sensitive MRI signal [39], have been emerging in the research.

Physical therapy

Physical exercises and physiotherapy (with or without cognitive behavior therapy) are recognized treatment strategies for low back pain [40]. Physical treatments are typically prescribed in early pain management, especially for patients with non-specific low back pain [40]. Physical therapy generally focuses on strengthening the truck muscles, reducing stiffness and restoring mobility, and may involve among other things a general aerobic

training, Pilates-like exercises or strength training [41-43]. Recent randomized control studies have reported comparable improvements outcomes between patients undergoing spinal fusion and physical therapy [44]; however, the implications of these findings may be restricted due to study limitations, such loss of follow-up, lack of appropriate control groups, no blinding and disparities in used metrics [45, 46].

Pharmacological treatment

Pharmacological treatments for LBP may be administered in combination with other treatments or as a sole treatment (e.g. if the physical treatment is insufficient), and is recommended for short-term pain relief [31].

Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly prescribed drugs for pain relief. NSAIDs target COX-1 and COX-2 - enzymes that produce pro-inflammatory prostaglandins, and therefore have an ability to e.g. reduce pain and decrease fever [47]. Some of the commonly known NSAIDs include aspirin and ibuprofen and were shown to provide temporary pain improvement for patients with acute and chronic LBP [47, 48].

Since paracetamol is a weak inhibitor of COX-1 and COX-2 and has negligible anti-inflammatory properties, it is generally not considered as NSAID [49]. Despite the fact of being commonly used for LBP, it has weak efficacy with questionable pain and disability reduction, and its effects are comparable to placebo [50].

Opioids, such as morphine, fentanyl, codeine and others, are a powerful class of drugs targeting opioid receptors situated on neuronal cell membranes [51]. Opioids are known analgesics and supportive evidence exists for their effectiveness in temporal reduction of chronic LBP. However, it was suggested that their effectiveness may be comparable with NSAIDs [51, 52]. In addition, ~20 % risk of opioids misuse was reported, resulting in 8-12 % addiction rate after long-term usage [53].

Treatments such as epidural steroid injections (**Figure 4**), although pharmacological treatments, could be also classified as invasive procedures due to their mode of application [31]. Epidural steroid injections allow for local application of an anesthetic to the lumbar epidural space [54] and are usually recommended for the treatment of inflammation of spinal nerve roots triggered by an IVD pathology, hence helping to relief the pain symptoms [55].

Epidural steroid injections are considered generally safe if administered by experienced medical personnel and have only a minor incidence rate for typically mild side effect (e.g. local trauma due to needle placement), which ranges between 1.6 % to 20 % [56, 57].

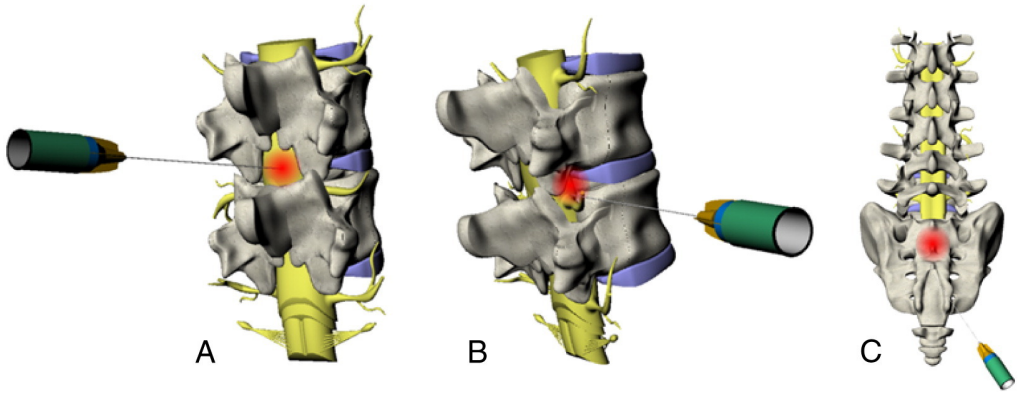


Figure 4. Application of an epidural injection: (**A**) interlaminar, (**B**) transforaminal and (**C**) caudal approaches. Grey: vertebral body; yellow: spinal nerve; blue: IVD; red: injection area. Reprinted from [58] with a permission from Elsevier.

Invasive treatments

Spinal fusion surgery is a viable and well-recognized treatment option, and currently the gold standard for the surgical treatment of severely degenerated IVDs, especially in case of DH. The procedure, which has its beginnings in the late 19th and early 20th century, has over the years developed from using a wire and steel rods into the application of more sophisticated implants composed of biocompatible metals, plastics and ceramics [59]. Fusion surgery aims at stabilizing the spine by eliminating motion between two or more vertebrae, allowing them to fuse into one solid block and thereby reducing pain caused by degenerative changes [32]. Over time, various approaches to performing fusion surgery have been developed, including posterolateral fusion, anterior lumbar interbody fusion (ALIF), posterior lumbar interbody fusion (PLIF) and minimally invasive versions, such as lateral lumbar interbody fusion (LLIF) [32]. Still, reported success rate vary between publications (~16-95 %) and complications (such as a degeneration to the adjacent segments) are commonly described [60, 61].

Spinal surgery approaches focused on preserving disc motion by the use of dynamic implants have gained increasing interests as an alternative to spinal fusion. During disc arthroplasty

(also known as total disc replacement), degenerated tissue is removed and replaced by an artificial disc [62]. In line with spinal fusion, total disc replacement is designed for patients with severely damaged or herniated IVDs [62]. Besides the benefit of preserved motion, the key reported advantage of disc arthroplasty over spinal fusion is a presumably faster recovery time, which leads to a faster return to work [62, 63]. However, disc arthroplasty has several contraindications (e.g. osteoporosis, scoliosis), hence limiting its applicability [63, 64]. Additionally, only moderate evidence exists that arthroplasty in fact reduces the risk of adjacent disc disease [62, 65].

Another surgical alternative, commonly used in the treatment of disc herniation, is the (micro-) discectomy – a procedure focused on the removal of the prolapsed IVD tissue. Discectomy was shown to be relatively successful at eliminating pain (including sciatica) and reducing disability, although with relatively high risks for reoccurrence [66].

These and other spinal surgical procedures have an ability to manage low back pain and/or restore the patient's mobility if IVD degeneration or herniation are at the source of the issue. However these surgical interventions are not recommended for non-specific low back pain, unless 2 years of other conservative treatments have failed [31].

2.4 In search for new therapies

Cell-based therapies

Recently, there has been an increasing interest in cell-based therapies for the treatment of low back pain and IVD degeneration [67]. The rationale behind cell-based therapies is to accelerate self-repair and/or promote the regenerative potential of degenerated IVD tissue by increasing the population of healthy NP cells and minimizing cell death [67]. In theory, cell-based therapies should be less aggressive than a spinal surgery, hence reducing risks and possibly costs. The procedure consists of an injection of cells (e.g. notochordal cells or stem cells derived from bone marrow or adipose tissue) or cell-biomaterial suspensions, where a biomaterial (e.g. pNIPAM) is used as a delivery vehicle, to the site of the degeneration [32, 68]. Although promising, cell-based therapies are still in clinical trials (for an overview please refer to [69] or consult https://clinicaltrials.gov/) and present many unknowns. Examples include selection of therapy-appropriate patients (e.g. early *versus* later stages of the IVD degeneration), sourcing of cells (e.g. autologous or allogenic, selection

of the harvest site), delivery technique (e.g. avoidance of cell leakage or minimizing disruption of the AF during injection), demonstration of safety (e.g. determining adverse effects) and efficacy (e.g. improvements of symptoms) [67, 68, 70].

Gene therapies

Gene therapies for IVD pathologies focus on the idea of transduction of genes implicated in IVD degeneration or homeostasis such as TGF-β1, SOX9, ADAMTS5 or growth factors [32, 71]. Currently, the research on IVD gene therapies is limited to *in vitro* studies [72] or *in vivo* animal models [73, 74]. CRISPR/Cas9 is a promising state-of-the-art technique, which has an ability to induce stable and permanent phenotype changes [75]. So far, CRISPR/Cas9 was mainly used to induce single gene mutations and generate disease animal models [75]. However, if applied in the spine research, it can support the characterization of IVD degeneration, promote the identification of new therapeutic targets and therefore aid in drug discovery [75].

2.5 Novel molecular biomarkers and therapeutic targets

Biological markers (biomarkers) are defined as a "*characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention*" [76]. Advanced screening tools such as genomics, proteomics, gene array or mass spectrometry allow for an extensive discovery of many targets from one biological sample [76]. Identification of novel molecular biomarkers for the detection of early signs of IVD degeneration or for broadening the scientific understanding of the etiology as well as epidemiology of symptomatic and asymptomatic low back pain may be crucial for the development of future treatments, especially those focused on personalized medicine [77], as well as for assessing treatment responses. A successful biomarker should be specific, sensitive and should correlate with patient- or condition-specific factors, as for example Pfirrmann or Modic grading systems, patient pain severity or intensity [77, 78]. However, in order to ensure a reliable outcome, the identification of new biomarkers for IVD degeneration and LBP should also go hand in hand with the technological improvements of imaging techniques and IVD degeneration grading systems. Moreover, study design and study execution aspects such as sample handling,

storage and preparation, patient selection (e.g. comorbidities) and analysis methods must be considered in order to reach a comprehensive conclusion [78].

Pro-inflammatory molecules

Cytokines are a family of small, pro-inflammatory proteins, which include interleukins (IL), interferons (IFN), chemokines (CC) and tumor necrosis factors (TNF) and can be released by various tissues, including IVD [79-81], and have been implicated in the development of inflammation [82]. Cytokines are used as biomarkers in multitude of conditions and diseases including sepsis, infections or tuberculosis [82]. Analytical approaches such as enzyme-linked immunosorbent assays (ELISA) or immunosensing can be used to directly measure cytokine activity from a biological sample, whereas mRNA levels can help predict the potential for cytokine production [82, 83]. As proteins released from the injured and inflamed tissue may enter the bloodstream, the blood serum biomarkers have attracted a lot of attention due to the low invasiveness of blood collection and its accessibility [84]. Interestingly, past studies have reported that serum protein and/or mRNA levels of IL-6, IL-8, TNF-α and RANTES correlate with pain intensity and/or severity [84, 85] as well as the severity of the IVD degeneration (e.g. IL-6, CCL5, CXCL6) [86, 87]. These findings may be useful for guiding treatment selection (e.g. prescription of anti-inflammatory drugs) as already current anti-inflammatory and anti-pain medication for the low back pain management target pro-inflammatory proteins such as IL-6, TNF-α [88, 89]. However, these and other pro-inflammatory targets are not specific to the intervertebral disc pathologies only and their detection may be affected by comorbidities [90].

Non-coding RNA molecules

Regulatory non-coding RNA molecules, which can be divided into two classes based on their length – small < 200 nucleotides (microRNAs, miRNAs) [91] and long > 200 nucleotides (lncRNAs) [92], have lately gained attention as potential biomarkers for multiple diseases (e.g. cancer, autoimmune disease) and conditions including IVD pathologies.

miRNAs have an ability to regulate gene expression and their alternations may contribute to normal physiological processes as well as to a development of a disease [91]. Hence, research on miRNAs can prove useful for unlocking the mechanism of painful IVD degeneration. It was demonstrated that miRNAs are present in the IVD with presumed

differences in expression between NP and AF tissue [93], and certain miRNAs (e.g. miR-17 [94] and other [95]) may be associated with IVD degeneration and pain. It was reported that various miRNAs may interplay with signaling pathways in the IVD [91] leading to differential expression of ECM and pro-inflammatory molecules. Upregulation of miR-625-5p for example may be TLR4/NF-κB signaling dependent and lead to the downregulation of COL1 [96]. A very recent study has identified miRNAs from serum of a mouse IVD degeneration model and showed that miR-26a-5p not only increases with IVD degeneration, but also promotes it by indirectly enhancing VEGF [97]. Similarly, miR-155-5p was reported to be downregulated during IVD degeneration in human NP cells, with serum expression levels correlating with the expression in tissue. Therefore, miR-155-5p may have a high potential to be used as a minimally invasive biomarker.

Similar to miRNAs, lncRNAs play a role in gene transcription, translation of protein-coding genes or epigenetic regulation [92]. lncRNAs were shown to be expressed in human IVDs, with altered expression during degeneration [92, 98, 99]. Another recent study has demonstrated a possible relationship between lncRNAs and miRNAs in the human IVD, where lncRNA-dependent up-regulation of taurine upregulated gene 1 (TUG1) was correlated with the down-regulation of miR-26a, hence contributing to IVD degeneration [100, 101].

However, very little is known about the mechanism of action as well as the function of lncRNAs and miRNAs, and future studies will have to determine their potential as IVD degeneration and LBP biomarkers and therapeutic targets [92, 95].

Transient receptor potential (TRP) channels

In the last decade, Transient receptor potential (TRP) channels have emerged as important modulators of tissue homeostasis in a multitude of cell types. However, their altered expression and/or regulation may contribute to inflammation and pain [102]. Thus, they constitute potential biomarkers and therapeutic targets for the detection and treatment of various conditions including join diseases [103, 104]. In mammals, TRP channels represent a family of 28 cation selective transmembrane receptors, which can be categorized by their structure into six protein subfamilies 1) TRP ankyrin (A), 2) TRP canonical (C), 3) TRP vanilloid (V), 4) TRP melastatin (M), 5) TRP polycystic (P) and 6) TRP mucolipin (ML) [105]. All TRP channels consists of six putative transmembrane spans (6 TM), intracellular

amino (N), carboxy (C) termini and the cation-permeable pore presumably located between the fifth (TM5) and the sixth (TM6) putative transmembrane spans [106]. The functional domain and the length of intracellular termini varies between the TRP subfamilies [106]. TRP channels either act as individual units or may form multimeric complexes within (e.g. TRPC1/C5 [107]) or across (e.g. TRPC1/TRPP2 [108]) distinctive subfamilies. TRP channels mediate the flux of ions (mainly sodium (Na^+) and calcium (Ca^{2+})) across the plasma membrane and to the cytoplasm, and respond to a wide range of processes and stimuli such as touch, taste, olfaction, temperature, osmolarity and mechanical stress. The activation modes for TRP channels can be divided into three subgroups 1) receptor activation *via* G protein–coupled receptors (GPCRs) and receptor tyrosine kinases that activate phospholipases C (PLCs), 2) ligand activation *via* binding of small organic (natural and synthetic) molecules or inorganic ions, and 3) direct activation – e.g. *via* mechanical force [105, 109].

TRP channels have been previously shown to be expressed in chondrocytes – a cell type found in cartilage and phenotypically similar to the NP cells [110, 111]. The TRPV4 channel was shown to transduce mechanical and osmotic loading in cartilage to e.g. regulate cartilage ECM synthesis in response to dynamic loading [112-114]. Another study has reported that cartilage-specific TRPV4 deletion decreases aging-associated osteoarthritis (OA) progression [115]. TRPA1 was speculated to mediate the development of OA-related degenerative changes, inflammation and pain [116-118]. Therefore suggesting that novel TRP channels inhibitors (e.g. for TRPV4 or TRPA1) may have a potential to reduce pain and inflammation [119]. However, TRP channels have been so far closely not investigated in the IVD. The very first study on this subject was published in 2016, where authors reported osmosensitive properties of TRPV4 in NP cells and speculated on its role as a mediator of inflammation during IVD degeneration [120]. Nonetheless, the presence, function, mechanism of action and therapeutic value of TRP channels in the IVD remains to be explored.

References

1. Middleditch, A. and J. Oliver, *Structure of the vertebral column*, in *Functional Anatomy of the Spine*. 2005, Butterworth-Heinemann. p. 1-62.
2. Mathis, J.M., *Spine Anatomy*, in *Percutaneous Vertebroplasty and Kyphoplasty*, J.M. Mathis, H. Deramond, and S.M. Belkoff, Editors. 2006, Springer New York: New York, NY. p. 8-32.
3. Middleditch, A. and J. Oliver, *Intervertebral discs*, in *Functional Anatomy of the Spine*. 2005, Butterworth-Heinemann. p. 63-86.
4. Wang, Y., T. Videman, and M.C. Battie, *Lumbar vertebral endplate lesions: prevalence, classification, and association with age.* Spine (Phila Pa 1976), 2012. **37**(17): p. 1432-9.
5. Jackson, A.R., C.Y. Huang, and W.Y. Gu, *Effect of endplate calcification and mechanical deformation on the distribution of glucose in intervertebral disc: a 3D finite element study.* Comput Methods Biomech Biomed Engin, 2011. **14**(2): p. 195-204.
6. Nabil A. Ebraheim, A.H., Ming Lee, Rongming Xu, *Functional anatomy of the lumbar spine.* Seminars in Pain Medicine, 2004. **2**(3): p. 131-137.
7. Anderson, D.G. and C. Tannoury, *Molecular pathogenic factors in symptomatic disc degeneration.* Spine J, 2005. **5**(6 Suppl): p. 260S-266S.
8. Ding, F., Z.W. Shao, and L.M. Xiong, *Cell death in intervertebral disc degeneration.* Apoptosis, 2013. **18**(7): p. 777-85.
9. Wuertz, K., et al., *Inflammatory and catabolic signalling in intervertebral discs: the roles of NF-κB and MAP kinases.* European cells & materials, 2012. **23**(Ivd): p. 103-19; discussion 119-20.
10. Eisenstein, S. and S. Roberts, *The physiology of the disc and its clinical relevance.* J Bone Joint Surg Br, 2003. **85**(5): p. 633-6.
11. Feng, Y., B. Egan, and J. Wang, *Genetic Factors in Intervertebral Disc Degeneration.* Genes Dis, 2016. **3**(3): p. 178-185.
12. Katz, J.N., *Lumbar disc disorders and low-back pain: socioeconomic factors and consequences.* J Bone Joint Surg Am, 2006. **88 Suppl 2**: p. 21-4.
13. Fogelholm, R.R. and A.V. Alho, *Smoking and intervertebral disc degeneration.* Med Hypotheses, 2001. **56**(4): p. 537-9.
14. Jakoi, A.M., et al., *The Clinical Correlations between Diabetes, Cigarette Smoking and Obesity on Intervertebral Degenerative Disc Disease of the Lumbar Spine.* Asian Spine J, 2017. **11**(3): p. 337-347.
15. Fardon, D.F., et al., *Nomenclature and classification of lumbar disc pathology. Recommendations of the Combined task Forces of the North American Spine*

Society, American Society of Spine Radiology, and American Society of Neuroradiology. Spine (Phila Pa 1976), 2001. **26**(5): p. E93-E113.
16. Ahn, S.H., et al., *mRNA expression of cytokines and chemokines in herniated lumbar intervertebral discs.* Spine (Phila Pa 1976), 2002. **27**(9): p. 911-7.
17. Zhu, X., et al., *Contribution of chemokine CCL2/CCR2 signaling in the dorsal root ganglion and spinal cord to the maintenance of neuropathic pain in a rat model of lumbar disc herniation.* J Pain, 2014. **15**(5): p. 516-26.
18. Phillips, K.L., et al., *Potential roles of cytokines and chemokines in human intervertebral disc degeneration: interleukin-1 is a master regulator of catabolic processes.* Osteoarthritis Cartilage, 2015. **23**(7): p. 1165-77.
19. Valat, J.-P., et al., *Sciatica.* 2010. **24**(2): p. 241-252.
20. Dang, L. and Z. Liu, *A review of current treatment for lumbar disc herniation in children and adolescents.* Eur Spine J, 2010. **19**(2): p. 205-14.
21. Shimia, M., et al., *Risk factors of recurrent lumbar disk herniation.* Asian J Neurosurg, 2013. **8**(2): p. 93-6.
22. Suk, K.S., et al., *Recurrent lumbar disc herniation: results of operative management.* Spine (Phila Pa 1976), 2001. **26**(6): p. 672-6.
23. Miwa, S., et al., *Risk factors of recurrent lumbar disk herniation: a single center study and review of the literature.* J Spinal Disord Tech, 2015. **28**(5): p. E265-9.
24. Hudson, K.D., et al., *Recent advances in biological therapies for disc degeneration: tissue engineering of the annulus fibrosus, nucleus pulposus and whole intervertebral discs.* Curr Opin Biotechnol, 2013. **24**(5): p. 872-9.
25. Bendtsen, M., et al., *Biological challenges for regeneration of the degenerated disc using cellular therapies.* Acta Orthop, 2016. **87**(sup363): p. 39-46.
26. Hegmann, K.T., et al., *ACOEM practice guidelines: opioids for treatment of acute, subacute, chronic, and postoperative pain.* J Occup Environ Med, 2014. **56**(12): p. e143-59.
27. Balague, F., et al., *Non-specific low back pain.* Lancet, 2012. **379**(9814): p. 482-91.
28. Borenstein, D., *Does osteoarthritis of the lumbar spine cause chronic low back pain?* Curr Pain Headache Rep, 2004. **8**(6): p. 512-7.
29. Panjabi, M.M., *A hypothesis of chronic back pain: ligament subfailure injuries lead to muscle control dysfunction.* Eur Spine J, 2006. **15**(5): p. 668-76.
30. Maher, C., M. Underwood, and R. Buchbinder, *Non-specific low back pain.* Lancet, 2017. **389**(10070): p. 736-747.
31. Airaksinen, O., et al., *Chapter 4. European guidelines for the management of chronic nonspecific low back pain.* Eur Spine J, 2006. **15 Suppl 2**: p. S192-300.
32. Taher, F., et al., *Lumbar degenerative disc disease: current and future concepts of diagnosis and management.* Adv Orthop, 2012. **2012**: p. 970752.

33. Pfirrmann, C.W., et al., *Magnetic resonance classification of lumbar intervertebral disc degeneration.* Spine (Phila Pa 1976), 2001. **26**(17): p. 1873-8.
34. Modic, M.T., et al., *Degenerative disk disease: assessment of changes in vertebral body marrow with MR imaging.* Radiology, 1988. **166**(1 Pt 1): p. 193-9.
35. Rahme, R. and R. Moussa, *The modic vertebral endplate and marrow changes: pathologic significance and relation to low back pain and segmental instability of the lumbar spine.* AJNR Am J Neuroradiol, 2008. **29**(5): p. 838-42.
36. Dudli, S., et al., *Pathobiology of Modic changes.* Eur Spine J, 2016. **25**(11): p. 3723-3734.
37. Tonosu, J., et al., *The associations between magnetic resonance imaging findings and low back pain: A 10-year longitudinal analysis.* PLoS One, 2017. **12**(11): p. e0188057.
38. Burgstaller, J.M., et al., *Is There an Association Between Pain and Magnetic Resonance Imaging Parameters in Patients With Lumbar Spinal Stenosis?* Spine (Phila Pa 1976), 2016. **41**(17): p. E1053-62.
39. Bez, M., et al., *Molecular pain markers correlate with pH-sensitive MRI signal in a pig model of disc degeneration.* Sci Rep, 2018. **8**(1): p. 17363.
40. Hayden, J.A., et al., *Exercise therapy for treatment of non-specific low back pain.* Cochrane Database Syst Rev, 2005(3): p. CD000335.
41. Petersen, T., et al., *The effect of McKenzie therapy as compared with that of intensive strengthening training for the treatment of patients with subacute or chronic low back pain: A randomized controlled trial.* Spine (Phila Pa 1976), 2002. **27**(16): p. 1702-9.
42. Chatzitheodorou, D., et al., *A pilot study of the effects of high-intensity aerobic exercise versus passive interventions on pain, disability, psychological strain, and serum cortisol concentrations in people with chronic low back pain.* Phys Ther, 2007. **87**(3): p. 304-12.
43. Rydeard, R., A. Leger, and D. Smith, *Pilates-based therapeutic exercise: effect on subjects with nonspecific chronic low back pain and functional disability: a randomized controlled trial.* J Orthop Sports Phys Ther, 2006. **36**(7): p. 472-84.
44. Saltychev, M., M. Eskola, and K. Laimi, *Lumbar fusion compared with conservative treatment in patients with chronic low back pain: a meta-analysis.* Int J Rehabil Res, 2014. **37**(1): p. 2-8.
45. Rainville, J., R. Nguyen, and P. Suri, *Effective Conservative Treatment for Chronic Low Back Pain.* Semin Spine Surg, 2009. **21**(4): p. 257-263.
46. Pengel, L.H., et al., *Physiotherapist-directed exercise, advice, or both for subacute low back pain: a randomized trial.* Ann Intern Med, 2007. **146**(11): p. 787-96.

47. Roelofs, P.D., et al., *Nonsteroidal anti-inflammatory drugs for low back pain: an updated Cochrane review.* Spine (Phila Pa 1976), 2008. **33**(16): p. 1766-74.
48. Enthoven, W.T., et al., *Non-steroidal anti-inflammatory drugs for chronic low back pain.* Cochrane Database Syst Rev, 2016. **2**: p. CD012087.
49. Graham, G.G. and K.F. Scott, *Mechanism of action of paracetamol.* Am J Ther, 2005. **12**(1): p. 46-55.
50. Williams, C.M., et al., *Efficacy of paracetamol for acute low-back pain: a double-blind, randomised controlled trial.* Lancet, 2014. **384**(9954): p. 1586-96.
51. Chahl, L.A., *Opioids-mechanisms of action.* 1996.
52. Chaparro, L.E., et al., *Opioids compared with placebo or other treatments for chronic low back pain: an update of the Cochrane Review.* Spine (Phila Pa 1976), 2014. **39**(7): p. 556-63.
53. Vowles, K.E., et al., *Rates of opioid misuse, abuse, and addiction in chronic pain: a systematic review and data synthesis.* Pain, 2015. **156**(4): p. 569-76.
54. Peng, B.G., *Pathophysiology, diagnosis, and treatment of discogenic low back pain.* World J Orthop, 2013. **4**(2): p. 42-52.
55. Patel, K. and S. Upadhyayula, *Epidural Steroid Injections.* 2019.
56. Schneider, B., et al., *Safety of epidural steroid injections.* Expert Opin Drug Saf, 2016. **15**(8): p. 1031-9.
57. Karaman, H., et al., *The complications of transforaminal lumbar epidural steroid injections.* Spine (Phila Pa 1976), 2011. **36**(13): p. E819-24.
58. Blanquer, S.B., D.W. Grijpma, and A.A. Poot, *Delivery systems for the treatment of degenerated intervertebral discs.* Adv Drug Deliv Rev, 2015. **84**: p. 172-87.
59. Peek, R.D. and L.L. Wiltse, *History of spinal fusion*, in *Spinal fusion*. 1990, Springer. p. 3-8.
60. Penta, M. and R.D. Fraser, *Anterior lumbar interbody fusion. A minimum 10-year follow-up.* Spine (Phila Pa 1976), 1997. **22**(20): p. 2429-34.
61. Deyo, R.A., *Fusion surgery for lumbar degenerative disc disease: still more questions than answers.* Spine J, 2015. **15**(2): p. 272-4.
62. Anderson, P.A. and J.P. Rouleau, *Intervertebral disc arthroplasty.* Spine (Phila Pa 1976), 2004. **29**(23): p. 2779-86.
63. Moatz, B. and P.J. Tortolani, *Cervical disc arthroplasty: Pros and cons.* Surg Neurol Int, 2012. **3**(Suppl 3): p. S216-24.
64. Huang, R.C., et al., *The prevalence of contraindications to total disc replacement in a cohort of lumbar surgical patients.* Spine (Phila Pa 1976), 2004. **29**(22): p. 2538-41.
65. Latka, D., et al., *Safety and efficacy of cervical disc arthroplasty in preventing the adjacent segment disease: a meta-analysis of mid- to long-term outcomes in*

prospective, randomized, controlled multicenter studies. Ther Clin Risk Manag, 2019. **15**: p. 531-539.
66. Carlson, J.D., A.H. Burgher, and G.P. Jasper, *Endoscopic Discectomy*, in *Deer's Treatment of Pain*. 2019, Springer. p. 767-772.
67. Vasiliadis, E.S., et al., *Biologic treatment of mild and moderate intervertebral disc degeneration.* Mol Med, 2014. **20**: p. 400-9.
68. Smith, L.J., et al., *Advancing cell therapies for intervertebral disc regeneration from the lab to the clinic: Recommendations of the ORS spine section.* JOR Spine, 2018. **1**(4): p. e1036.
69. Sakai, D. and J. Schol, *Cell therapy for intervertebral disc repair: Clinical perspective.* J Orthop Translat, 2017. **9**: p. 8-18.
70. Loibl, M., et al., *Controversies in regenerative medicine: Should intervertebral disc degeneration be treated with mesenchymal stem cells?* JOR Spine, 2019. **2**(1): p. e1043.
71. Priyadarshani, P., Y. Li, and L. Yao, *Advances in biological therapy for nucleus pulposus regeneration.* Osteoarthritis Cartilage, 2016. **24**(2): p. 206-12.
72. Bucher, C., et al., *Nonviral Gene Delivery of Growth and Differentiation Factor 5 to Human Mesenchymal Stem Cells Injected into a 3D Bovine Intervertebral Disc Organ Culture System.* Stem Cells Int, 2013. **2013**: p. 326828.
73. Liu, Y., et al., *Lentivirus-mediated TGF-beta3, CTGF and TIMP1 gene transduction as a gene therapy for intervertebral disc degeneration in an in vivo rabbit model.* Exp Ther Med, 2016. **11**(4): p. 1399-1404.
74. Leckie, S.K., et al., *Injection of AAV2-BMP2 and AAV2-TIMP1 into the nucleus pulposus slows the course of intervertebral disc degeneration in an in vivo rabbit model.* Spine J, 2012. **12**(1): p. 7-20.
75. Krupkova, O., et al., *The potential of CRISPR/Cas9 genome editing for the study and treatment of intervertebral disc pathologies.* JOR Spine, 2018. **1**(1): p. e1003.
76. Biomarkers Definitions Working, G., *Biomarkers and surrogate endpoints: preferred definitions and conceptual framework.* Clin Pharmacol Ther, 2001. **69**(3): p. 89-95.
77. Gruber, H.E. and E.N. Hanley, *Do we need biomarkers for disc degeneration?* Biomark Insights, 2007. **1**: p. 131-3.
78. Drucker, E. and K. Krapfenbauer, *Pitfalls and limitations in translation from biomarker discovery to clinical utility in predictive and personalised medicine.* EPMA J, 2013. **4**(1): p. 7.
79. Burke, J.G., et al., *Intervertebral discs which cause low back pain secrete high levels of proinflammatory mediators.* J Bone Joint Surg Br, 2002. **84**(2): p. 196-201.

80. Gawri, R., et al., *High mechanical strain of primary intervertebral disc cells promotes secretion of inflammatory factors associated with disc degeneration and pain.* Arthritis Res Ther, 2014. **16**(1): p. R21.
81. Zhang, Y., et al., *Intervertebral Disc Cells Produce Interleukins Found in Patients with Back Pain.* Am J Phys Med Rehabil, 2016. **95**(6): p. 407-15.
82. Monastero, R.N. and S. Pentyala, *Cytokines as Biomarkers and Their Respective Clinical Cutoff Levels.* Int J Inflam, 2017. **2017**: p. 4309485.
83. Liu, G., et al., *Recent advances in cytokine detection by immunosensing.* Biosens Bioelectron, 2016. **79**: p. 810-21.
84. Khan, A.N., et al., *Inflammatory biomarkers of low back pain and disc degeneration: a review.* Ann N Y Acad Sci, 2017. **1410**(1): p. 68-84.
85. Pedersen, L.M., et al., *Serum levels of the pro-inflammatory interleukins 6 (IL-6) and -8 (IL-8) in patients with lumbar radicular pain due to disc herniation: A 12-month prospective study.* Brain Behav Immun, 2015. **46**: p. 132-6.
86. Weber, K.T., et al., *Serum levels of the proinflammatory cytokine interleukin-6 vary based on diagnoses in individuals with lumbar intervertebral disc diseases.* Arthritis Res Ther, 2016. **18**: p. 3.
87. Grad, S., et al., *Systemic blood plasma CCL5 and CXCL6: Potential biomarkers for human lumbar disc degeneration.* Eur Cell Mater, 2016. **31**: p. 1-10.
88. Pimentel, D.C., et al., *Anti-tumor necrosis factor antagonists in the treatment of low back pain and radiculopathy: a systematic review and meta-analysis.* Pain Physician, 2014. **17**(1): p. E27-44.
89. Ohtori, S., et al., *Efficacy of epidural administration of anti-interleukin-6 receptor antibody onto spinal nerve for treatment of sciatica.* Eur Spine J, 2012. **21**(10): p. 2079-84.
90. de Gonzalo-Calvo, D., et al., *Differential inflammatory responses in aging and disease: TNF-alpha and IL-6 as possible biomarkers.* Free Radic Biol Med, 2010. **49**(5): p. 733-7.
91. Zhou, X., et al., *The roles and perspectives of microRNAs as biomarkers for intervertebral disc degeneration.* J Tissue Eng Regen Med, 2017. **11**(12): p. 3481-3487.
92. Chen, W.K., et al., *lncRNAs: novel players in intervertebral disc degeneration and osteoarthritis.* Cell Prolif, 2017. **50**(1).
93. Ohrt-Nissen, S., et al., *Characterization of miRNA expression in human degenerative lumbar disks.* Connect Tissue Res, 2013. **54**(3): p. 197-203.
94. Hasvik, E., et al., *Up-regulation of circulating microRNA-17 is associated with lumbar radicular pain following disc herniation.* Arthritis Res Ther, 2019. **21**(1): p. 186.

95. Wang, C., et al., *MicroRNAs: New players in intervertebral disc degeneration.* Clin Chim Acta, 2015. **450**: p. 333-41.
96. Shen, L., et al., *TLR4/NF-kappaB axis signaling pathway-dependent up-regulation of miR-625-5p contributes to human intervertebral disc degeneration by targeting COL1A1.* Am J Transl Res, 2019. **11**(3): p. 1374-1388.
97. Fan, Y., et al., *Serum miRNAs are potential biomarkers for the detection of disc degeneration, among which miR-26a-5p suppresses Smad1 to regulate disc homeostasis.* J Cell Mol Med, 2019. **23**(10): p. 6679-6689.
98. Wan, Z.Y., et al., *Aberrantly expressed long noncoding RNAs in human intervertebral disc degeneration: a microarray related study.* Arthritis Res Ther, 2014. **16**(5): p. 465.
99. Chen, Y., et al., *Potential Role of lncRNAs in Contributing to Pathogenesis of Intervertebral Disc Degeneration Based on Microarray Data.* Med Sci Monit, 2015. **21**: p. 3449-58.
100. Chen, J., et al., *Role of LncRNA TUG1 in intervertebral disc degeneration and nucleus pulposus cells via regulating Wnt/beta-catenin signaling pathway.* Biochem Biophys Res Commun, 2017. **491**(3): p. 668-674.
101. Tang, N., et al., *LncRNA TUG1 Promotes the Intervertebral Disc Degeneration and Nucleus Pulposus Cell Apoptosis Though Modulating miR-26a/HMGB1 Axis and Regulating NF-κB Activation.* 2019.
102. Akopian, A., *Role of TRP ion channels in physiology and pathology.* Semin Immunopathol, 2016. **38**(3): p. 275-6.
103. Nilius, B., *TRP channels in disease.* Biochim Biophys Acta, 2007. **1772**(8): p. 805-12.
104. Krupkova, O., J. Zvick, and K. Wuertz-Kozak, *The role of transient receptor potential channels in joint diseases.* Eur Cell Mater, 2017. **34**: p. 180-201.
105. Ramsey, I.S., M. Delling, and D.E. Clapham, *An introduction to TRP channels.* Annu Rev Physiol, 2006. **68**: p. 619-47.
106. Owsianik, G., et al., *Permeation and selectivity of TRP channels.* Annu Rev Physiol, 2006. **68**: p. 685-717.
107. Strubing, C., et al., *TRPC1 and TRPC5 form a novel cation channel in mammalian brain.* Neuron, 2001. **29**(3): p. 645-55.
108. Bai, C.X., et al., *Formation of a new receptor-operated channel by heteromeric assembly of TRPP2 and TRPC1 subunits.* EMBO Rep, 2008. **9**(5): p. 472-9.
109. Christensen, A.P. and D.P. Corey, *TRP channels in mechanosensation: direct or indirect activation?* Nat Rev Neurosci, 2007. **8**(7): p. 510-21.

110. Minogue, B.M., et al., *Transcriptional profiling of bovine intervertebral disc cells: implications for identification of normal and degenerate human intervertebral disc cell phenotypes.* Arthritis Res Ther, 2010. **12**(1): p. R22.
111. Gavenis, K., et al., *Expression of ion channels of the TRP family in articular chondrocytes from osteoarthritic patients: changes between native and in vitro propagated chondrocytes.* Mol Cell Biochem, 2009. **321**(1-2): p. 135-43.
112. O'Conor, C.J., et al., *TRPV4-mediated mechanotransduction regulates the metabolic response of chondrocytes to dynamic loading.* Proc Natl Acad Sci U S A, 2014. **111**(4): p. 1316-21.
113. Phan, M.N., et al., *Functional characterization of TRPV4 as an osmotically sensitive ion channel in porcine articular chondrocytes.* Arthritis Rheum, 2009. **60**(10): p. 3028-37.
114. Eleswarapu, S.V. and K.A. Athanasiou, *TRPV4 channel activation improves the tensile properties of self-assembled articular cartilage constructs.* Acta Biomater, 2013. **9**(3): p. 5554-61.
115. O'Conor, C.J., et al., *Cartilage-Specific Knockout of the Mechanosensory Ion Channel TRPV4 Decreases Age-Related Osteoarthritis.* Sci Rep, 2016. **6**: p. 29053.
116. Moilanen, L.J., et al., *Monosodium iodoacetate-induced inflammation and joint pain are reduced in TRPA1 deficient mice--potential role of TRPA1 in osteoarthritis.* Osteoarthritis Cartilage, 2015. **23**(11): p. 2017-26.
117. Nummenmaa, E., et al., *Transient receptor potential ankyrin 1 (TRPA1) is functionally expressed in primary human osteoarthritic chondrocytes.* Arthritis Res Ther, 2016. **18**(1): p. 185.
118. Fernandes, E.S., et al., *Environmental cold exposure increases blood flow and affects pain sensitivity in the knee joints of CFA-induced arthritic mice in a TRPA1-dependent manner.* Arthritis Res Ther, 2016. **18**: p. 7.
119. Kanju, P., et al., *Small molecule dual-inhibitors of TRPV4 and TRPA1 for attenuation of inflammation and pain.* Sci Rep, 2016. **6**: p. 26894.
120. Walter, B.A., et al., *Reduced tissue osmolarity increases TRPV4 expression and pro-inflammatory cytokines in intervertebral disc cells.* European cells & materials, 2016. **32**(7): p. 123-36.

Chapter 3

3 Inflammaging in the intervertebral disc

Inflammaging in the intervertebral disc

Aleksandra Sadowska[1], Oliver Nic Hausmann[2], Karin Wuertz-Kozak[1,3,4,5]

[1]ETH Zurich, Zurich, Switzerland

[2]Klinik St. Anna-Hirslanden, Neuro- and Spine Center, Lucerne, Switzerland

[3]Department of Health Sciences, University of Potsdam, Potsdam, Germany

[4]Schön Klinik München Harlaching, Spine Center, Munich, Germany

[5]Academic Teaching Hospital and Spine Research Institute, Paracelsus Private Medical University Salzburg, Salzburg, Austria

Published as:

Sadowska, A., Hausmann, O. N., & Wuertz-Kozak, K. (2018).
Inflammaging in the intervertebral disc. *Clinical and Translational Neuroscience*.
https://doi.org/10.1177/2514183X18761146

Abstract:

Degeneration of the intervertebral disc – triggered by ageing, mechanical stress, traumatic injury, infection, inflammation and other factors – has a significant role in the development of low back pain. Back pain not only has a high prevalence, but also a major socio-economic impact. With the ageing population, its occurrence and costs are expected to grow even more in the future. Disc degeneration is characterized by matrix breakdown, loss in proteoglycans and thus water content, disc height loss and an increase in inflammatory molecules. The accumulation of cytokines, such as interleukin (IL)-1β, IL-8 or tumor necrosis factor (TNF)-α, together with age-related immune deficiency, leads to the so-called inflammaging – low-grade, chronic inflammation with a crucial role in pain development. Despite the relevance of these molecular processes, current therapies target symptoms, but not underlying causes. This review describes the biological and biomechanical changes that occur in a degenerated disc, discusses the connection between disc degeneration and inflammaging, highlights factors that enhance the inflammatory processes in disc pathologies and suggests future research avenues.

Keywords: Intervertebral disc, chronic inflammation, inflammaging, senescence, mechanical loading, matrix fragmentation, obesity, Propionibacterium acnes

3.1 The concept of inflammaging

During the course of life, humans are exposed to numerous internal and external damaging agents, including products of metabolic stress, UV light or pathogens. The body can counterbalance the detrimental effects that these stressors exert by various mechanisms (DNA repair, cell apoptosis/autophagy, etc.), at least to a certain degree [1]. Induction of immune and inflammatory processes are also a part of the body's toolbox to shield itself from these types of dangers, but to ensure healthy aging, later neutralization of these inflammatory processes is required. However, there is cumulative data indicating a decrease in the counteraction capacity with increasing age [1]. Consequently, pathology-associated aging that is linked to an imbalance between inflammatory and anti-inflammatory networks occurs. This imbalance is further promoted by age-related immune deficiency termed immunosenescence, which entails a reduced capability of the body to effectively combat stressors [2].

The resulting low-grade, chronic inflammation was termed inflammaging by Franceschi *et al.* in the year 2000 [3]. Since then, inflammaging (also known as inflamm-ageing [4]) was found to induce endocrine, metabolic, and nutritional changes that likewise promote pro-inflammatory conditions [5]. Importantly, recent research highlighted that inflammaging is based on a complex relationship between pro- and anti-inflammatory markers, including the activation of counter-regulatory mechanism [6]. Inflammaging has been described as an important contributor to numerous age-related diseases, including osteoarthritis, Alzheimer's disease, atherosclerosis, heart disease or type II diabetes [4]. In the musculoskeletal system, age-related changes include loss of bone mass as well as degradation of cartilage and intervertebral disc (IVD) tissues [7]. As the term inflammaging has recently also emerged in the context of disc pathologies [8], the subsequent chapters will provide an overview of the current state of the art.

3.2 Aging and degeneration of the intervertebral disc

Degeneration of the IVD is a major contributor to the low back pain (LBP) [9]. Due to the lifetime prevalence of 84% [10], LBP – and thus also disc degeneration – has a major socio-economic impact. In fact, the financial burden related to LBP is approximately 2% of the national gross domestic product in various countries [11, 12].

IVD degeneration is an age-related process, with an early onset [8, 13]. Aging IVDs are characterized by a shift from anabolism towards catabolism, with a consequent matrix breakdown, loss in hydration in the nucleus pulposus (NP) and reduction in disc height. These changes not only result in altered biomechanics, but also neovascularization and neoinnervation, at least in the annulus fibrosus (AF) [14]. With aging and the loss of tissue homeostasis, cells are exposed to damaging factors (such as damaged matrix products, toxins or oxidative stress), leading to increased cell death and senescence [15, 16].

The prevalence of IVD degeneration is vast. A recent cross-sectional population study with 1043 volunteers showed that 40% of subjects under 30 years had IVD degeneration, with an increase to over 90% in the 50 to 55-year-old group [17]. Due to ongoing aging of the population, the numbers of those with IVD degeneration – and thus the associated socio-economic impact - will even further increase in the future. However, it has to be noted that only a subpopulation develops painful disc degeneration, whereas it is asymptomatic in approximately two thirds of the population [18, 19]. As described in more detail in the later chapters, inflammation and inflammaging contributes to disc-related LBP.

3.3 Inflammation and degenerative disc disease

Inflammation has been described as the major pathological contributor to the development of painful disc degeneration, also termed degenerative disc disease (DDD; **Figure 1**). While local inflammation arising within the IVD tissue has been extensively studies over the past decade, first evidence also points to a role of systemic inflammation in DDD. **Table 1** summarizes the pro-inflammatory molecules that are involved in the developed of DDD.

Systemic Inflammation

With technical advancements in the cost-effective analysis of proteins, clinicians and researchers have become increasingly interested in the identification of biomarkers in the biological fluids of patients, predominantly blood. Biomarkers can be of tremendous value in early detection of diseases and prognosis, but also management and monitoring.

Studies investigating serum samples demonstrated an age-dependent increase in numerous inflammatory cytokines, including interleukin (IL)-6, and tumor necrosis factor-α (TNF-α), supporting the notion of age-associated chronic, low-grade inflammation and hence inflammaging [20, 21]. Interestingly, a meta-analysis of eight studies, including 263 subjects with IVD degeneration (bulging, protrusion, or sequestration) and 129 healthy controls, demonstrated an association between IL-6 serum levels and the occurrence of IVD degeneration, with higher levels in those affected [22]. Recent work by Weber *et al.* not only confirmed that serum IL-6 correlated with age, but also highlighted that levels were significantly higher in subjects with low back pain arising from disc herniation, DDD or spinal stenosis, compared with non-affected controls [23]. As participants were controlled for age during recruitment, these findings point towards a general presence of inflammaging, but with disease-specific alterations in the cytokine expression patterns. Furthermore, higher IL-6 serum levels were reported as an indicator of inferior recovery in patients with lumbar radicular pain due to lumbar disc herniation over the course of one year, as demonstrated by Oswestry Disability Index and visual analogue scale [24]. Aside from IL-6, IL-8 has also been suggested as a potential biomarker in disc herniation (DH) and the level of associated pain, but possibly also in DDD without occurrence of IVD protrusion [25, 26].

In summary, these results indicate that systemic inflammation not only represents a link between aging and IVD pathology, but also disc-related LBP.

Local Inflammation

Past research has provided ample evidence for the expression of inflammatory mediators in the IVD, including IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-17, TNF-α, prostaglandin E2 (PGE2) and interferon-γ (IFN-γ) [27, 28], partially with an age- and degeneration-dependent expression profile [29-31]. Importantly, not only invading cells of the immune

system, e.g. in case of herniation, can produce pro-inflammatory cytokines. IVD cells themselves are also a source of pro-inflammatory cytokines, with some differences between NP and AF cells, e.g. with regard to IL-1α, IL-1β, IL-6, IL-17 and TNF-α [8, 31, 32].

Several of these inflammatory mediators are major pathological markers, with higher levels in "diseased" IVDs. Higher levels of TNF-α and IL-8 were measured in DDD samples compared to DH samples and this likely contributes to more severe back pain commonly observed in DDD patients [33]. On the other hand, DH samples - which are characterized by enhanced macrophage infiltration and rare lymphocyte infiltration - possess higher levels of IL-4, IL-6, IL-12 and IFN-γ *than DDD samples* [32].

The presence of these inflammatory mediators not only aggravates IVD degeneration, e.g. by inducing the expression of matrix degrading enzymes, by enhancing cellular senescence, as well as by inhibiting extracellular matrix (ECM) synthesis [28, 30, 34, 35], but also plays a crucial role in pain development. In fact, there is clear evidence that a variety of pro-inflammatory cytokines are upregulated in patients with higher pain sensation (such as IL-1β, IL-6, IL-8, IL-10, TNF-α and IFN-γ [36-39]) – albeit with differences between studies likely associated with patient selection and consequent dissimilarities in pathology inclusion criteria.

The mechanisms of cytokine-induced pain sensation are complex [27, 40, 41]. During DH, pain sensation can be due to mechano-chemical irritation of spinal nerves caused by inflammatory nature of the extruded NP material (radiculopathy) and/or nerve infiltration into the compromised disc (nociception). In cases of DDD, LBP develops through newly invading nociceptive nerve fibers, either via nociceptive or neuropathic mechanisms. Pro-inflammatory cytokines and chemokines also induce immune cell infiltration into degenerated IVDs and these cells further aggravate the inflammatory status. Additionally, resident and infiltrating cells release neurogenic factors that not only facilitate nerve ingrowth into the IVD, but also induce the expression of pain associated cation channels in the dorsal root ganglion (e.g. ASIC3, TRPV1).

Figure 1. Concept of inflammaging in the IVD.

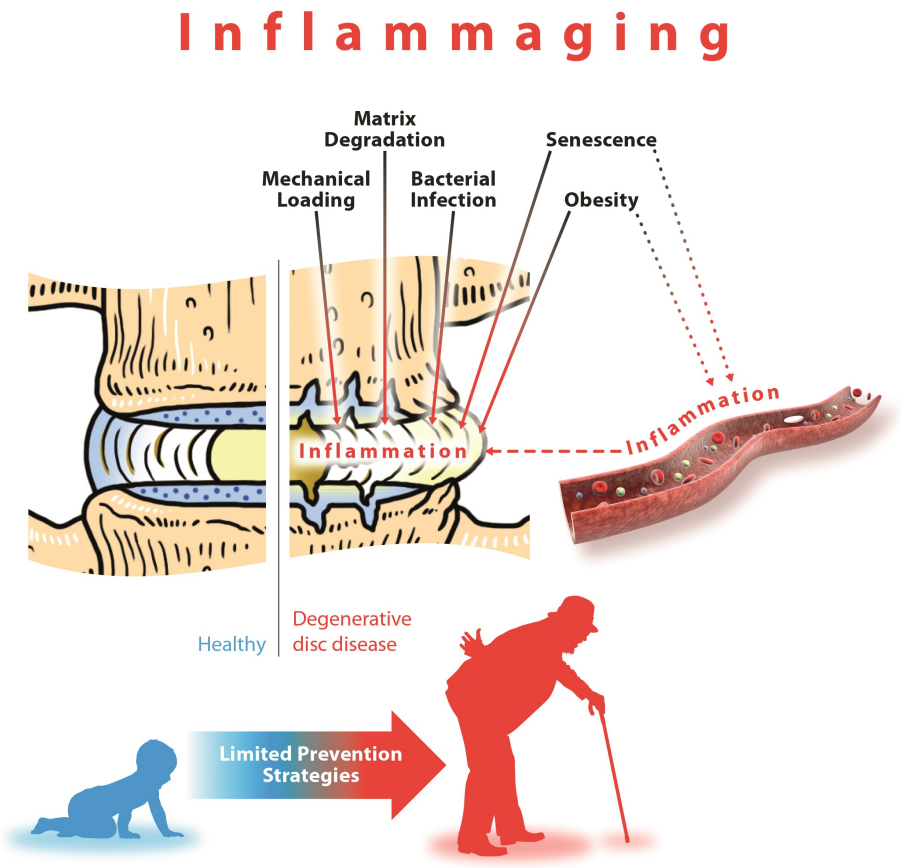


Table 1. Pro-inflammatory cytokines involved in the DDD. For more information, please refer to the cited literature.

	IL-1α	IL-1β	IL-2	IL-4	IL-6	IL-8	IL-10	IL-12	IL-15	IL-17	TNF-α	PGE2	IFN-γ
Systemically increased					[20, 21, 23, 25]	[25]					[21]		
Locally increased	[27, 31]	[27, 28, 31, 39, 41]	[27, 28]	[27, 28, 32]	[8, 27, 28, 32, 36]	[8, 27, 28, 33, 36, 38]	[27, 28, 38]	[32]	[8]	[27, 28, 32]	[8, 27-30, 33-36, 41]	[27, 28]	[27, 28, 32, 37]

3.4 Aging-related inducers of inflammation in the IVD

It is likely that several biological processes ranging from senescence, mechanical loading, matrix degradation, bacterial infection to obesity contribute to inflammaging in the IVD (**Figure 1**). Some examples of these processes are explained below.

Senescence

Senescence is the biological aging of cells that is associated with a cessation in cell division, yet continuous metabolic activity. Numerous methods and markers are used to identify senescent cells, ranging from senescence-associated β-galactosidase (SA-β-gal) staining over telomeres shortening to induction in the expression of cyclin-dependent kinase inhibitors p16INK4a or p21(Waf1/Cip1). Using these techniques, senescent cells were shown to accumulate during IVD aging and specifically in degenerated and herniated IVDs [42-45]. Two general forms of senescence exist, typically termed as (telomere-dependent) replicative senescence and stress-induced premature senescence and both can be present in the IVD cells [46, 47]. Both types of senescence share similar phenotypic features, including a shift toward an immunogenic phenotype. This phenotype is generally known as senescence-associated secretory phenotype (SASP) and is characterized by a pro-inflammatory secretome (the collection of proteins secreted by a cell), with enhanced expression of IL-6, IL-8 and IFN-γ in IVD cells [48]. Furthermore, senescent cells are responsible for enhanced catabolism and ECM degradation through stimulation of matrix metalloproteinases MMP-1, -2, -3, -9 and -13 [44, 48]. Therefore, senescence is likely an important contributor to premature disc aging and inflammaging (**Figure 1**) [44, 49].

Mechanical loading

The IVD is a mechanically complex tissue, in which hydrostatic pressure/compression and osmotic stresses predominate in the NP and tensile and shear stresses preside in AF. IVD cells have been shown to respond to mechanical stresses in a dose-, frequency-, duration- and zone-specific manner [15, 50, 51]. Moderate physiological levels of stress produce anabolic responses, hyper-physiological stresses bias towards catabolism and reduced viability, but also induce inflammation [52-58].

Importantly, aging and consequent degeneration alter the load distribution in IVDs and result in higher compressive axial and tensile radial strains [59]. Areas of peak stresses hence exist within degenerated IVDs. Cells located in these regions will experience disproportionally high loads during normal physiological activities, hence responding with catabolism and inflammation rather than anabolism (**Figure 1**). In fact, previous research has demonstrated that cellular responses to physical stress are graded by the degree of tissue degeneration[55]. Transient Receptor Potential (TRP) channels, a family of multimodal cation channels, may represent a molecular link between mechanical loading and inflammation in the IVD. TRP channels, which have been described to play a crucial role in mechanosensing, but also in transmission of inflammation and pain, have recently been detected in the IVD [8, 60, 61]. The mechanism of locally altered load distribution, together with potentially altered mechanotransduction mechanisms (e.g. vial altered TRP channel expression with aging and degeneration) [8], helps to rationalize why mechanical loading originating from normal daily activities may result in low-grade inflammation in aging IVDs.

Matrix degradation

Age-associated degeneration of the IVD is characterized by degradation of the ECM, leading not only to a decrease in total proteoglycan and collagen content, but also to altered expression and synthesis of other matrix components [62, 63]. Specific enzymes, e.g. reactive oxygen species, can induce fragmentation of these ECM proteins. A number of these fragments have been described to be biologically active and some possess inflammatory properties (**Figure 1**). In fact, fragments of hyaluronic acid were already shown to induce catabolic and inflammatory responses in IVD cells [64].

Aside from hyaluronic acid fragments, additional matrix cleavage products occurring during aging and degeneration of the IVD may contribute to IVD inflammaging, albeit likely in a size-specific manner. Fragmentation of the small, leucine-rich proteoglycan biglycan takes place in pathological human IVDs [65] and these fragments were shown to induce pro-inflammatory processes in e.g. macrophages [66, 67]. Our own unpublished data indicate that fibronectin fragments - which are present in degenerating IVDs [68, 69] - also induce inflammation in IVD cells, similar to numerous other cell types [70]. Other possible fragmentation products that may play a role in mediating IVD inflammaging include those of versican, decorin, elastin or laminin [67, 71, 72].

Bacterial Infection

Propionibacterium acnes (*P. acnes*) infection is one of the hypothesized causes of the development of chronic low-grade IVD infection and Modic changes [73]. Due to IVD aging and altered IVD biomechanics, clefts and tears occurring in the outer layer of the AF promote neovascularization and allow for easier bacterial invasion. Importantly, this new microenvironment can enhance the growth of anaerobic bacteria [74].

Previously, bacterial infection with, for example, P. acnes was believed to arise from epidural injections and contaminations during surgeries or tissue collection [75]. However, recent studies were able to demonstrate that an infection with P. acnes is likely independent of these factors [76-79].

Although *P. acnes* infection is associated with chronic inflammation in IVDs (**Figure 1**), with stimulation of various pro-inflammatory cytokines (e.g. IL-8, macrophage inflammatory protein (MIP)-1α, TNF-α) [80], the exact mechanism of inflammation induction remains unknown. However, recent evidences point towards activation of Toll-like receptors (TLR) 2 and 4 and subsequent induction of the NF-kB pathway [80, 81].

Obesity

Obesity is linked not only to a large number of comorbidities, including a significant association with the incidence of type II diabetes, various types of cancer, cardiovascular diseases, asthma or osteoarthritis [82, 83], but also with the development of disc degeneration and chronic back pain [84, 85]. A recent meta-analysis calculated that the risk of LBP increased by approximately two fold in obese patients, but with a relatively weak statistical association (OR 1.8) [86]. Interestingly, when patient selection criteria were tightened, focusing solely on morbidly obese people (body mass index (BMI) > 40), the prevalence of LBP was significantly higher in the obese group compared to a control group with normal weight[87], demonstrating that relevant weight thresholds may exist. The underlying pathophysiological mechanism leading to disc degeneration, DDD and LBP in obese patients is believed to be three-fold:

Firstly, increased mechanical loading originating from the excessive body weight in obese patients is thought to contribute to reduced disc height (i.e. disc space narrowing), increased

severity of disc degeneration and a higher number of degenerated levels in the lumbar spine [88-90]. As described above, alterations in the loading patterns, especially in case of coexisting degeneration of the IVD, can lead to inflammatory responses and may play a part in the observed interrelationship between obesity, disc height, and recent pain [88].

Secondly, overweight or obese patients may have a higher prevalence of LBP due to enhanced systemic expression of fat-derived inflammatory mediators. Adipocytes can produce cytokines, especially with aging when they undergo a phenotypic shift towards a SASP, resulting in enhanced expression of pro-inflammatory cytokines and adipokines, including leptin [91-93]. Leptin is a noteworthy adipokine as its expression levels are not only associated with body fat content [94], but also with pain levels or pain sensitivity [95], such as osteoarthritic pain or neuropathic pain [96-98]. Systemic leptin is hence discussed as a biomarker for pain prediction in various pathologies [95, 99, 100] and a first study published recently highlighted its potential application in predicting the duration of LBP [101].

Thirdly, atherosclerosis or high serum lipid levels may have a negative impact on IVD nutrition by impairing the diffusion of nutrients through the adjacent vertebrae into the IVD [102]. Interestingly, high cholesterol not only leads to an accumulation of fat in endplates and vertebral bodies as recently described by Sasani et al., but also results in a higher prevalence of disc degeneration [103] - likely due to nutritional deficits [104].

In summary, the biomechanical and biological consequences of obesity seem to contribute to IVD inflammaging (**Figure 1**). When taking into account that the prevalence of obesity has almost doubled over the past thirty years, this mechanism is likely to gain increasing importance in the years and decades to come [105].

3.5 A clinician's perspective

Until today, clinicians are predominantly guided by the patient history, as well as by signs and symptoms in combination with neuroradiological imaging, especially magnetic resonance imaging (MRI). Neurosurgeons and orthopedic spine surgeons are trained with a mechanistic understanding of the prevalent pathologies. Therefore, mechanical approaches are usually offered to patients suffering from disc pathologies. This may include selective

microsurgical removal of space occupying disc material, decompression of the spinal canal or in some cases fusion of the degenerated segment.

This review article shall help to increase the awareness that - aside from mechanical factors - local inflammation and specifically inflammaging play a crucial role in disc pathologies, including DDD. The pathological mechanisms may differ depending on the pro-inflammatory profile, likely reflected in the degree of pain experienced by patients [106]. This has indeed a strong potential to change clinical practice from diagnosis to treatment: Current efforts aim at combining technological developments with advancements in biology (including inflammation), thereby building the foundation for improved diagnosis. This will not only be limited to improved MRI analysis [107], but may also entail tracing [108] and assessment of biomarkers [109, 110]. Furthermore, precise diagnosis will allow for more personalized treatments, e.g. through neutralization of specific cytokines [111]. In the future, combination therapies that not only modulate inflammation, but also restore the ECM can be envisioned possibly by combining pharmaceuticals with, for example, stem cell application [112]. Stem cells may not only counteract the degeneration-associated loss in ECM due to their anabolic function, but may furthermore contribute to modulating inflammation via their anti-inflammatory and immune-modulatory capacity [113]. Gene therapy or genome editing (such as CRIPRS/Cas) could be used to mobilize the host cells and potentially provide long-lasting results [114, 115].

This review clearly demonstrates that the mechanism of inflammaging are highly complex. Despite the recent speculations on the role of *P. acnes* infection in inflammation and pain development in the IVD, biology-driven treatments cannot solely rely on use of antibiotics to combat low-grade infection with *P. acnes* bacterium. Future research will be needed to better understand the interaction, crosstalk and association between the numerous cytokines involved in inflammaging and their role in the development of DDD and other disc pathologies. Increased knowledge will be crucial for the development of effective – and potentially personalized - molecular treatments targeting inflammaging.

References

1. Franceschi, C., et al., *Inflammaging and anti-inflammaging: a systemic perspective on aging and longevity emerged from studies in humans.* Mech Ageing Dev, 2007. **128**(1): p. 92-105.
2. Castle, S.C., *Clinical relevance of age-related immune dysfunction.* Clinical Infectious Diseases, 2000. **31**(2): p. 578-585.
3. Franceschi, C., et al., *Inflamm-aging. An evolutionary perspective on immunosenescence.* Ann N Y Acad Sci, 2000. **908**: p. 244-54.
4. Xia, S.J., et al., *An Update on Inflamm-Aging: Mechanisms, Prevention, and Treatment.* Journal of Immunology Research, 2016.
5. Greene, M.A. and R.F. Loeser, *Aging-related inflammation in osteoarthritis.* Osteoarthritis Cartilage, 2015. **23**(11): p. 1966-71.
6. Morrisette-Thomas, V., et al., *Inflamm-aging does not simply reflect increases in pro-inflammatory markers.* Mechanisms of Ageing and Development, 2014. **139**: p. 49-57.
7. Roberts, S., et al., *Ageing in the musculoskeletal system.* Acta Orthop, 2016. **87**(sup363): p. 15-25.
8. Sadowska, A., et al., *Inflammaging in cervical and lumbar degenerated intervertebral discs: analysis of proinflammatory cytokine and TRP channel expression.* Eur Spine J, 2017. **in press**.
9. Luoma, K., et al., *Low back pain in relation to lumbar disc degeneration.* Spine (Phila Pa 1976), 2000. **25**(4): p. 487-92.
10. Balague, F., et al., *Non-specific low back pain.* Lancet, 2012. **379**(9814): p. 482-491.
11. Colombini, D., et al., *Manual Lifting: A Guide to the Study of Simple and Complex Lifting Tasks.* 2013: CRC Press.
12. Wieser, S., et al., *Cost of low back pain in Switzerland in 2005.* Eur J Health Econ, 2011. **12**(5): p. 455-67.
13. Boos, N., et al., *Classification of age-related changes in lumbar intervertebral discs.* Spine, 2002. **27**(23): p. 2631-2644.
14. Gruber, H.E. and E.N. Hanley, *Analysis of aging and degeneration of the human intervertebral disc - Comparison of surgical specimens with normal controls.* Spine, 1998. **23**(7): p. 751-757.
15. Iatridis, J.C., et al., *Region-Dependent Aggrecan Degradation Patterns in the Rat Intervertebral Disc Are Affected by Mechanical Loading In Vivo.* Spine, 2011. **36**(3): p. 203-209.

16. Hasty, P., et al., *Aging and genome maintenance: Lessons from the mouse?* Science, 2003. **299**(5611): p. 1355-1359.
17. Cheung, K.M.C., et al., *Prevalence and Pattern of Lumbar Magnetic Resonance Imaging Changes in a Population Study of One Thousand Forty-Three Individuals.* Spine, 2009. **34**(9): p. 934-940.
18. Greenberg, J.O. and R.G. Schnell, *Magnetic resonance imaging of the lumbar spine in asymptomatic adults. Cooperative study--American Society of Neuroimaging.* J Neuroimaging, 1991. **1**(1): p. 2-7.
19. Jensen, M.C., et al., *Magnetic-Resonance-Imaging of the Lumbar Spine in People without Back Pain.* New England Journal of Medicine, 1994. **331**(2): p. 69-73.
20. Ershler, W.B., *Interleukin-6: a cytokine for gerontologists.* J Am Geriatr Soc, 1993. **41**(2): p. 176-81.
21. Bruunsgaard, H., *Effects of tumor necrosis factor-alpha and interleukin-6 in elderly populations.* Eur Cytokine Netw, 2002. **13**(4): p. 389-91.
22. Deng, X., et al., *Elevated interleukin-6 expression levels are associated with intervertebral disc degeneration.* Exp Ther Med, 2016. **11**(4): p. 1425-1432.
23. Weber, K.T., et al., *Serum levels of the proinflammatory cytokine interleukin-6 vary based on diagnoses in individuals with lumbar intervertebral disc diseases.* Arthritis Res Ther, 2016. **18**: p. 3.
24. Schistad, E.I., et al., *Association between baseline IL-6 and 1-year recovery in lumbar radicular pain.* European Journal of Pain, 2014. **18**(10): p. 1394-1401.
25. Pedersen, L.M., et al., *Serum levels of the pro-inflammatory interleukins 6 (IL-6) and-8 (IL-8) in patients with lumbar radicular pain due to disc herniation: A 12-month prospective study.* Brain Behavior and Immunity, 2015. **46**: p. 132-136.
26. Brisby, H., et al., *Proinflammatory cytokines in cerebrospinal fluid and serum in patients with disc herniation and sciatica.* European Spine Journal, 2002. **11**(1): p. 62-66.
27. Risbud, M.V. and I.M. Shapiro, *Role of cytokines in intervertebral disc degeneration: pain and disc content.* Nat Rev Rheumatol, 2014. **10**(1): p. 44-56.
28. Wuertz, K. and L. Haglund, *Inflammatory mediators in intervertebral disk degeneration and discogenic pain.* Global Spine J, 2013. **3**(3): p. 175-84.
29. Bachmeier, B.E., et al., *Analysis of tissue distribution of TNF-alpha, TNF-alpha-receptors, and the activating TNF-alpha-converting enzyme suggests activation of the TNF-alpha system in the aging intervertebral disc.* Signal Transduction Pathways, Pt D, 2007. **1096**: p. 44-54.
30. Wang, C., et al., *Tumor necrosis factor-alpha: a key contributor to intervertebral disc degeneration.* Acta Biochimica Et Biophysica Sinica, 2017. **49**(1): p. 1-13.

31. Le Maitre, C.L., A.J. Freemont, and J.A. Hoyland, *The role of interleukin-1 in the pathogenesis of human intervertebral disc degeneration.* Arthritis Res Ther, 2005. **7**(4): p. R732-45.
32. Shamji, M.F., et al., *Proinflammatory Cytokine Expression Profile in Degenerated and Herniated Human Intervertebral Disc Tissues.* Arthritis and Rheumatism, 2010. **62**(7): p. 1974-1982.
33. Lee, S., et al., *Comparison of growth factor and cytokine expression in patients with degenerated disc disease and herniated nucleus pulposus.* Clin Biochem, 2009. **42**(15): p. 1504-11.
34. Purmessur, D., et al., *A role for TNF alpha in intervertebral disc degeneration: A non-recoverable catabolic shift.* Biochemical and Biophysical Research Communications, 2013. **433**(1): p. 151-156.
35. Kang, R., et al., *Intervertebral disc degenerative changes after intradiscal injection of TNF-alpha in a porcine model.* European Spine Journal, 2015. **24**(9): p. 2010-2016.
36. Sutovsky, J., et al., *Cytokine and chemokine profile changes in patients with lower segment lumbar degenerative spondylolisthesis.* Int J Surg, 2017. **43**: p. 163-170.
37. Cuellar, J.M., et al., *Cytokine evaluation in individuals with low back pain using discographic lavage.* Spine J, 2010. **10**(3): p. 212-8.
38. Zhang, Y., et al., *Intervertebral Disc Cells Produce Interleukins Found in Patients with Back Pain.* Am J Phys Med Rehabil, 2016. **95**(6): p. 407-15.
39. Kepler, C.K., et al., *Expression and relationship of proinflammatory chemokine RANTES/CCL5 and cytokine IL-1beta in painful human intervertebral discs.* Spine (Phila Pa 1976), 2013. **38**(11): p. 873-80.
40. Navone, S.E., et al., *Inflammatory mediators and signalling pathways controlling intervertebral disc degeneration.* Histol Histopathol, 2017. **32**(6): p. 523-542.
41. Johnson, Z.I., et al., *Disc in flames: Roles of TNF-alpha and IL-1beta in intervertebral disc degeneration.* Eur Cell Mater, 2015. **30**: p. 104-16; discussion 116-7.
42. Kim, K.W., et al., *Senescence mechanisms of nucleus pulposus chondrocytes in human intervertebral discs.* Spine J, 2009. **9**(8): p. 658-66.
43. Roberts, S., et al., *Senescence in human intervertebral discs.* European Spine Journal, 2006. **15**: p. S312-S316.
44. Le Maitre, C.L., A.J. Freemont, and J.A. Hoyland, *Accelerated cellular senescence in degenerate intervertebral discs: a possible role in the pathogenesis of intervertebral disc degeneration.* Arthritis Research & Therapy, 2007. **9**(3).

45. Gruber, H.E., et al., *Senescence in cells of the aging and degenerating intervertebral disc - Immunolocalization of senescence-associated beta-galactosidase in human and sand rat discs.* Spine, 2007. **32**(3): p. 321-327.
46. Dimozi, A., et al., *Oxidative stress inhibits the proliferation, induces premature senescence and promotes a catabolic phenotype in human nucleus pulposus intervertebral disc cells.* Eur Cell Mater, 2015. **30**: p. 89-102; discussion 103.
47. Jeong, S.W., J.S. Lee, and K.W. Kim, *In vitro lifespan and senescence mechanisms of human nucleus pulposus chondrocytes.* Spine J, 2014. **14**(3): p. 499-504.
48. Vamvakas, S.S., E. Mavrogonatou, and D. Kletsas, *Human nucleus pulposus intervertebral disc cells becoming senescent using different treatments exhibit a similar transcriptional profile of catabolic and inflammatory genes.* European Spine Journal, 2017. **26**(8): p. 2063-2071.
49. Vo, N.V., et al., *Molecular mechanisms of biological aging in intervertebral discs.* J Orthop Res, 2016. **34**(8): p. 1289-306.
50. Chan, S.C.W., et al., *Region Specific Response of Intervertebral Disc Cells to Complex Dynamic Loading: An Organ Culture Study Using a Dynamic Torsion-Compression Bioreactor.* Plos One, 2013. **8**(8).
51. Setton, L.A. and J. Chen, *Mechanobiology of the intervertebral disc and relevance to disc degeneration.* Journal of Bone and Joint Surgery-American Volume, 2006. **88a**: p. 52-57.
52. Neidlinger-Wilke, C., et al., *Regulation of gene expression in intervertebral disc cells by low and high hydrostatic pressure.* European Spine Journal, 2006. **15**: p. S372-S378.
53. Sowa, G.A., et al., *Alterations in gene expression in response to compression of nucleus pulposus cells.* Spine Journal, 2011. **11**(1): p. 36-43.
54. Gilbert, H.T.J., et al., *The involvement of interleukin-1 and interleukin-4 in the response of human annulus fibrosus cells to cyclic tensile strain: an altered mechanotransduction pathway with degeneration.* Arthritis Research & Therapy, 2011. **13**(1).
55. Gilbert, H.T.J., J.A. Hoyland, and S.J. Millward-Sadler, *The Response of Human Anulus Fibrosus Cells to Cyclic Tensile Strain Is Frequency-Dependent and Altered with Disc Degeneration.* Arthritis and Rheumatism, 2010. **62**(11): p. 3385-3394.
56. Gawri, R., et al., *High mechanical strain of primary intervertebral disc cells promotes secretion of inflammatory factors associated with disc degeneration and pain.* Arthritis Research & Therapy, 2014. **16**(1).
57. Walter, B.A., et al., *Complex loading affects intervertebral disc mechanics and biology.* Osteoarthritis and Cartilage, 2011. **19**(8): p. 1011-1018.

58. Miyagi, M., et al., *ISSLS Prize Winner: Disc Dynamic Compression in Rats Produces Long-Lasting Increases in Inflammatory Mediators in Discs and Induces Long-Lasting Nerve Injury and Regeneration of the Afferent Fibers Innervating Discs A Pathomechanism for Chronic Discogenic Low Back Pain.* Spine, 2012. **37**(21): p. 1810-1818.
59. O'Connell, G.D., E.J. Vresilovic, and D.M. Elliott, *Human intervertebral disc internal strain in compression: the effect of disc region, loading position, and degeneration.* J Orthop Res, 2011. **29**(4): p. 547-55.
60. Walter, B.A., et al., *Reduced tissue osmolarity increases TRPV4 expression and pro-inflammatory cytokines in intervertebral disc cells.* Eur Cell Mater, 2016. **32**: p. 123-36.
61. Krupkova, O., J. Zvick, and K. Wuertz-Kozak, *The role of transient receptor potential channels in joint diseases.* Eur Cell Mater, 2017. **34**: p. 180-201.
62. Urban, J.P. and S. Roberts, *Degeneration of the intervertebral disc.* Arthritis Res Ther, 2003. **5**(3): p. 120-30.
63. Singh, K., et al., *Age-related changes in the extracellular matrix of nucleus pulposus and anulus fibrosus of human intervertebral disc.* Spine (Phila Pa 1976), 2009. **34**(1): p. 10-6.
64. Quero, L., et al., *Hyaluronic acid fragments enhance the inflammatory and catabolic response in human intervertebral disc cells through modulation of toll-like receptor 2 signalling pathways.* Arthritis Res Ther, 2013. **15**(4): p. R94.
65. Brown, S., et al., *A comparative evaluation of the small leucine-rich proteoglycans of pathological human intervertebral discs.* Eur Spine J, 2012. **21 Suppl 2**: p. S154-9.
66. Schaefer, L., et al., *The matrix component biglycan is proinflammatory and signals through toll-like receptor-4 and-2 in macrophages.* Naunyn-Schmiedebergs Archives of Pharmacology, 2006. **372**: p. 21-21.
67. Moreth, K., R.V. Iozzo, and L. Schaefer, *Small leucine-rich proteoglycans orchestrate receptor crosstalk during inflammation.* Cell Cycle, 2012. **11**(11): p. 2084-2091.
68. Ruel, N., et al., *Fibronectin Fragments and the Cleaving Enzyme ADAM-8 in the Degenerative Human Intervertebral Disc.* Spine, 2014. **39**(16): p. 1274-1279.
69. Anderson, D.G., et al., *Fibronectin Splicing Variants in Human Intervertebral Disc and Association With Disc Degeneration.* Spine, 2010. **35**(17): p. 1581-1588.
70. Arroyo, A.G. and M.L. Iruela-Arispe, *Extracellular matrix, inflammation, and the angiogenic response.* Cardiovascular Research, 2010. **86**(2): p. 226-235.

71. Adair-Kirk, T.L. and R.M. Senior, *Fragments of extracellular matrix as mediators of inflammation.* International Journal of Biochemistry & Cell Biology, 2008. **40**(6-7): p. 1101-1110.
72. Almine, J.F., et al., *Elastin sequences trigger transient proinflammatory responses by human dermal fibroblasts.* Faseb Journal, 2013. **27**(9): p. 3455-3465.
73. Albert, H.B., et al., *Does nuclear tissue infected with bacteria following disc herniations lead to Modic changes in the adjacent vertebrae?* European Spine Journal, 2013. **22**(4): p. 690-696.
74. Albert, H.B., et al., *Modic changes, possible causes and relation to low back pain.* Med Hypotheses, 2008. **70**(2): p. 361-8.
75. McLorinan, G.C., et al., *Propionibacterium acnes wound contamination at the time of spinal surgery.* Clinical Orthopaedics and Related Research, 2005(437): p. 67-73.
76. Zhou, Z.Z., et al., *Relationship between annular tear and presence of Propionibacterium acnes in lumbar intervertebral disc.* European Spine Journal, 2015. **24**(11): p. 2496-2502.
77. Capoor, M.N., et al., *Propionibacterium acnes biofilm is present in intervertebral discs of patients undergoing microdiscectomy.* Plos One, 2017. **12**(4).
78. Dudli, S., et al., *Inflammatory response of disc cells against Propionibacterium acnes depends on the presence of lumbar Modic changes.* Eur Spine J, 2017.
79. Arndt, J., et al., *Bacteriology of Degenerated Lumbar Intervertebral Disks.* Journal of Spinal Disorders & Techniques, 2012. **25**(7): p. E211-E216.
80. Yuan, Y., et al., *Association between chronic inflammation and latent infection of Propionibacterium acnes in non-pyogenic degenerated intervertebral discs: a pilot study.* Eur Spine J, 2017.
81. Lwin, S.M., I. Kimber, and J.P. McFadden, *Acne, quorum sensing and danger.* Clin Exp Dermatol, 2014. **39**(2): p. 162-7.
82. Guh, D.P., et al., *The incidence of co-morbidities related to obesity and overweight: A systematic review and meta-analysis.* Bmc Public Health, 2009. **9**.
83. Schelbert, K.B., *Comorbidities of Obesity.* Primary Care, 2009. **36**(2): p. 271-+.
84. Jakoi, A.M., et al., *The Clinical Correlations between Diabetes, Cigarette Smoking and Obesity on Intervertebral Degenerative Disc Disease of the Lumbar Spine.* Asian Spine Journal, 2017. **11**(3): p. 337-347.
85. Shiri, R., et al., *The Association Between Obesity and Low Back Pain: A Meta-Analysis.* American Journal of Epidemiology, 2010. **171**(2): p. 135-154.
86. Dario, A.B., et al., *The relationship between obesity, low back pain, and lumbar disc degeneration when genetics and the environment are considered: a systematic review of twin studies.* Spine Journal, 2015. **15**(5): p. 1106-1117.

87. Melissas, J., E. Volakakis, and A. Hadjipavlou, *Low-back pain in morbidly obese patients and the effect of weight loss following surgery.* Obes Surg, 2003. **13**(3): p. 389-93.
88. Urquhart, D.M., et al., *Obesity Is Associated With Reduced Disc Height in the Lumbar Spine but Not at the Lumbosacral Junction.* Spine, 2014. **39**(16): p. E962-E966.
89. Liuke, M., et al., *Disc degeneration of the lumbar spine in relation to overweight.* International Journal of Obesity, 2005. **29**(8): p. 903-908.
90. Samartzis, D., et al., *The association of lumbar intervertebral disc degeneration on magnetic resonance imaging with body mass index in overweight and obese adults: A population-based study.* Arthritis and Rheumatism, 2012. **64**(5): p. 1488-1496.
91. Kirkland, J., et al., *Aging, Fat Tissue, Inflammation, and Cellular Senescence.* Gerontologist, 2012. **52**: p. 492-492.
92. Tchkonia, T., et al., *Fat tissue, aging, and cellular senescence.* Aging Cell, 2010. **9**(5): p. 667-684.
93. Samartzis, D., et al., *Disk degeneration and low back pain: are they fat-related conditions?* Global Spine J, 2013. **3**(3): p. 133-44.
94. Ahima, R.S., *Revisiting leptin's role in obesity and weight loss.* J Clin Invest, 2008. **118**(7): p. 2380-3.
95. Younger, J., et al., *Association of Leptin with Body Pain in Women.* J Womens Health (Larchmt), 2016. **25**(7): p. 752-60.
96. Gandhi, R., et al., *The synovial fluid adiponectin-leptin ratio predicts pain with knee osteoarthritis.* Clinical Rheumatology, 2010. **29**(11): p. 1223-1228.
97. Lim, G., et al., *Spinal leptin contributes to the pathogenesis of neuropathic pain in rodents.* J Clin Invest, 2009. **119**(2): p. 295-304.
98. Lubbeke, A., et al., *Do synovial leptin levels correlate with pain in end stage arthritis?* Int Orthop, 2013. **37**(10): p. 2071-9.
99. Galletti, F., et al., *Circulating leptin levels predict the development of metabolic syndrome in middle-aged men: an 8-year follow-up study.* J Hypertens, 2007. **25**(8): p. 1671-7.
100. Osorio, C.F., et al., *Leptin and leptin receptor expressions in prostate tumors may predict disease aggressiveness?* Acta Cirurgica Brasileira, 2014. **29**: p. 44-48.
101. Lippi, G., et al., *The serum concentrations of leptin and MCP-1 independently predict low back pain duration.* Clinical Chemistry and Laboratory Medicine, 2017. **55**(9): p. 1368-1374.
102. Kauppila, L.I., *Atherosclerosis and Disc Degeneration/Low-Back Pain - A Systematic Review.* European Journal of Vascular and Endovascular Surgery, 2009. **37**(6): p. 661-670.

103. Sasani, M., et al., *Effect of a hypercholesterolemia as a starting factor on spinal degeneration in rabbits and role of Vitamin E (alpha-tocopherol).* Surg Neurol Int, 2016. **7**: p. 36.
104. Urban, J.P.G., S. Smith, and J.C.T. Fairbank, *Nutrition of the intervertebral disc.* Spine, 2004. **29**(23): p. 2700-2709.
105. Finucane, M.M., et al., *National, regional, and global trends in body-mass index since 1980: systematic analysis of health examination surveys and epidemiological studies with 960 country-years and 9.1 million participants.* Lancet, 2011. **377**(9765): p. 557-567.
106. Leung, Y.Y., et al., *Synovial fluid pro-inflammatory profile differs according to the characteristics of knee pain.* Osteoarthritis Cartilage, 2017. **25**(9): p. 1420-1427.
107. Waldenberg, C., et al., *MRI histogram analysis enables objective and continuous classification of intervertebral disc degeneration.* Eur Spine J, 2017.
108. Xiao, L., et al., *A Novel Modality for Functional Imaging in Acute Intervertebral Disk Herniation via Tracking Leukocyte Infiltration.* Mol Imaging Biol, 2017. **19**(5): p. 703-713.
109. Haneder, S., et al., *Assessment of glycosaminoglycan content in intervertebral discs using chemical exchange saturation transfer at 3.0 Tesla: preliminary results in patients with low-back pain.* Eur Radiol, 2013. **23**(3): p. 861-8.
110. Kandahari, A.M., et al., *Recognition of Immune Response for the Early Diagnosis and Treatment of Osteoarthritis.* J Immunol Res, 2015. **2015**: p. 192415.
111. Rider, P., Y. Carmi, and I. Cohen, *Biologics for Targeting Inflammatory Cytokines, Clinical Uses, and Limitations.* Int J Cell Biol, 2016. **2016**: p. 9259646.
112. Sakai, D., *Future perspectives of cell-based therapy for intervertebral disc disease.* Eur Spine J, 2008. **17 Suppl 4**: p. 452-8.
113. Krock, E., D.H. Rosenzweig, and L. Haglund, *The Inflammatory Milieu of the Degenerate Disc: Is Mesenchymal Stem Cell-based Therapy for Intervertebral Disc Repair a Feasible Approach?* Curr Stem Cell Res Ther, 2015. **10**(4): p. 317-28.
114. Nishida, K., et al., *Gene therapy approach for disc degeneration and associated spinal disorders.* Eur Spine J, 2008. **17 Suppl 4**: p. 459-66.
115. Farhang, N., et al., *(*) CRISPR-Based Epigenome Editing of Cytokine Receptors for the Promotion of Cell Survival and Tissue Deposition in Inflammatory Environments.* Tissue Eng Part A, 2017. **23**(15-16): p. 738-749.

Chapter 4

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

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

Aleksandra Sadowska[1*], Ermioni Touli[2*], Wolfgang Hitzl[3], Helen Greutert[1], Stephen J. Ferguson[1], Karin Wuertz-Kozak[1,3,4,5#] & Oliver N. Hausmann[2#]

[1]Institute for Biomechanics, ETH Zurich, Zurich, Switzerland.
[2]Neuro and Spine Center, Hirslanden Klinik St. Anna, Lucerne, Switzerland.
[3]Paracelsus Medical University, Strubergasse 21, 5020, Salzburg, Austria.
[4]Spine Center, Schön Clinic Munich Harlaching, Munich, Germany.
[5]Department of Health Sciences, University of Potsdam, Potsdam, Germany.

[* #] These authors contributed equally.

Published as:
Sadowska, A., *et al.* Inflammaging in cervical and lumbar degenerated intervertebral discs: analysis of proinflammatory cytokine and TRP channel expression.
Eur Spine J 27, 564–577 (2018). https://doi.org/10.1007/s00586-017-5360-8

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

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

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

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

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

4.1 Introduction

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

Disc degeneration (DDD) is an age-related process that occurs early in life and is associated with dehydration and fibrosis of the NP, as well as formation of tears and clefts in the AF. Factors known to be associated with DDD include mechanical loading, genetics, and nutritional deprivation [2]. Certain individuals experience age-related chronic inflammation of the IVD, termed “inflammaging”, which is characterized by up-regulation of proinflammatory cytokines such as Interleukin-6 (IL-6) [3]. Furthermore, osteophytes and spondylosis can develop, and the posterior longitudinal ligament and the ligamentum flavum may bulge into the spinal canal [1, 4]. On the molecular level, degenerative processes in the disc are characterized by a shift in the collagen synthesis profile and increased expression

of catabolic enzymes, such as matrix metalloproteinases (MMP), with a subsequent loss of proteoglycans [1]. The proteolytic disintegration of aggrecan, which is promoted by cytokine-regulated aggrecanases (ADAMTS-4 and -5) [4, 5], reduces the disc's barrier function to nerve ingrowth [6, 7]. Microvessels can also invade the degenerating disc, increasing the production of the neurotrophin nerve growth factor (NGF) and thus the incidence of neural structures within the tissue [7].

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

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

degeneration, inflammation and pain and hence be of mechanistic relevance in disc inflammaging.

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

4.2 Materials and methods

Sample Collection

A total of 51 disc samples were obtained from 45 patients (18 men, 27 women, mean age = 52 [age range 16-79 years]) undergoing elective spinal surgery in the cervical (n = 24) or lumbar (n =2 1) region. Cervical samples were collected as entire discs due to the tissue size. Lumbar discs were intraoperatively excised as NP (n = 15) and/or AF (n = 12) samples, followed by macroscopic tissue evaluation. 22 patients suffered from disc herniation and 23 from degenerative disc disease. 26 individuals underwent one level discectomy and 19 individuals multi level discectomy. Assessment of the disease state was performed using Pfirrmann grading (IVD degeneration) and Modic grading (endplate changes). Informed consent for sample collection was obtained from each patient and the study was approved through the local ethics committee (Ethics Committee of the Canton Lucerne/Switzerland, #1007). Detailed patient information is given in **Table 1**.

Table 1. Patient information.

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

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

RNA Isolation

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

Gene Expression Analysis

Two micrograms of RNA was used to synthesize cDNA in a total volume of 60 µl, using the reverse transcription kit (4374966, Applied Biosystems). For samples with lower yields, the reverse transcription was conducted at reduced concentrations.

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

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

Table 2. TaqMan primers used for qPCR analysis.

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

Statistical Analysis

Data consistency was checked and data were screened for outliers by using quantile plots and normality using Kolmogorov–Smirnov test. Due to the given distributions, generalized linear models were not applicable and, hence, Wilcoxon-matched pairs test, Mann–Whitney U test and Spearman correlation were used to analyze continuously distributed data. Cross tabulation tables with Fisher's exact test, Pearson's Chi square test, marginal homogeneity test and McNemar's test were used to analyze cross tabulation tables. One-factorial ANOVA with unpaired and two-sided Student's t tests as post hoc tests were used to test means among different groups. Whisker plots with medians and 25% and 75% quantiles as well as

scatter plots were used to illustrate the results. All reported tests were two sided, and p values < 0.05 were considered to be statistically significant.

For the comparison of lumbar and cervical gene expression patterns, the entire data set as well as a patient matched data set was analyzed. Matching was conducted with regard to IVD degeneration grade, age, surgical extent and pathology, with the highest focus on IVD degeneration grade and subsequently decreasing importance.

All statistical analyses in this report were performed using STATISTICA 13 (Hill, T. & Lewicki, P. Statistics: Methods and Applications. StatSoft, Tulsa, OK) and PASW 22 (IBM SPSS Statistics for Windows, Version 21.0., Armonk, NY) and StatXact 10 (Cytel Software 2013, Cambridge MA, USA).

4.3 Results

Expression Levels: Overview

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

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

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

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

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

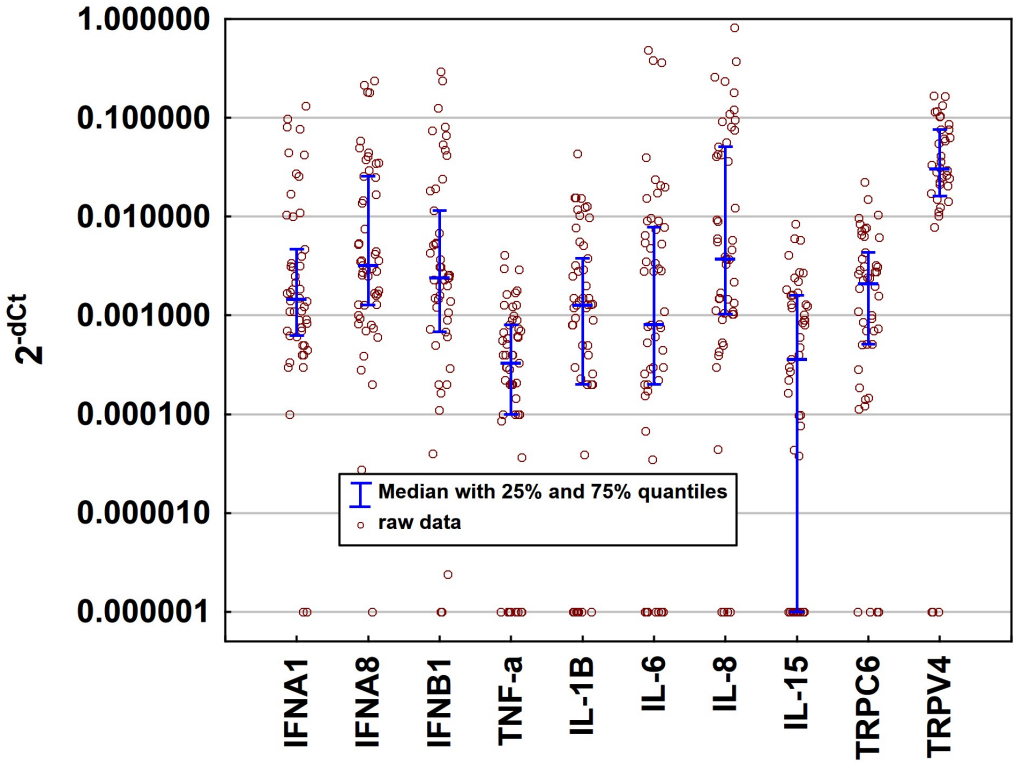


Figure 1. Gene expression of the selected cytokines and TRP channels in all specimens (n=51, 24x cervical, 15x lumbar AF, 12x lumbar NP), calculated as $2^{-\Delta Ct}$ values (relative to GAPDH).

Expression Levels: Correlation with Age, Degeneration Grade and Modic Grade

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

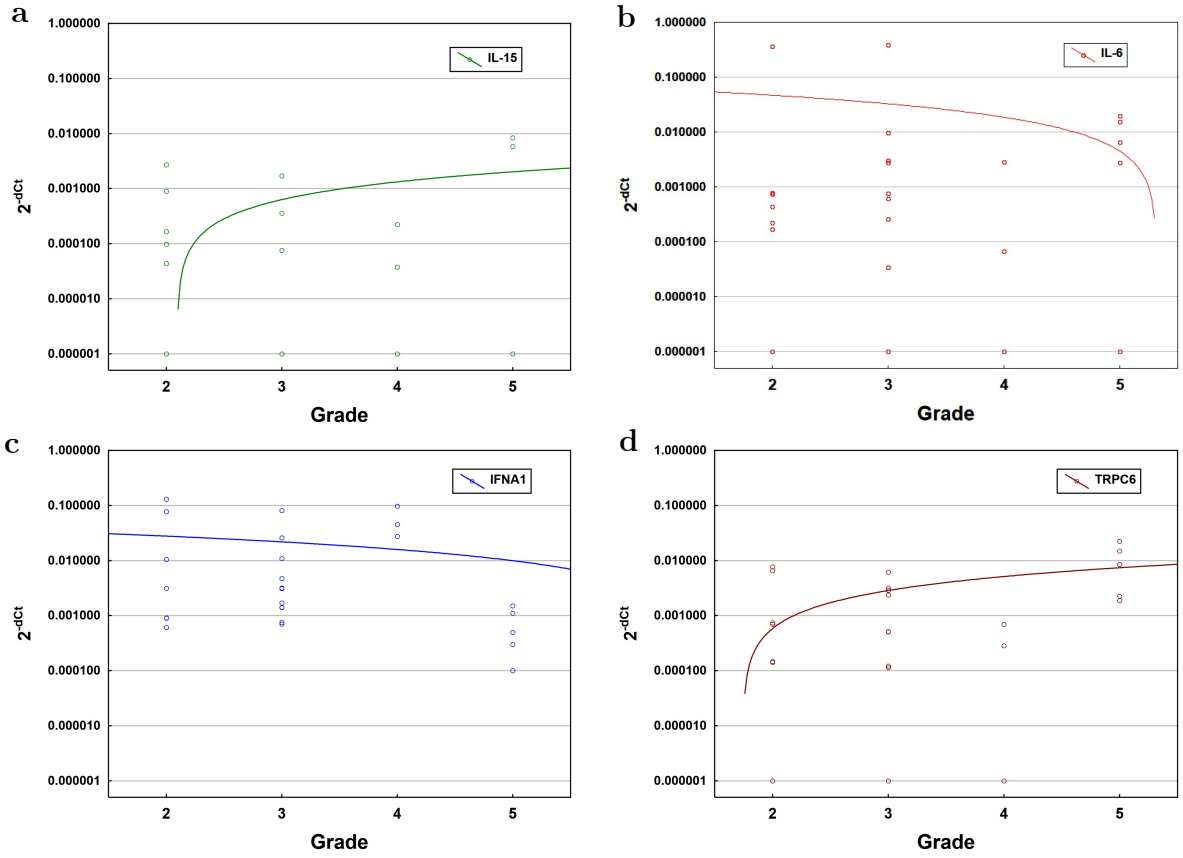


Figure 2. Correlations between gene expression of IL-15 (**a**), IL-6 (**b**), IFNA1 (**c**), and TRPC6 (**d**) and the degeneration grade. Results were calculated as $2^{-\Delta Ct}$ values (relative to GAPDH). Note: gene expression is plotted on a logarithmic scale.

Expression Levels: Differences with Extent of Surgery, Pathology and Gender

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

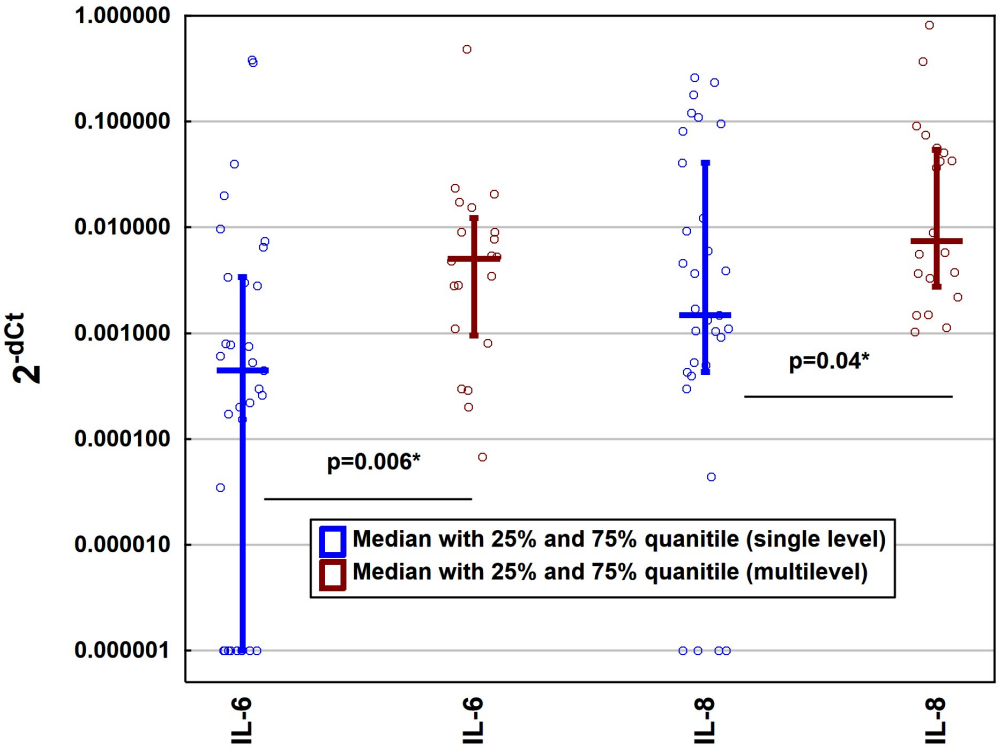


Figure 3. Statistically significant differences in the gene expression of IL-6 and IL-8 between patients undergoing single-level (n=31) and multi-level (n=20) discectomy. Results were calculated as $2^{-\Delta Ct}$ values (relative to GAPDH).

Expression Levels: Differences with Spinal Region (Cervical/Lumbar)

When comparing the gene expression in cervical and lumbar samples over the entire sample population (n = 51), significant differences were found for the cytokines IFNA1 (cervical < lumbar, p = 0.038), IL-6 (cervical > lumbar, p = 0.0375), IL-15 (p = 0.0001, cervical > lumbar) and the ion channel TRPC6 (p = 0.045, cervical > lumbar) (**Figure 4a**).

As these genes were also found to be regulated by the IVD degeneration grade (IL-15, IL-6, IFNA1, TRPC6), age (IL-15) and surgical extent (IL-6), we aimed to further elucidate whether differences were truly related to spinal region or confounded by dissimilarities in age and IVD degeneration grade in the cervical and lumbar group, using a sample matching approach. When analyzing 28 well-matched samples (14 x cervical and 14 x lumbar), we were able to confirm the difference in IL-15 expression in the cervical and lumbar IVDs (p=0.013, **Figure 4b**), whereas the difference in IFNA1 expression did not reach

significance in this grouping (p=0.056, data not shown), possibly due to lower statistical power arising from the reduced sample size. IL-6 and TRPC6 showed no differences after matching was conducted.

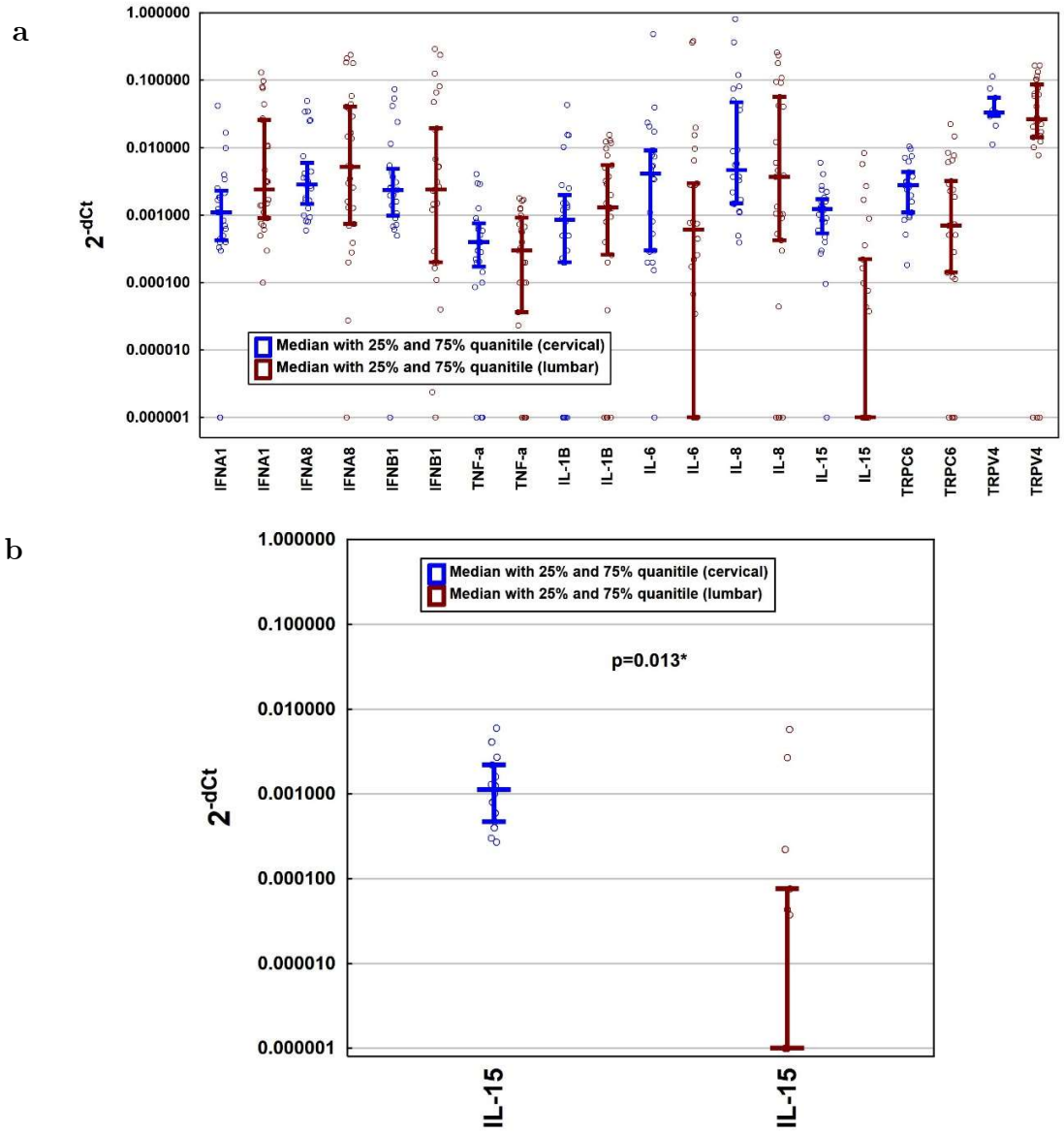


Figure 4. Differences in gene expression between cervical (n = 24) and lumbar (n = 27) samples (entire sample population), with statistically significant results for IFNA1, IL-6, IL-15 and TRPC6 (**a**) and confirmation of statistical difference in gene expression of IL-15 between well-matched cervical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

Expression Levels: Differences with Disc Region (NP/AF)

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

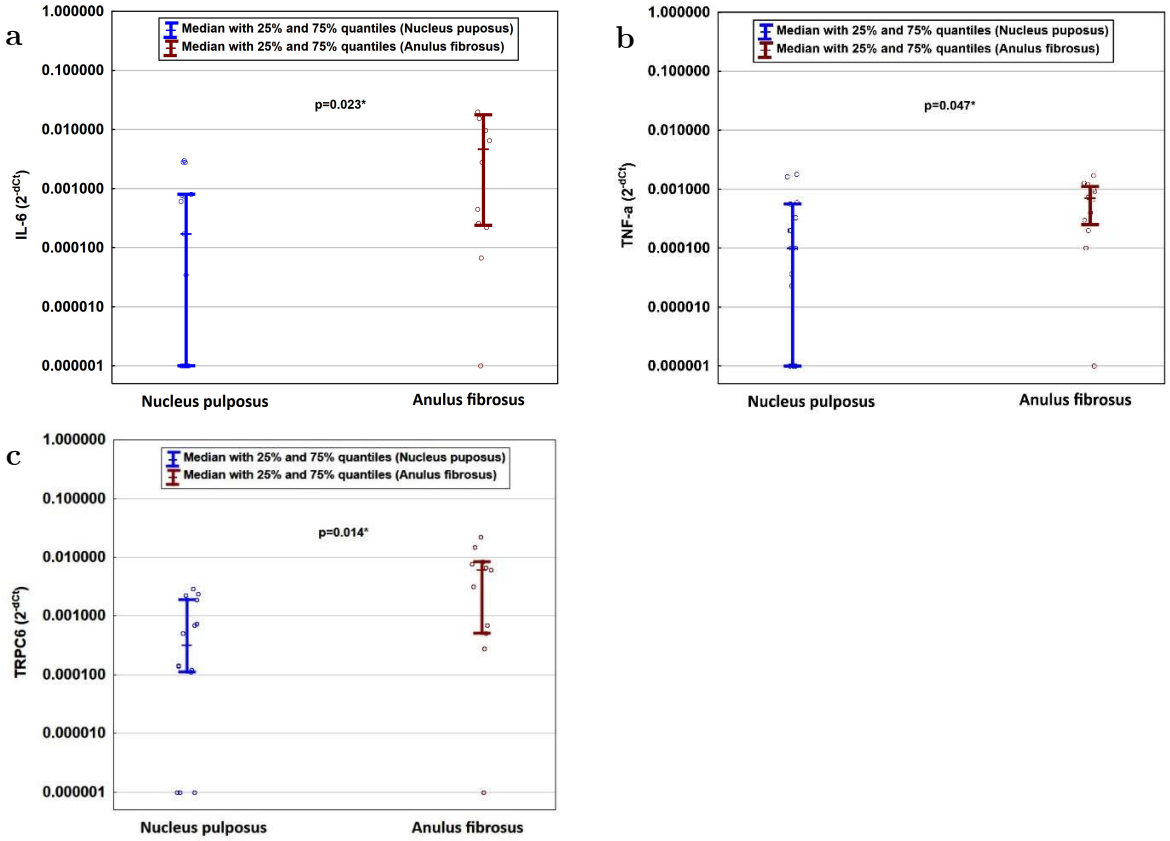


Figure 5. Statistically significant differences in gene expression of IL-6 (**a**), TNF-α (**b**) and TRPC6 (**c**) between AF (n = 12) and NP (n = 15) samples in lumbar discs. Results were calculated as $2^{-\Delta Ct}$ values (relative to GAPDH).

Expression Levels: Correlation between Genes

To test whether the expression of target genes is intra-correlated, a Spearman correlation test was conducted on the entire data set. Numerous genes were found to be correlated (for all results see **Table 4**), with the strongest correlation for the gene pairs IFNA8 and IFNB1 ($n = 51$, $r = 0.93$, $p < 0.0001$, **Figure 6a**), IL-6 and IL-8 ($n = 51$, $r = 0.81$, $p < 0.0001$,

Figure 6b), IFNA1 and IFNB1 (n = 50, r = 0.78, p < 0.0001, **Figure 6c**), IL-1β and TNF-α (n = 51, r = 0.78, p < 0.0001, **Figure 6d**), IFNA1 and IFNA8 (n = 50, r = 0.74, p < 0.0001, **Figure 6e**), IL-15 and TRPC6 (n = 46, r = 0.64, p < 0.0001, **Figure 6f**), IL6 and TRPC6 (n = 46, r = 0.59, p < 0.0001, **Figure 6g**), IFNA8 and TRPC6 (n = 46, r = -0.57, p < 0.0001, **Figure 6h**), and IL-1β and IL-8 (n = 51, r = 0.56, p < 0.0001, **Figure 6i**).

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

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

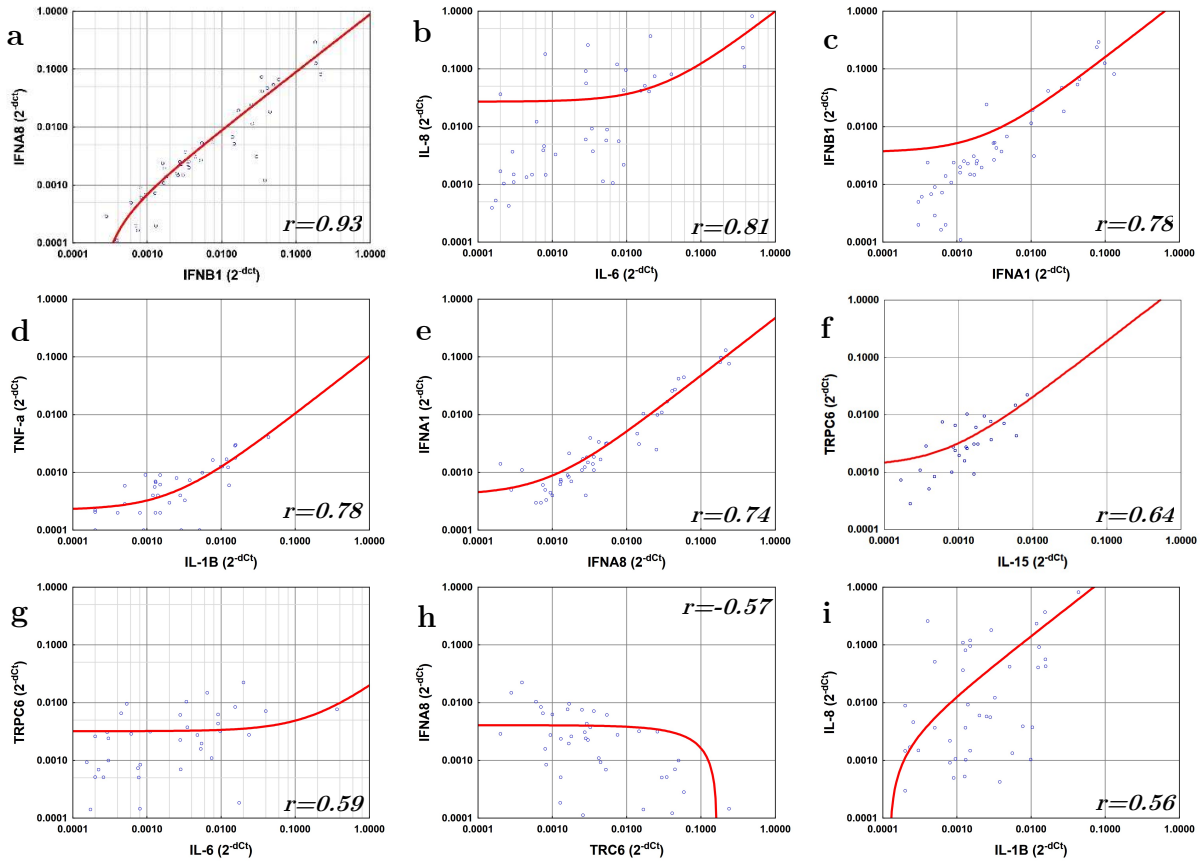


Figure 6. Statistically significant correlations between the expression of IFNA8 and IFNB1 (**a**), IL-6 and IL-8 (**b**), IFNA1 and IFNB1 (**c**), IL-1B and TNF-α (**d**), IFNA1 and IFNA8 (**e**), IL-15 and TRPC6 (**f**), IL-6 and TRPC6 (**g**), IFNA8 and TRPC6 (**h**) as well as IL-1β and IL-8 (**i**).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

4.4 Discussion

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

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

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

Aside from IL-8, IL-6 was also expressed to a higher level in multi-level surgery patients in the herein presented study. IL-6 is an inflammatory cytokine with multiple biological effects [25]. After tissue injury, it promotes monocyte differentiation into macrophages and activates maturation of B- and T-lineage lymphocytes [31], thereby stimulating the production of immunoglobulin via B-lymphocytes [32]. By binding to the non-signaling membrane-bound IL-6 receptor (mIL-6R) and subsequently interacting with membrane protein gp130, it activates a variety of intracellular signaling pathways, including the Janus-activated kinase/signal transducer activator of transcription (JAK/STAT), the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), and the

phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) [33]. Importantly, IL-6 is believed to play a major role in the pathogenesis of spinal neuropathic pain, specifically in symptomatic radiculopathy (by inducing PGE2- mediated allodynia in experimental rat models) and peripheral nerve injury [25, 31, 34, 35]. This notion is supported by studies demonstrating that lumbar radicular pain induced by disc herniation is associated with elevated IL-6 serum levels [29, 36]. The functional role of IL-6 in spinal pain is further supported through therapeutic studies. A recent study demonstrated that epidural injection of an anti-IL-6R monoclonal antibody, tocilizumab, onto the spinal nerve alleviated radicular leg pain, numbness, and low back pain without causing adverse events in 60 patients with lumbar spinal stenosis-induced sciatica [37]. Interestingly, we could demonstrate that the expression of IL-6 was dependent on the disc degeneration grade, the zonal region of the IVD (AF versus NP) and the spinal level (cervical versus lumbar). However, the latter effect was no longer present in matched samples, indicating that non-uniform distribution of the degeneration grade may have been the underlying cause of the difference seen in the entire (non-matched) dataset.

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

in TRPC6-null mice in a study of lung inflammation [41]. Aside from IL-6, we could also demonstrate mild to moderate correlations between TRPC6 and other cytokines, such as TNF-α.

TNF-α is a type II transmembrane protein secreted by macrophages and also other cell types (including disc cells [42]), which binds its receptors TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR. In the disc, TNF-α is thought to participate in the initiation of the inflammatory cascade [43], as it is associated with an increase in the levels of other cytokines, such as IL-1B and IL-6 [44]. These reports are in agreement with our results that show a positive correlation between the expression of TNF-α and the cytokines IL-1β, IL-6, IL-8 and IL-15. In our study, relatively low levels of TNF-α were measured, possibly due to the advanced stage of degeneration of many samples [45]. Our results indicate a higher expression of TNF-α in the AF than the NP. In fact, previous reports have provided contradictory findings on the zonal expression of TNF-α, describing it to be either higher or lower in the AF compared to the NP [46-48]. As TNF-α is implicated in neurogenic, radicular and low back pain [49], correlating the duration of symptoms with TNF-α expression would have been of interest, but this information was not available in the patient history. Importantly, recent evidence suggests therapeutic effectiveness of TNF-α inhibitors for disc herniation-associated radicular pain through inhibition [50]. Hence, although the role of TNF-α in disc herniation is established, its implication in disc degeneration is still somewhat unclear [48].

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

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

IL-15 is fundamental in the immune response as it modulates the activation and proliferation of natural killer- T- and B cells [55-57]. While little evidence exists on is relevance in disc disease, it is essential in cancer pathology, with promotion of malignancies like multiple myeloma, cutaneous T-cell lymphoma and large granular lymphocytes leukemia [58, 59]. Moreover, IL-15 is found in skeletal muscles where studies have suggested its involvement in autoimmune myositis progression [60]. IL-15 also plays a crucial role in autoimmune diseases by promoting the effect of cytotoxic $CD8^+$ T cells, including rheumatoid arthritis, inflammatory bowel disease, and multiple sclerosis, and is hence suggested as a therapeutic target [61, 62]. We found IL-15 to be expressed in a degeneration- and age-dependent manner, but with relatively high variability over the entire dataset. However, rather than being coincidental, the observed variability is likely related to the fact that IL-15 expression is significantly lower in lumbar disc samples compared to cervical samples. Importantly, even after matching samples with regard to the IVD degeneration grade and age - to exclude these factors as confounders - the significant difference in IL-15 expression between the cervical and lumbar disc was maintained, indicating that IL-15 is indeed degeneration, age and region dependent. Interestingly, it was hypothesized that mechanical stress (related to obesity and resistance training) may regulate IL-15 expression and activity in skeletal muscle fibers [63, 64]. Therefore, the different stress magnitudes in lumbar and cervical discs may have affected the expression of IL-15 – a mechanism that could be further explored in *in vitro* loading experiments. Similarly, IL-15 was recently shown to have a significant role in the pathogenesis of osteoarthritis, with expression levels being dependent on the disease stage (albeit with different pattern than in the IVD) [65, 66]. While our study only provides descriptive evidence for the potential relevance of IL-15 in degenerative disc disease, mechanistic investigations are required to demonstrate its pathological relevance.

Interferons are a multigene family of inducible cytokines that are divided into two groups, type I IFN (IFN-α, IFN-β, and IFN-ω) and type II (IFN-γ). Although interferons are generally considered to be important modulators of the immune system, no data currently exist with regard to DDD, apart from IFN-γ [13, 67]. We present first data on the occurrence

of members of the IFN-α (IFNA1, IFNA8) and IFN-β (IFNB1) subfamily in diseased discs. Interestingly, IFNB1 (similar to IL-1β and TNF-α) has been suggested to promote autophagy for certain cell types [68] and elevated autophagy has recently been described in degenerated AFs [69]. Our results demonstrate that IFNA1, IFNA8 and IFNB1 are intra-correlated and are expressed at significantly higher levels than TNF-α, yet at levels similar to IL-6 and IL-8. Moreover, we were able to show a negative correlation of IFNA1 with the degree of disc degeneration as well as enhanced expression in lumbar samples compared with cervical samples. However, when cervical and lumbar samples were matched with regard to the IVD degeneration grade and age, no significant difference in IFNA1 expression (with $p = 0.056$) was found. This may be due to the reduction in sample size and hence lowered statistical power. On the other hand, it could also indicate that IFNA1 expression is influenced primarily by degeneration and age, and only secondarily by spinal region and its associated factors, such as altered biomechanical loading situations and differences in diffusion distances and hence cellular nutrition. Although our study has provided first indications of a significant role of investigated members of the interferon type I family in disc diseases, further studies are warranted to determine their therapeutic potential [70].

In summary, this study demonstrated clear differences in mRNA expression for several of the analyzed parameters and genes. Future studies could focus on either confirming results on the protein level or on analyzing (on a larger set of NP and AF samples) whether the investigated genes are affected by increasing age, degeneration and/or Modic grade when using a zone-specific evaluation approach. Furthermore, investigating the pro-inflammatory crosstalk between endplates, vertebral bone marrow and the IVD could provide further insight into the role of Modic changes in disc inflammaging and the development of low back pain. Clear technical limitations that weaken the implications of this study are the lack of (1) healthy (non-degenerated) disc tissue, of (2) patient-specific pain scores as well as of (3) collection of data on additional environmental factors, such as smoking or body weight, which were previously shown to be of importance in the degenerative processes of the disc.

4.5 Conclusion

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

Ethical approval:

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

Funding:

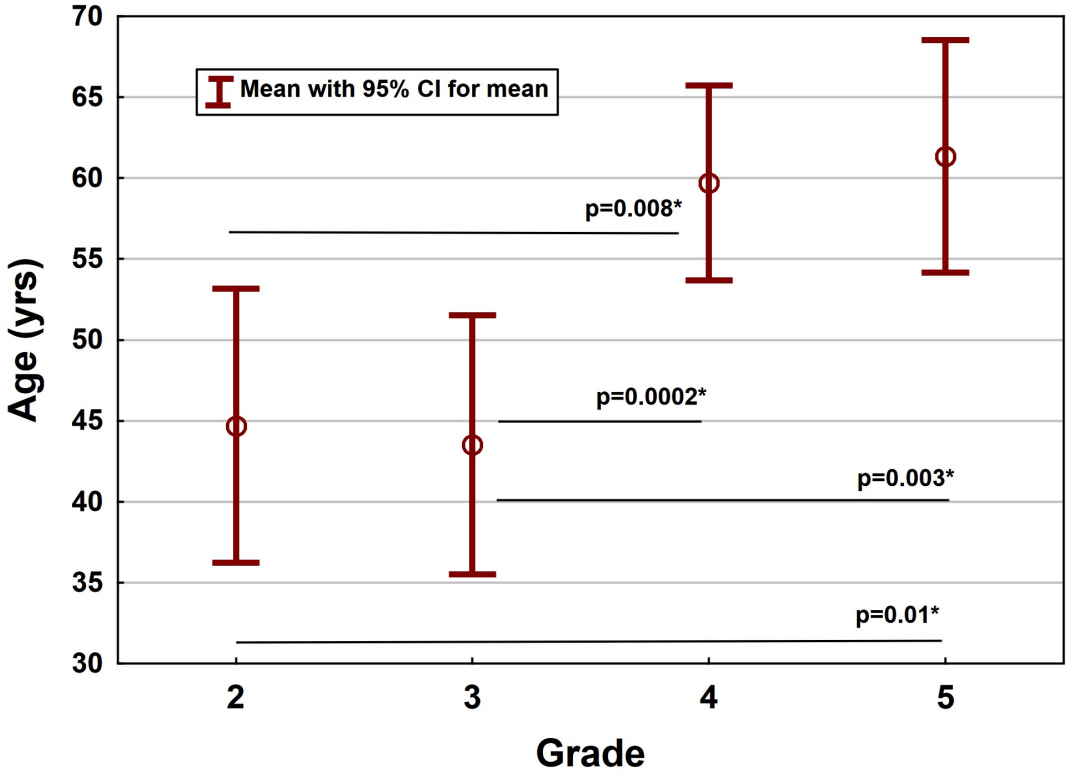
The study was financially supported by the Swiss Neuro Foundation (Bern/Switzerland), the Swiss National Science Foundation (SNF PP00P2_163678/1) as well as the Spine Society of Europe (Eurospine 2016_4).

Conflict of interest:

All authors declare that they have no conflict of interest.

Supplementary Material

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



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

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

*Results significant at $p<0.05$

References

1. Urban, J.P. and S. Roberts, *Degeneration of the intervertebral disc.* Arthritis Res Ther, 2003. **5**(3): p. 120-30.
2. Modic, M.T. and J.S. Ross, *Lumbar degenerative disk disease.* Radiology, 2007. **245**(1): p. 43-61.
3. Franceschi, C. and M. Bonafe, *Centenarians as a model for healthy aging.* Biochemical Society Transactions, 2003. **31**: p. 457-461.
4. Choi, Y.S., *Pathophysiology of degenerative disc disease.* Asian Spine J, 2009. **3**(1): p. 39-44.
5. Wang, J., et al., *TNF-alpha and IL-1beta promote a disintegrin-like and metalloprotease with thrombospondin type I motif-5-mediated aggrecan degradation through syndecan-4 in intervertebral disc.* J Biol Chem, 2011. **286**(46): p. 39738-49.
6. Melrose, J., et al., *Increased nerve and blood vessel ingrowth associated with proteoglycan depletion in an ovine anular lesion model of experimental disc degeneration.* Spine (Phila Pa 1976), 2002. **27**(12): p. 1278-85.
7. Freemont, A.J., et al., *Nerve growth factor expression and innervation of the painful intervertebral disc.* J Pathol, 2002. **197**(3): p. 286-92.
8. Cheung, K.M., et al., *Prevalence and pattern of lumbar magnetic resonance imaging changes in a population study of one thousand forty-three individuals.* Spine (Phila Pa 1976), 2009. **34**(9): p. 934-40.
9. Walker, B.F., *The prevalence of low back pain: a systematic review of the literature from 1966 to 1998.* J Spinal Disord, 2000. **13**(3): p. 205-17.
10. Crockett, M.T., et al., *Modic Type 1 Vertebral Endplate Changes: Injury, Inflammation, or Infection?* American Journal of Roentgenology, 2017. **209**(1): p. 167-170.
11. Dudli, S., D. Haschtmann, and S.J. Ferguson, *Fracture of the vertebral endplates, but not equienergetic impact load, promotes disc degeneration in vitro.* Journal of Orthopaedic Research, 2012. **30**(5): p. 809-816.
12. Kanna, R.M., et al., *Prevalence, Patterns, and Genetic Association Analysis of Modic Vertebral Endplate Changes.* Asian Spine Journal, 2017. **11**(4): p. 594-600.
13. Risbud, M.V. and I.M. Shapiro, *Role of cytokines in intervertebral disc degeneration: pain and disc content.* Nat Rev Rheumatol, 2014. **10**(1): p. 44-56.
14. Gruber, H.E., et al., *Interleukin 1-beta upregulates brain-derived neurotrophic factor, neurotrophin 3 and neuropilin 2 gene expression and NGF production in annulus cells.* Biotech Histochem, 2012. **87**(8): p. 506-11.
15. Shen, C., et al., *Autophagy in rat annulus fibrosus cells: evidence and possible implications.* Arthritis Res Ther, 2011. **13**(4): p. R132.

16. Broeckx, S., et al., *Regenerative therapies for equine degenerative joint disease: a preliminary study.* PLoS One, 2014. **9**(1): p. e85917.
17. Navone, S.E., et al., *Inflammatory mediators and signalling pathways controlling intervertebral disc degeneration.* Histol Histopathol, 2017. **32**(6): p. 523-542.
18. Wuertz, K. and L. Haglund, *Inflammatory mediators in intervertebral disk degeneration and discogenic pain.* Global Spine J, 2013. **3**(3): p. 175-84.
19. Johnson, Z.I., et al., *Disc in flames: Roles of TNF-alpha and IL-1beta in intervertebral disc degeneration.* Eur Cell Mater, 2015. **30**: p. 104-16; discussion 116-7.
20. Krupkova, O., J. Zvick, and K. Wuertz-Kozak, *The role of transient receptor potential channels in joint diseases.* Eur Cell Mater, 2017. **in review**.
21. Schumacher, M.A., *Transient receptor potential channels in pain and inflammation: therapeutic opportunities.* Pain Pract, 2010. **10**(3): p. 185-200.
22. Zhang, Y.J., et al., *Intervertebral Disc Cells Produce Interleukins Found in Patients with Back Pain.* American Journal of Physical Medicine & Rehabilitation, 2016. **95**(6): p. 407-415.
23. Phan, M.N., et al., *Functional characterization of TRPV4 as an osmotically sensitive ion channel in porcine articular chondrocytes.* Arthritis Rheum, 2009. **60**(10): p. 3028-37.
24. Walter, B.A., et al., *Reduced tissue osmolarity increases TRPV4 expression and pro-inflammatory cytokines in intervertebral disc cells.* Eur Cell Mater, 2016. **32**: p. 123-36.
25. Zhou, Y.Q., et al., *Interleukin-6: an emerging regulator of pathological pain.* Journal of Neuroinflammation, 2016. **13**.
26. Qazi, B.S., K. Tang, and A. Qazi, *Recent advances in underlying pathologies provide insight into interleukin-8 expression-mediated inflammation and angiogenesis.* Int J Inflam, 2011. **2011**: p. 908468.
27. Poveda, L., et al., *Peroxynitrite Induces Gene Expression in Intervertebral Disc Cells.* Spine, 2009. **34**(11): p. 1127-1133.
28. Brisby, H., et al., *Proinflammatory cytokines in cerebrospinal fluid and serum in patients with disc herniation and sciatica.* European Spine Journal, 2002. **11**(1): p. 62-66.
29. Pedersen, L.M., et al., *Serum levels of the pro-inflammatory interleukins 6 (IL-6) and -8 (IL-8) in patients with lumbar radicular pain due to disc herniation: A 12-month prospective study.* Brain, Behavior, and Immunity, 2015. **46**(Supplement C): p. 132-136.

30. Lee, S., et al., *Comparison of growth factor and cytokine expression in patients with degenerated disc disease and herniated nucleus pulposus.* Clin Biochem, 2009. **42**(15): p. 1504-11.
31. Shamji, M.F., et al., *Proinflammatory Cytokine Expression Profile in Degenerated and Herniated Human Intervertebral Disc Tissues.* Arthritis and Rheumatism, 2010. **62**(7): p. 1974-1982.
32. Hirano, T., et al., *Complementary-DNA for a Novel Human Interleukin (Bsf-2) That Induces Lymphocytes-B to Produce Immunoglobulin.* Nature, 1986. **324**(6092): p. 73-76.
33. Scheller, J., C. Garbers, and S. Rose-John, *Interleukin-6: From basic biology to selective blockade of pro-inflammatory activities.* Seminars in Immunology, 2014. **26**(1): p. 2-12.
34. Dubovy, P., et al., *Bilateral elevation of interleukin-6 protein and mRNA in both lumbar and cervical dorsal root ganglia following unilateral chronic compression injury of the sciatic nerve.* Journal of Neuroinflammation, 2013. **10**.
35. Arruda, J.L., et al., *Intrathecal anti-IL-6 antibody and IgG attenuates peripheral nerve injury-induced mechanical allodynia in the rat: possible immune modulation in neuropathic pain.* Brain Research, 2000. **879**(1-2): p. 216-225.
36. Weber, K.T., et al., *Serum levels of the proinflammatory cytokine interleukin-6 vary based on diagnoses in individuals with lumbar intervertebral disc diseases.* Arthritis Research & Therapy, 2016. **18**: p. 3.
37. Ohtori, S., et al., *Efficacy of epidural administration of anti-interleukin-6 receptor antibody onto spinal nerve for treatment of sciatica.* European Spine Journal, 2012. **21**(10): p. 2079-2084.
38. Sherwood, J., et al., *TRPC6 plays a role in CXCR2-mediated chondrocyte phenotypic stability.* Osteoarthritis and Cartilage. **23**: p. A152-A153.
39. Franco-Obregon A, K.H., Greutert H, Wernas T, Egli M, Cambria E, Sekiguchi M, Boos N, Hausmann O, Ferguson S, Wuertz-Kozak K *The balance of Transient Receptor Potential Channel TRPC6 to TRPC1 determines ageing and mechanotransduction in intervertebral disc cells.* 2017. European cells & materials 34. Suppl. 1: 38.
40. Kurahara, L.H., et al., *Significant contribution of TRPC6 channel-mediated Ca2+ influx to the pathogenesis of Crohn's disease fibrotic stenosis.* J Smooth Muscle Res, 2016. **52**(0): p. 78-92.
41. Tauseef, M., et al., *TLR4 activation of TRPC6-dependent calcium signaling mediates endotoxin-induced lung vascular permeability and inflammation.* J Exp Med, 2012. **209**(11): p. 1953-68.

42. Seguin, C.A., et al., *Tumor necrosis factor alpha modulates matrix production and catabolism in nucleus pulposus tissue.* Spine, 2005. **30**(17): p. 1940-1948.
43. Purmessur, D., et al., *A role for TNF alpha in intervertebral disc degeneration: A non-recoverable catabolic shift.* Biochemical and Biophysical Research Communications, 2013. **433**(1): p. 151-156.
44. Kang, R., et al., *Intervertebral disc degenerative changes after intradiscal injection of TNF-alpha in a porcine model.* European Spine Journal, 2015. **24**(9): p. 2010-2016.
45. Bachmeier, B.E., et al., *Analysis of tissue distribution of TNF-alpha, TNF-alpha-receptors, and the activating TNF-alpha-converting enzyme suggests activation of the TNF-alpha system in the aging intervertebral disc.* Ann N Y Acad Sci, 2007. **1096**: p. 44-54.
46. Sutovsky, J., et al., *Cytokine and chemokine profile changes in patients with lower segment lumbar degenerative spondylolisthesis.* Int J Surg, 2017. **43**: p. 163-170.
47. Wang, C., et al., *Tumor necrosis factor-alpha: a key contributor to intervertebral disc degeneration.* Acta Biochim Biophys Sin (Shanghai), 2017. **49**(1): p. 1-13.
48. Le Maitre, C.L., J.A. Hoyland, and A.J. Freemont, *Catabolic cytokine expression in degenerate and herniated human intervertebral discs: IL-1beta and TNFalpha expression profile.* Arthritis Res Ther, 2007. **9**(4): p. R77.
49. Leung, L. and C.M. Cahill, *TNF-α and neuropathic pain - a review.* Journal of Neuroinflammation, 2010. **7**: p. 27-27.
50. Murata, Y., et al., *Selective inhibition of tumor necrosis factor-alpha prevents nucleus pulposus-induced histologic changes in the dorsal root ganglion.* Spine, 2004. **29**(22): p. 2477-2484.
51. Le Maitre, C.L., A.J. Freemont, and J.A. Hoyland, *The role of interleukin-1 in the pathogenesis of human intervertebral disc degeneration.* Arthritis Research & Therapy, 2005. **7**(4): p. R732-R745.
52. Millward-Sadler, S.J., et al., *Regulation of catabolic gene expression in normal and degenerate human intervertebral disc cells: implications for the pathogenesis of intervertebral disc degeneration.* Arthritis Research & Therapy, 2009. **11**(3).
53. Gorth, D.J., et al., *IL-1ra delivered from poly(lactic-co-glycolic acid) microspheres attenuates IL-1 beta-mediated degradation of nucleus pulposus in vitro.* Arthritis Research & Therapy, 2012. **14**(4).
54. Wu, C.C., et al., *Regenerative potentials of platelet-rich plasma enhanced by collagen in retrieving pro-inflammatory cytokine-inhibited chondrogenesis.* Biomaterials, 2011. **32**(25): p. 5847-5854.

55. Armitage, R.J., et al., *Il-15 Has Stimulatory Activity for the Induction of B-Cell Proliferation and Differentiation.* Journal of Immunology, 1995. **154**(2): p. 483-490.
56. Kennedy, M.K., et al., *Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice.* Journal of Experimental Medicine, 2000. **191**(5): p. 771-780.
57. Cooper, M.A., et al., *In vivo evidence for a dependence on interleukin 15 for survival of natural killer cells.* Blood, 2002. **100**(10): p. 3633-3638.
58. Tinhofer, I., et al., *Expression of functional interleukin-15 receptor and autocrine production of interleukin-15 as mechanisms of tumor propagation in multiple myeloma.* Blood, 2000. **95**(2): p. 610-618.
59. Chen, J., et al., *Increased serum soluble IL-15R alpha levels in T-cell large granular lymphocyte leukemia.* Blood, 2012. **119**(1): p. 137-143.
60. Huang, P.L., et al., *Skeletal muscle interleukin 15 promotes CD8(+) T-cell function and autoimmune myositis.* Skeletal Muscle, 2015. **5**.
61. Croce, M., et al., *Immunotherapeutic applications of IL-15.* Immunotherapy, 2012. **4**(9): p. 957-969.
62. Mishra, A., L. Sullivan, and M.A. Caligiuri, *Molecular Pathways: Interleukin-15 Signaling in Health and in Cancer.* Clinical Cancer Research, 2014. **20**(8): p. 2044-2050.
63. Nielsen, A.R., et al., *Expression of interleukin-15 in human skeletal muscle – effect of exercise and muscle fibre type composition.* The Journal of Physiology, 2007. **584**(Pt 1): p. 305-312.
64. Quinn, L.S. and B.G. Anderson, *Interleukin-15, IL-15 Receptor-Alpha, and Obesity: Concordance of Laboratory Animal and Human Genetic Studies.* Journal of Obesity, 2011. **2011**: p. 456347.
65. Scanzello, C.R., et al., *Local cytokine profiles in knee osteoarthritis: elevated synovial fluid interleukin-15 differentiates early from end-stage disease.* Osteoarthritis Cartilage, 2009. **17**(8): p. 1040-8.
66. Tao, Y., et al., *Expression and correlation of matrix metalloproteinase-7 and interleukin-15 in human osteoarthritis.* Int J Clin Exp Pathol, 2015. **8**(8): p. 9112-8.
67. Cuellar, J.M., et al., *Cytokine evaluation in individuals with low back pain using discographic lavage.* Spine Journal, 2010. **10**(3): p. 212-218.
68. Ambjorn, M., et al., *IFNB1/interferon-beta-induced autophagy in MCF-7 breast cancer cells counteracts its proapoptotic function.* Autophagy, 2013. **9**(3): p. 287-302.

69. Gruber, H.E., et al., *Autophagy in the Degenerating Human Intervertebral Disc In Vivo Molecular and Morphological Evidence, and Induction of Autophagy in Cultured Annulus Cells Exposed to Proinflammatory Cytokines-Implications for Disc Degeneration.* Spine, 2015. **40**(11): p. 773-782.
70. Kazezian, Z., et al., *Injectable hyaluronic acid down-regulates interferon signaling molecules, IGFBP3 and IFIT3 in the bovine intervertebral disc.* Acta Biomater, 2017. **52**: p. 118-129.

Appendix I

This section contains earlier unpublished data generated during the work presented in chapter 4, which contains the manuscript entitled "*Inflammaging in cervical and lumbar degenerated intervertebral discs: analysis of proinflammatory cytokine and TRP channel expression*".

Expression levels: pain intensity and chronicity

For the background information as well as the materials and methods sections, please consult chapter 4.

Definitions

Pain intensity – the severity of pain sensation as evaluated by the patients.

Duration of symptoms:

- *Acute* is defined as pain and/or other symptoms lasting less than two months.
- *Sub-acute* is defined as pain and/or other symptoms lasting between two to 12 months.
- *Chronic* is defined as pain and/or other symptoms lasting over one year.

Results

The mRNA expression of IL-15 (**Figure A1 A**), IL-1B (**Figure A1 B**), IL-8 (**Figure A1 D**), TNF-α (**Figure A1 E**) and TRPC6 (**Figure A1 F**) tended to decrease with pain intensity. The mRNA expression of IFNA1 was the highest in the intense group, relatively low in the moderate group and almost at the baseline levels in the disabling group (**Figure A1 C**). Similarly to it, the mRNA expression of IL-1β (**Figure A1 B**) and IL-8 (**Figure A1 D**) was as well at baseline levels in the disabling group. In contrast, the mRNA expression of TRPV4 tended to increase with the pain intensity and was the highest in the disabling group. (**Figure A1 G**). Out of all tested targets, only the mRNA expression of IL-15 showed statistically significant differences between the duration of symptoms (between sub-acute and chronic group) and was lowest in the sub-acute group (**Figure A1 H**).

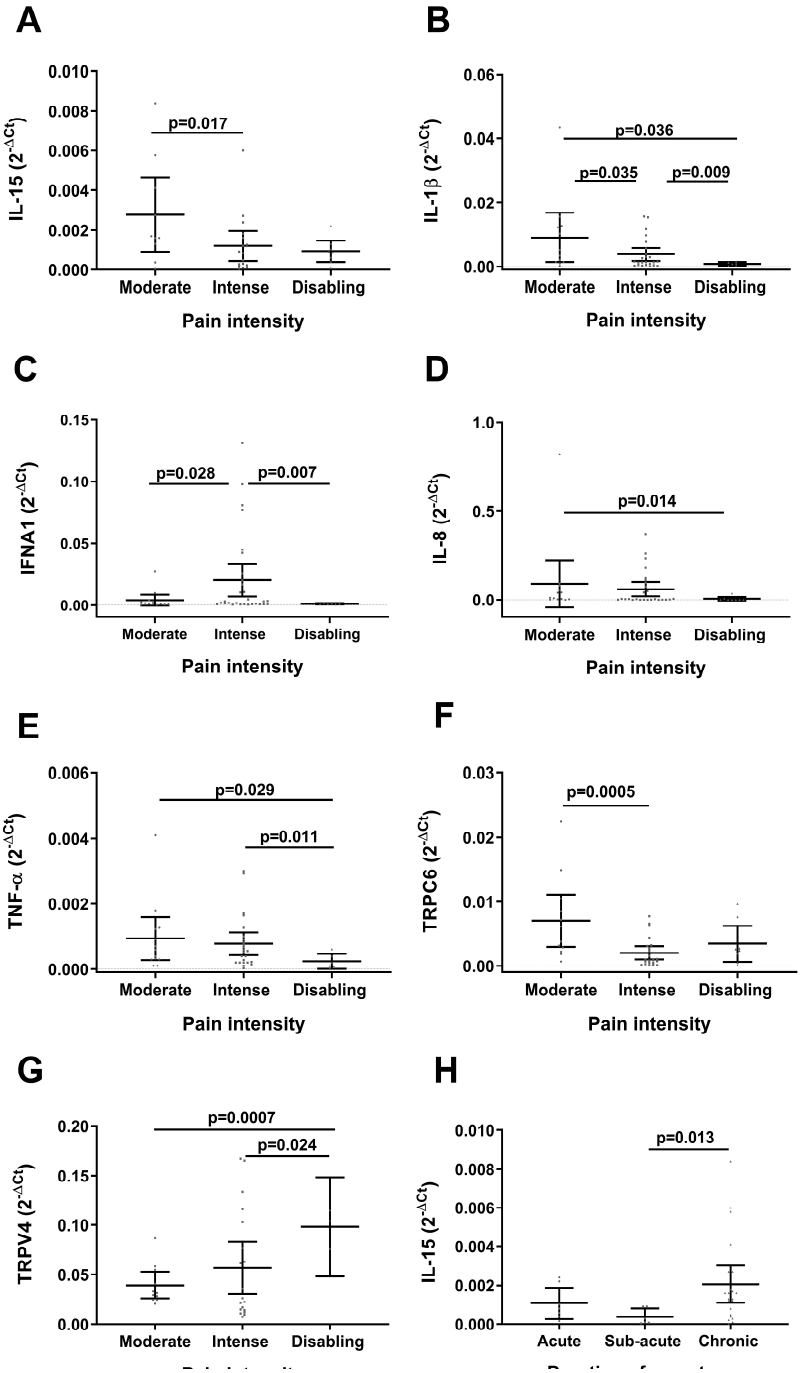


Figure A1. mRNA expression of IL-15 (**A**, n=35/51), IL-1β (**B**, n=40/51), IFNA1 (**C**, n=49/51), IL-8 (**D**, n=47/51), TNF-α (**E**, n=43/51), TRPC6 (**F**, n=42/51) and TRPV4 (**G**, n=33/51) *versus* the pain intensity, as well as, IL-15 (**H**, n=35/51) *versus* the duration of symptoms. The corresponding n-number present the number of samples in which the

target was detected over the total number of tested samples (n=51). Results were calculated as $2^{-\Delta Ct}$ values (relative to GAPDH). Error bars present mean with 95% confidence intervals.

Discussion

IL-15 plays a central role in the development of chronic diseases mediated by the immune system [1]. Our data presented in chapter 4 [2], together with the hereby presented gene expression data, indicate that IL-15 may be a key cytokine in discogenic pain as well as a promising therapeutic target. Elevated protein levels of IL-15 were detected in the serum of patients suffering from long-term (over two years) rheumatoid arthritis [3]. However, in another study, serum levels of IL-15 did not depend on the pain severity in knee osteoarthritis (OA) patients, although IL-15 was significantly increased in OA patients (n = 226) as compared to healthy controls (n = 106) [4]. IL-1β, IFNA1, IL-8 and TNF-α are pro-inflammatory molecules involved in the development of painful disc degeneration and were discussed in chapter 4. Elevated levels of IL-1β were detected in the NP tissue of painful disc herniation patients (DH), but no correlation was found between IL-1β levels and pain intensity before or post-surgery [5]. In contrast, our data suggest that IL-1β is expressed at higher levels in lower pain intensity groups. Increased levels of IL-8 measured in cerebrospinal fluid (CSF) were shown to be associated with the increasing pain intensity in patients suffering from disc herniation (DH) [6]. In contrary, here we presented that IL-8 gene expression was the highest in the patients who described their pain as moderate and therefore presenting a reverse trend to the one from the earlier study [6]. Differences between the reports may originate from the use of different samples (degenerated IVD tissue vs. CSF);furthermore, hereby presented data were not controlled for the pathophysiology (degenerative disc disease patients and DH patients were combined together). TNF-α is believed to be the key pain mediator in discogenic pain and the inhibition of TNF-α was demonstrated to aid pain sensation in low back pain patients [7, 8]. Increased serum levels of TNF-α were associated with chronic low back pain [9]. Our data suggest that TNF-α gene expression increases with pain intensity. Interestingly, a positive correlation between pain intensity and protein expression of the TNF receptor - TNFR1 was found in the NP tissue of DH patients at 12 months post-surgery [10]. Furthermore, in the same study the protein expression of TNFR2 was negatively correlated with the pain score in the AF tissue [10], hence possibly indicating zonal differences. TRPC6 showed a similar expression trend to our later data [11] (presented in the chapter 5). However, in the later study, we could

also demonstrate that the TRPC6 expression was increasing with the duration of symptoms [11] – a finding that could not be replicated here. Moreover, here we could present that the mRNA expression of TRPV4 steadily increases with the pain intensity and this finding could not be confirmed in our later study, which showed a generally stable expression of TRPV4, and which additionally did not depend on any other patient/tissue specific factor [11]. Such differences may be due to the donor variations and different sample sizes (total and within the assembled groups). To conclude, these data support the hypothesis that IL-15 and TRPC6 are promising therapeutic targets, however future studies will need to determine the role of TRPV4 in disc degeneration. Donor to donor variation, a lack of healthy controld and unequally balanced sample numbers in each group were a clear limitation in this study.

References

1. Di Sabatino, A., et al., *Role of IL-15 in immune-mediated and infectious diseases.* Cytokine Growth Factor Rev, 2011. **22**(1): p. 19-33.
2. Sadowska, A., et al., *Inflammaging in cervical and lumbar degenerated intervertebral discs: analysis of proinflammatory cytokine and TRP channel expression.* Eur Spine J, 2017. **in press**.
3. Gonzalez-Alvaro, I., et al., *Increased serum levels of interleukin-15 in rheumatoid arthritis with long- term disease.* Clin Exp Rheumatol, 2003. **21**(5): p. 639-42.
4. Sun, J.M., et al., *Serum interleukin-15 levels are associated with severity of pain in patients with knee osteoarthritis.* Dis Markers, 2013. **35**(3): p. 203-6.
5. Andrade, P., et al., *Elevated IL-1beta and IL-6 levels in lumbar herniated discs in patients with sciatic pain.* Eur Spine J, 2013. **22**(4): p. 714-20.
6. Palada, V., et al., *Characterization of neuroinflammation and periphery-to-CNS inflammatory cross-talk in patients with disc herniation and degenerative disc disease.* Brain Behav Immun, 2019. **75**: p. 60-71.
7. Johnson, Z.I., et al., *Disc in flames: Roles of TNF-alpha and IL-1beta in intervertebral disc degeneration.* Eur Cell Mater, 2015. **30**: p. 104-16; discussion 116-7.
8. Tobinick, E.L. and S. Britschgi-Davoodifar, *Perispinal TNF-alpha inhibition for discogenic pain.* Swiss Med Wkly, 2003. **133**(11-12): p. 170-7.
9. Zu, B., et al., *Serum Levels of the Inflammatory Cytokines in Patients with Lumbar Radicular Pain Due to Disc Herniation.* Asian Spine J, 2016. **10**(5): p. 843-849.
10. Andrade, P., et al., *Tumor necrosis factor-alpha levels correlate with postoperative pain severity in lumbar disc hernia patients: opposite clinical effects between tumor necrosis factor receptor 1 and 2.* Pain, 2011. **152**(11): p. 2645-52.
11. Sadowska, A., et al., *Differential regulation of TRP channel gene and protein expression by intervertebral disc degeneration and back pain.* Sci Rep, 2019. **9**(1): p. 18889.

Chapter 5

5 Differential regulation of TRP channel gene and protein expression by intervertebral disc degeneration and back pain

Differential regulation of TRP channel gene and protein expression by intervertebral disc degeneration and back pain

A. Sadowska[1], W. Hitzl[2,3,4], A.Karol[5], P. Jaszczuk[6], H. Cherif[7,8,9], L. Haglund[7,8,9,]
O. N. Hausmann[6#], K. Wuertz-Kozak[#1,10,11,12]

[1]Institute for Biomechanics, D-HEST, ETH Zurich, Zurich, Switzerland
[2]Research Office - Biostatistics, Paracelsus Medical University, Salzburg, Austria
[3]Department of Ophthalmology and Optometry, Paracelsus Medical University, Salzburg, Austria
[4]Research Program Experimental Ophthalmology and Glaucoma Research, Paracelsus Medical University Salzburg, Austria
[5]Musculoskeletal Research Unit (MSRU), Department of Molecular Mechanisms of Disease (DMMD), Vetsuisse Faculty, University of Zurich, Zurich, Switzerland
[6]Klinik St. Anna-Hirslanden, Neuro- and Spine Center, Lucerne, Switzerland
[7]Orthopaedic Research Laboratory, Montreal, Canada
[8]McGill Scoliosis and Spine Research Group, Montreal, Canada
[9]The Shriners Hospital for Children, Montreal, Canada
[10]Academic Teaching Hospital and Spine Research Institute, Paracelsus Medical University, Salzburg, Austria
[11]Spine Center, Schön Clinic Munich Harlaching, Munich, Germany
[12]Department of Health Sciences, University of Potsdam, Potsdam, Germany

[#] These authors contributed equally.

Published as:
Sadowska, A., *et al.* Differential regulation of TRP channel gene and protein expression by intervertebral disc degeneration and back pain. Sci Rep 9, 18889 (2019). https://doi.org/10.1038/s41598-019-55212-9

Abstract:
Intervertebral disc (IVD) degeneration and consequent low back pain (LBP) are common and costly pathological processes that require improved treatment strategies. Transient Receptor Potential (TRP) channels constitute a family of multimodal ion channels that have recently emerged as contributors to disc pathologies and were thus proposed as potential therapeutic targets, although limited data on their presence and function in the IVD exist. The purpose of this study was to determine the mRNA and protein expression of TRP channels in non-degenerated and degenerated human IVD tissue (with different pain intensity and chronicity) using gene array, conventional qPCR and immunohistochemistry. We could demonstrate that 26 out of 28 currently known TRP channels are expressed in the IVD on the mRNA level, thereby revealing novel therapeutic candidates from the TRPC, TRPM and TRPML subfamilies. TRPC6, TRPM2 and TRPML1 displayed enhanced gene and protein expression in degenerated IVDs as compared to non-degenerated IVDs. Additionally, the gene expression of TRPC6 and TRPML1 was influenced by the IVD degeneration grade. Pain intensity and/or chronicity influenced the gene and/or protein expression of TRPC6, TRPM2 and TRML1. Interestingly, decreased gene expression of TRPM2 was observed in patients treated with steroids. This study supports the importance of TRP channels in IVD homeostasis and pathology and their possible application as pharmacological targets for the treatment of IVD degeneration and LBP. However, the exact function and activation of the highlighted TRP channels will have to be determined in future studies.

5.1 Introduction

The intervertebral disc (IVD) is a mechanosensitive tissue that lies between adjacent vertebrae in the spinal column. The mechanical properties of the IVD are greatly defined by its biochemical composition, with the highly hydrated nucleus pulposus (NP) in the center, surrounded by the annulus fibrous (AF) [1]. The primary function of the IVD is to transmit loads arising from muscle activity and body weight, with hydrostatic pressure/compression and osmotic stresses predominating in the NP and tensile/shear stresses in the AF [1]. With its low cellularity (5000 cells/mm^3 in the NP [2]), avascular structure (with a consequent lack of nutrients and oxygen), high daily mechanical loads and an inability to repair itself, the IVD is prone to early degeneration. Degeneration is associated with a loss in extracellular matrix (ECM) components, specifically proteoglycans,

a consequent loss of tissue hydration as well as tissue weakening, including clefts and tears [3]. These changes not only influence the mechanical properties of the IVD, but also lead to high stress zones and hence altered IVD mechanobiology.

In a subgroup of those affected by IVD degeneration, inflammatory processes take place within the IVD tissue. Inflammation has been described as a major contributor to the development of painful disc degeneration (also known as degenerative disc disease (DDD)), hence presumably distinguishing symptomatic from asymptomatic IVD degeneration [4]. On the molecular level, DDD can be characterized by an up-regulation of pro-inflammatory cytokines such as interleukin (IL)-6, IL-1B and tumor necrosis factor (TNF)-α [3]. Moreover, mechanical loading can also induce inflammation, depending on its type, applied magnitude, duration and frequency [3, 5, 6]. Similarly, altered IVDs osmolarity can contribute to the tissue inflammation by modulating pro-inflammatory mediators and pathways (e.g. mitogen-activated protein (MAP) kinases, T-cells 5/tonicity response element-binding protein (NFAT5/TonEBP)) [7, 8] and molecules (e.g. IL-6, IL-1B) [9].

Although inflammation, as well as, mechanical and osmotic stress have been identified as important factors in the development of painful disc degeneration and hence back pain, the exact pathobiological mechanism remain to this day unknown. However, a superfamily of multimodal ion channels, the so-called transient receptor potential (TRP) channels, have recently emerged as potential contributors to disc pathologies [10]. TRP channels are of utmost interest in IVD research as they are regulated by a diverse range of stimuli, including mechanical and osmotic stress, and furthermore modulate inflammatory responses and mediate a variety of sensations, including pain. Stimulation of a TRP channel will cause its activation (i.e. opens channels pore), leading to ion movement and resulting in elevated cytosolic intracellular calcium. Applied stimuli (e.g. shear stress) can change a channel's molecular distribution, hence altering its membrane trafficking and spatial/temporal distribution, which in turn can influence its activity threshold levels [11, 12]. Very recent studies highlighted TRPC6 and TRPV4 as possible contributors to IVD health and disease [9, 13-15], but numerous other members of the various TRP families (ankyrin TRPA, canonical TRPC, vanilloid TRPV, melastatin TRPM, mucolipin TRPML and polycystic TRPP) have not yet been investigated.

To gain a better insight into the role of TRP channels in the IVD and low back pain, the purpose of this study was to identify the presence of all currently known TRP channels in

non-degenerated and degenerated human IVDs with varying pain intensity and chronicity, and to highlight their possible involvement in IVD pathologies.

5.2 Results

TRP channel mRNA expression: Gene array

In the initial step, a wide screening of all 28 TRP channels was investigated in 8 IVD samples (4 degenerated: 2x NP and 2x AF, assessed with Pfirrmann classification [16]; and 4 non-degenerated: 2x NP and 2x AF, assessed with Thompson classification [17]) using a gene array. Out of 28 tested TRP channels, two targets (TRPC5 and TRPM5), were not detectable in any of the samples included in the gene array. For patient information, see **Tables 1** and **2**.

In the non-degenerated IVDs, TRPC1, TRPC2, TRPM7, TRPML1-3, PKD1, TRPP1 (PKD2), TRPV1, TRPV3 and TRPV4 were detected in both, NP and AF samples, whereas other TRP channels were only detectable in either region: TRPC6 (AF), TRPM4 (NP), TRPM6 (NP), and TRPV5 (NP). Furthermore, TRPA1, TRPC3, TRPC4, TRPC7, TRPM1-M3, TRPM8, TRPP2 (PKD2L1), TRPP3 (PKD2L2), TRPV2 and TRPV6 were undetectable in the selected non-degenerated IVD samples.

In the degenerated IVDs, the highest gene expression, detected in both NP and AF, was measured for TRPC1, TRPM7, PKD1, TRPP1 (PKD2) and TRPV4. TRPC7 was detected only in the AF (2 out of 2 degenerated AF samples) and TRPM1 only in the NP (2 out of 2 degenerated NP samples). TRPP3 (PKD2L2) could be detected only in one out of two AF sample.

Fig. 1 presents overall (combined NP and AF) gene expression as measured on the gene array normalized to YWHAZ (measured Ct values for all tested TRP targets in the gene array screening are presented in the **Supplementary Material Table S1**).

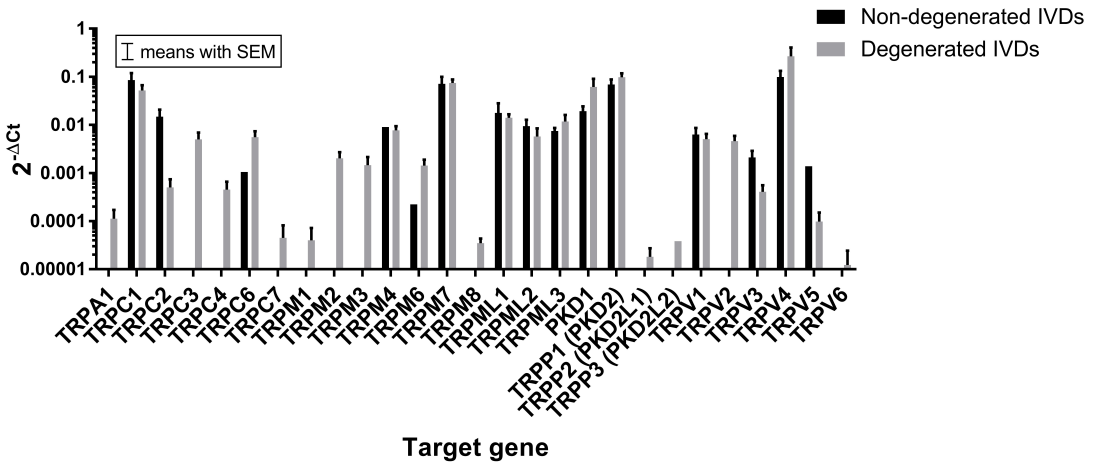


Fig. 1 The overall TRP channels gene expression as detected during the array screening in the non-degenerated (n=4, black bars) and degenerated (n=4, grey bars) human IVD tissue. Data are presented on a logarithmic scale as $2^{-\Delta Ct}$ values (relative to YWHAZ).

TRP channel expression (mRNA): Disease state and degeneration grade

Based on the gene array results, 12 targets (TRPC1, TRPC3, TRPC4, TRPC6, TRPM2, TRPM7, TRPML1, TRPML2, PKD1, TRPP1 (PKD2), TRPV1, TRPV4) were chosen for further analysis on the additional 26 IVD samples (8 non-degenerated IVDs: 4x NP and 4x AF; 18 degenerated: 8x AF, 8x NP, 2x mix tissue) using conventional qPCR (see **Table 3**). When combining data from all 34 samples, TRPV4 was the most highly expressed target in both non-degenerated and degenerated IVDs, followed by TRPC1, TRPP2, PKD1, and TRPM7 (**Fig. 2**). None of the investigated targets differed between the NP and AF zones ($p>0.05$, data not shown).

When comparing the expression of the TRP channels in the non-degenerated and degenerated IVDs, statistically significant differences were found in the mRNA expression levels of TRPC6 (**Fig. 3a**, p=0.001), TRPM2 (**Fig. 3b**, p=0.0002) and TRPML1 (**Fig. 3c**, p=0.03). In all three targets, mRNA levels were higher in the degenerated IVD samples than in the non-degenerated samples.

Within the degenerated IVDs (n=22), the expression of several TRP channels was significantly affected by the degeneration grade: TRPC4 (**Fig. 4a**, grade 3 > 4, p=0.03), TRPC6 (**Fig. 4b**, 5 >2 p=0.03, 5 > 3, p = 0.03, 5 > 4 p=0.01), TRPM7 (**Fig. 4c**, 5 > 3, p=0.04) and TRPML1 (**Fig. 4d**, 2 > 4, p=0.01).

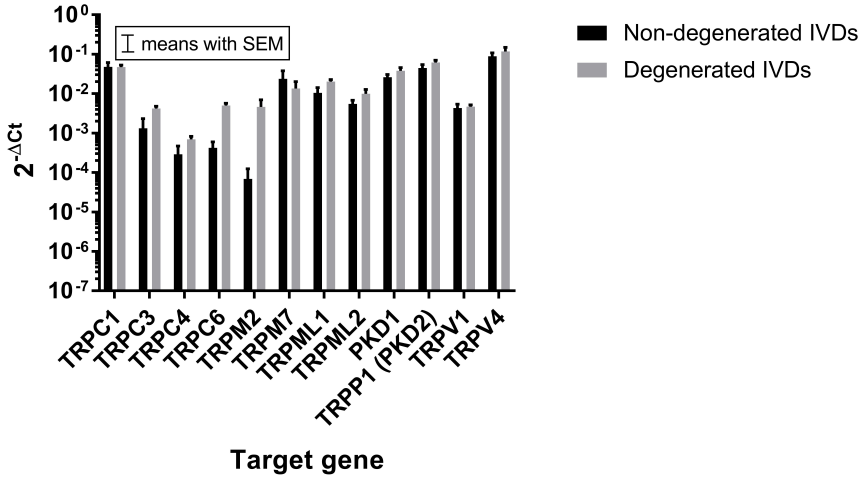


Fig. 2 The overall gene expression of the selected TRP channels in the non-degenerated (n=12, black bars) and degenerated (n=22, grey bars) IVD tissue. Data presented on a logarithmic scale as $2^{-\Delta Ct}$ values (relative to YWHAZ).

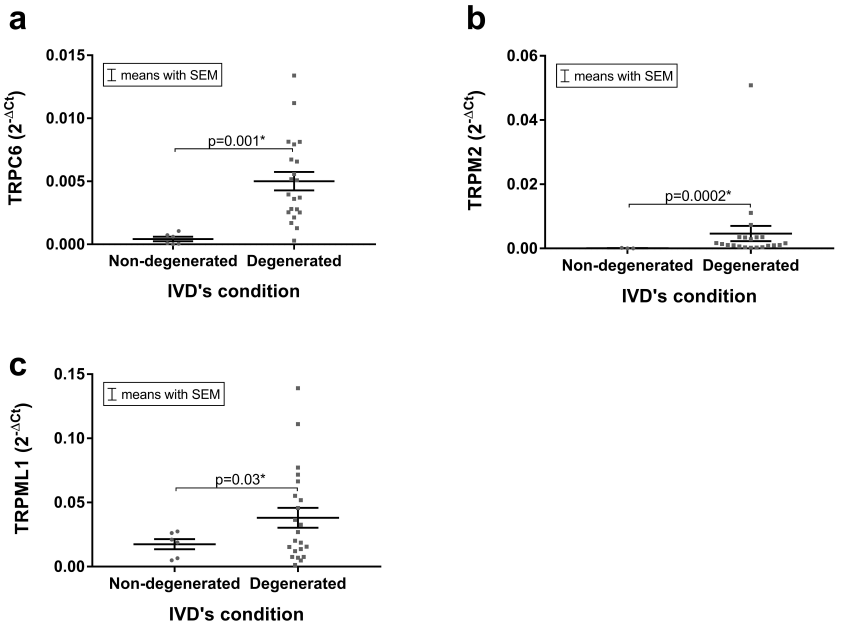


Fig. 3 The mRNA expression of **a)** TRPC6, **b)** TRPM2 and **c)** TRPML1 differed significantly between non-degenerated and degenerated human IVDs. Data presented as $2^{-\Delta Ct}$ values (relative to YWHAZ). Asterisks indicates a statistically significant difference between the groups indicated (*$p < 0.05$).

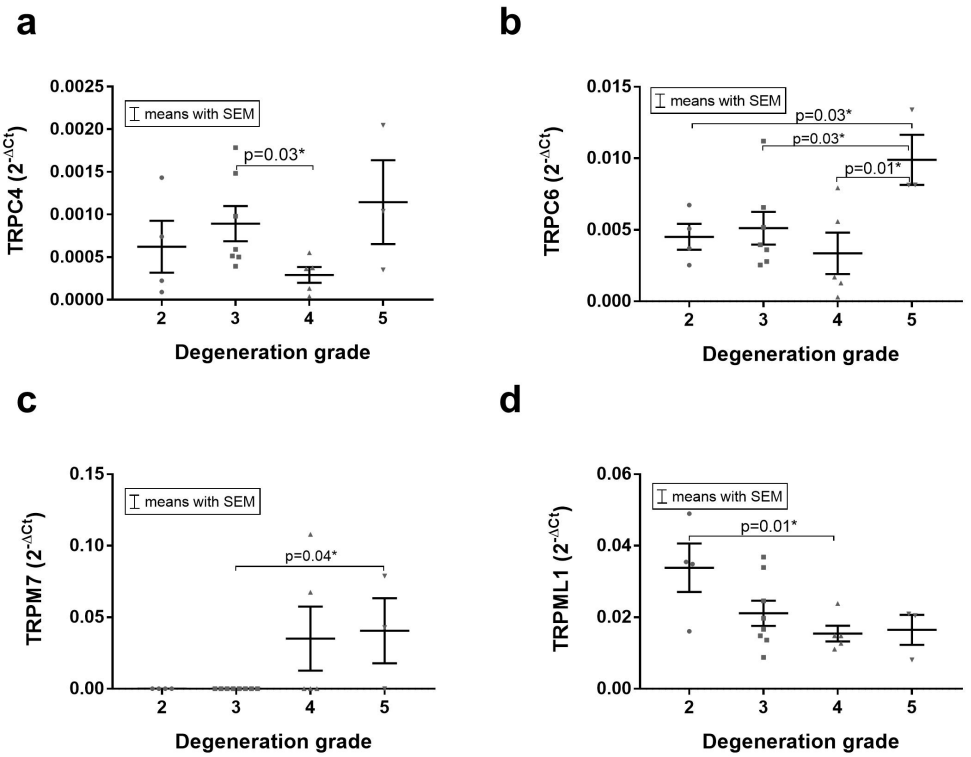


Fig. 4 The mRNA expression of **a)** TRPC4, **b)** TRPC6, **c)** TRPM7 and **d)** TRPML1 differed significantly between different degeneration grades. Data presented as $2^{-\Delta Ct}$ values (relative to YWHAZ). Asterisks indicates a statistically significant difference between the groups indicated (*$p < 0.05$).

TRP channel expression (mRNA): Modic changes

Modic changes represent a classification of pathological changes to the endplates, where 0 indicates no pathological changes, I is characterized by a vertebral bone edema, and II by fatty replacement of the bone marrow[18]. Modic changes may be associated with the pain development[19]. We hence tested whether the presence of Modic changes affects TRP channel expression and found a significant effect for TRPC4 (**Fig. 5a**, I > II, p=0.01) and TRPC6 (**Fig. 5b**, 0 > I p=0.02, 0> II p=0.007).

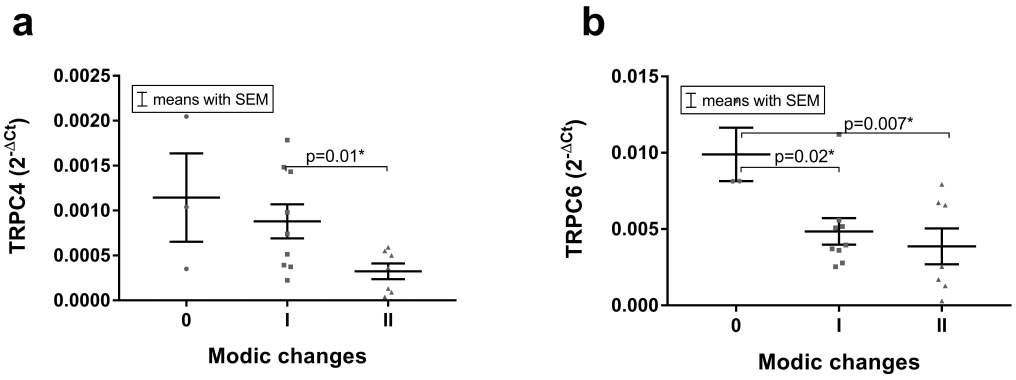


Fig. 5 The mRNA expression levels of **a)** TRPC4 (I > II, p=0.01) and **b)** TRPC6 (0 > I p=0.02, and > II p=0.007) were found to be affected by Modic changes (0 n=3, I n=10 and II n=7). Data presented as $2^{-\Delta Ct}$ values (relative to YWHAZ). Asterisks indicates a statistically significant difference between the groups indicated (*p < 0.05).

TRP channel expression (mRNA): Pain intensity, pain duration and steroid use

Degenerated IVD samples obtained from patients who assessed their pain as moderate were characterized by significantly higher TRPC6 expression levels compared to those obtained from patients with intense pain (p=0.007, **Fig. 6a**) or disabling pain (p=0.03, **Fig. 6a**). Interestingly, TRPC6 expression tended to increase with the duration of pain symptoms, but no statistical significance was found between the groups (p=0.058, **Fig. 6d**). Expression of TRPM2 and TRPML1 was found to be the highest in the IVDs obtained from patients who defined their pain as intense and was found to be statistically different from the IVDs obtained from patients with disabling (TRPM2 p=0.04 **Fig. 6b**, TRPML1 p=0.04 **Fig. 6c**). In addition, TRPM2 mRNA expression was significantly decreased in IVD samples obtained from patients who received steroid treatment (p=0.04, **Fig. 7**).

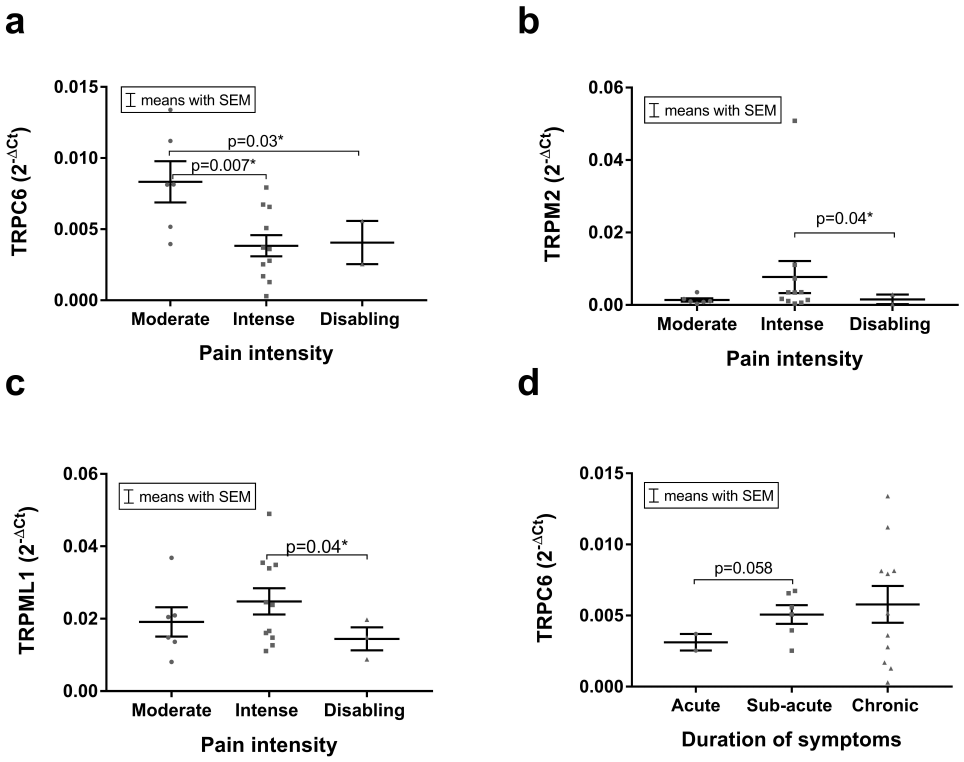


Fig. 6 The mRNA expression of **a)** TRPC6 was significantly higher in the moderate pain group compared to the intense (p=0.007) and disabling (p=0.03) pain groups, whereas the mRNA expression of **b)** TRPM2 and **c)** TRPML1 was statistically different between the intense and disabling pain groups (p=0.04). Although no statistical differences were found between the duration of symptoms (acute: >2 months, sub-acute: 2-12 months, and chronic: > 1 year), **d)** the TRPC6 mRNA expression tended to increase with the duration of pain symptoms. Data presented as $2^{-\Delta Ct}$ values (relative to YWHAZ). Asterisks indicates a statistically significant difference between the groups indicated (*$p < 0.05$).

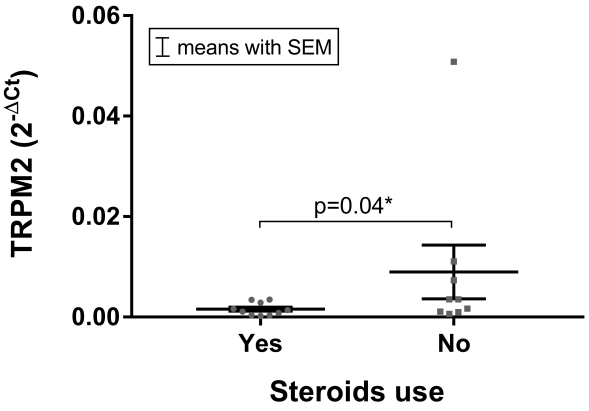


Fig. 7 The mRNA expression of TRPM2 was significantly lower ($p = 0.04$) in patients who received a steroid treatment. Data presented as $2^{-\Delta Ct}$ values (relative to YWHAZ). Asterisks indicates a statistically significant difference between the groups indicated ($*p < 0.05$).

TRP channels protein expression: Immunohistochemistry

In the next step, TRPC6, TRPM2 and TRPML1 expression changes observed during degeneration progression on the mRNA level (**Fig. 2**, **3a-c**, **4b**, **4d**, **5b**, **6a-d** and **7**) were investigated on the protein level by immunohistochemistry. A total of seven surgical symptomatic degenerated IVD tissue samples, two non-degenerated young IVD samples and one aged asymptomatic (non-painful) IVD sample with mild multifocal degenerative changes were examined per target. Details regarding patient information and a complete evaluation, as well as the applied scoring system can be found in **Tables 4**, **5**, **6** and **7**, respectively.

TRPM2 protein signal intensity was weak or absent in young non-degenerated IVD samples (**Fig. 8a**) and it was increased in asymptomatic (**Fig. 8b**) and symptomatic (**Fig. 8c-d**) degenerated IVD samples, where it reached the highest average combined score (6.71 ±1.11) out of the three investigated targets. Overall, in the symptomatic degenerated IVD samples, more positively stained cells were detected as compared to the asymptomatic aged and young IVD samples (4.71 ±0.49 vs. 2.0 ±1.73). Taken together, these findings reflect degeneration-driven expression pattern observed earlier on the gene level (**Fig. 2** and **Fig. 3b**).

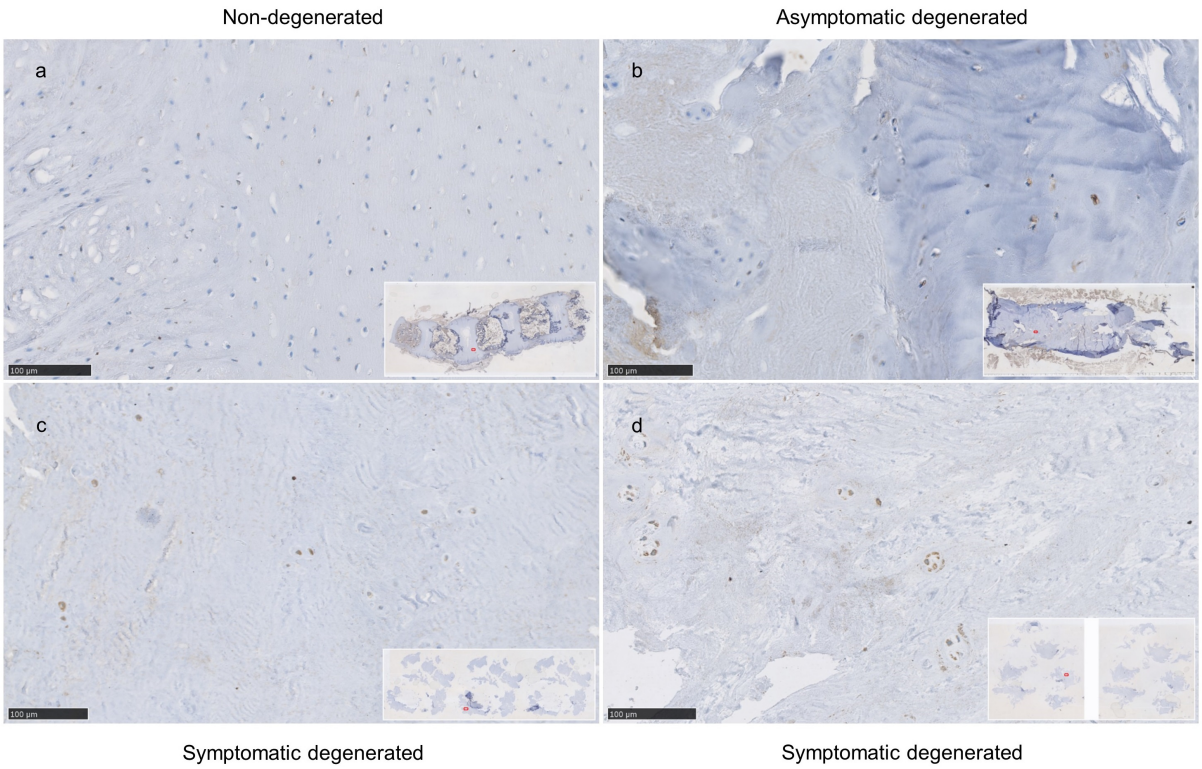


Fig. 8 Immunohistochemical staining against TRPM2 in **a**) non-degenerated IVD section obtained from a young donor (n.49) and **b**) asymptomatic (non-painful) IVD section with mild multifocal degenerative changes (n.48) showing weak staining positivity as compared to **c-d**) symptomatic degenerated IVD sections obtained from patients undergoing a low back surgery (**c**, n.37, Pfirrmann degeneration grade=3) and (**d**, n.46, degeneration grade =4). Scale bar is 100 µm.

TRPC6 protein expression levels were overall comparable to TRPM2, thus reflecting our gene expression data (**Fig. 2**). While no expression was observed in non-degenerated young samples (**Fig. 9a**), the observed staining intensity was similarly high in symptomatic degenerated IVD samples , showing a moderate signal positivity in up to 20% of the cells, and the asymptomatic aged sample (**Fig. 9b-d**), presenting moderate to strong positivity in most of the cells. In the examined symptomatic degenerated IVD tissue samples, TRPC6 was highly expressed in 6 out of 7 samples (6.00 ±2.38), with an expression pattern comparable to the TRPC6 gene expression results on degeneration grade and Modic score (**Fig. 3a** and **Fig. 5b**). One sample was considered to be negative for the TRCP6 protein expression with the overall score value 2, which was a result of lower samples quality with insufficient amount of preserved tissue structure.

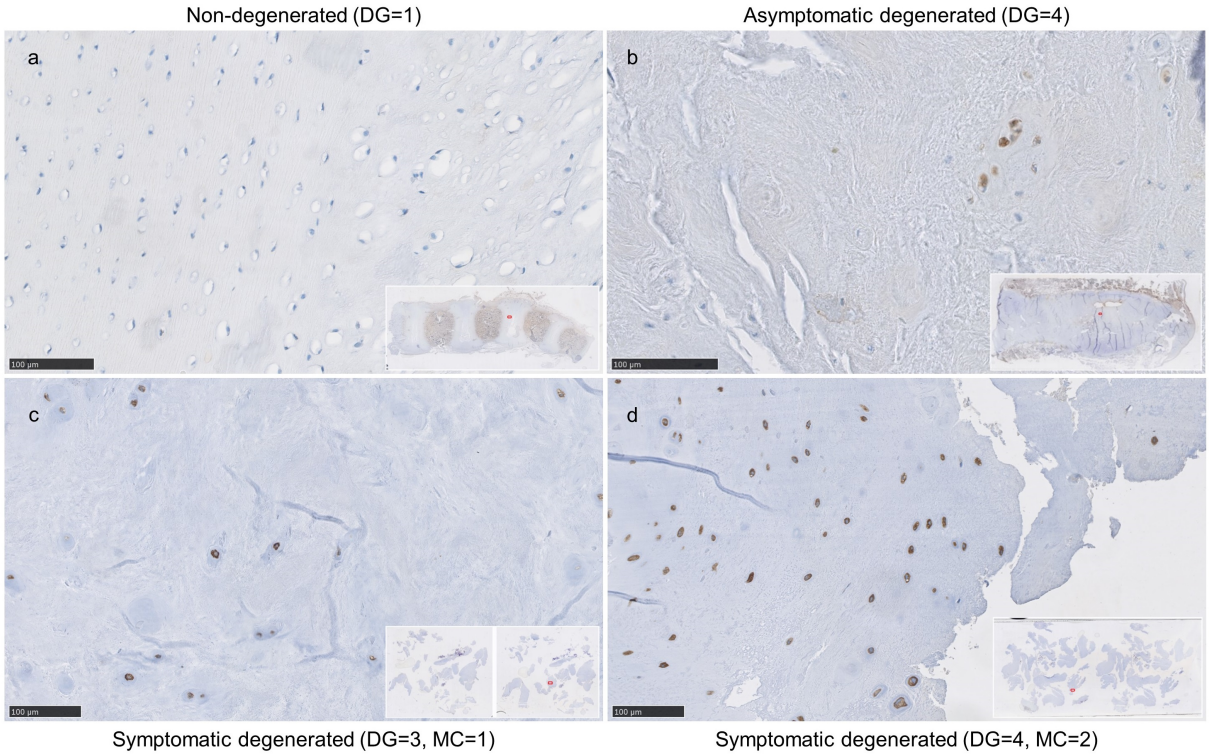


Fig. 9 Immunohistochemical staining against TRPC6 in **a**) non-degenerated IVD section obtained from a young donor (n.49) presenting a TRPC6 protein expression. IVD sections illustrating increased expression with age and degeneration: b) asymptomatic (non-painful) IVD section with mild multifocal degenerative changes (n.48), **c-d**) symptomatic degenerated IVD sections obtained from patients undergoing low back surgery (**c**, n.40, Pfirrmann degeneration grade (DG) = 3, Modic changes (MC) = 1) and (**d**, n.45, Pfirrmann degeneration grade (DG) = 4, Modic changes (MC) = 2). Cadaveric samples were assessed with Thompson classification and degenerated biopsies with Pfirrmann classification. Scale bar is 100 µm.

TRPML1 protein expression was weak to absent in asymptomatic and non-degenerated IVD samples (**Fig. 10e-f**). In symptomatic degenerated IVD samples TRPML1 protein expression was observed in 6 out of 7 cases (**Fig. 10a-d**), with an average final score of 4.57 ±1.72, and was characterized by weak to moderate signal positivity and mostly in areas, where clusters of cells were present. One sample was negative for TRPML1 presenting only single cells with a faint signal (score 2). In general, the TRPML1 signal intensity was weaker compared to TRPC6 and TRPM2, and the positivity was limited to a lower number of cells. In contrast, on the gene level TRPML1 expression was slightly higher than TRPC6

and TRPM2 expression (**Fig. 2**). Within the symptomatic degenerated IVD samples, TRPML1 tended to be expressed overall higher in samples collected from patients experiencing intense pain (**Fig. 10a-b**) in comparison to disabling pain (**Fig. 10c-d**), and asymptomatic and non-degenerated samples (**Fig. 10e-f**), hence illustrating a similar pattern as observed on the gene level (**Fig. 6c**).

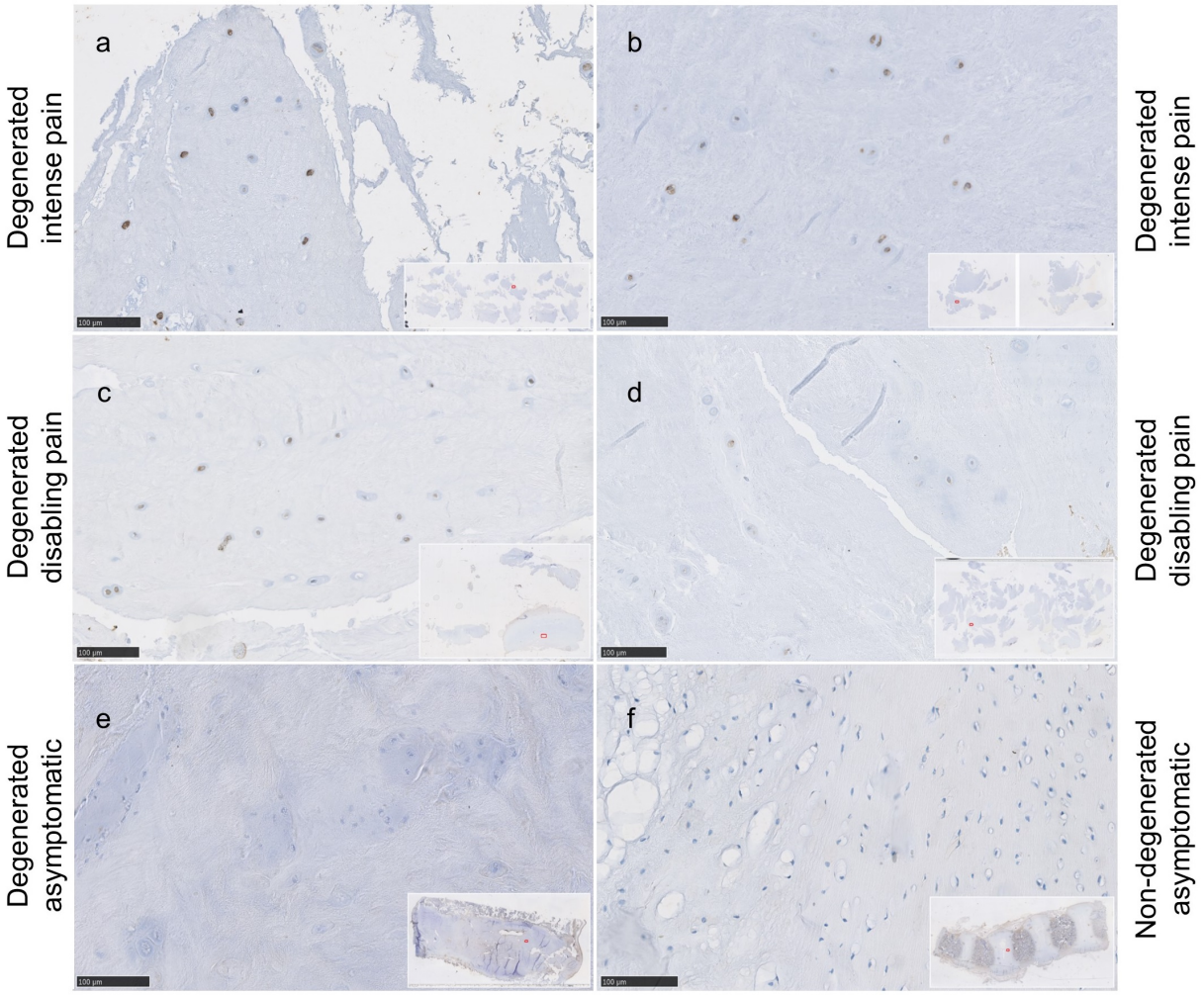


Fig. 10 Immunohistochemical staining against TRPML1 in symptomatic degenerated IVD sections with high TRPML1 staining intensity in **a-b)** obtained from patients suffering from intense low back pain (n.37 and n.39 respectively) as compared to sections with moderate staining intensity **c-d)** obtained from patients suffering from disabling low back pain (n.36 and n.38 respectively); and sections negative for TRPML1: **e)** asymptomatic (non-painful) IVD section with mild multifocal degenerative changes (n.48) and **f)** non-degenerated IVD (n.49). Scale bar is 100 µm.

5.3 Discussion

Degeneration of the intervertebral disc is one of the leading causes of low back pain, which not only lessens the patients' quality of life, but also constitutes a high financial burden on the society. With the aging population, the occurrence, as well as costs of IVD degeneration and LBP are predicted to grow. Hence, it is crucial to gain a better insight into the molecular processes of IVD degeneration along with a potential to develop new and targeted treatment strategies. Recently, transient resistant potential (TRP) channels, which constitute a family of ion channels that were shown to play an important role in various diseases [20], have emerged as potential targets [10], but their exact function in the IVD remains unknown. This study is the first one to report a complete screening of TRP channels in non-degenerated and degenerated IVDs. Our results confirm the important role of the TRPC subfamily in the IVD, but also highlight previously unexplored targets stemming from e.g. the TRPM or TRPML subfamily.

According to our results and in line with previously reported findings [13], the highest observed expressed TRP channel overall in the IVD is TRPV4. However, no significant differences were found for any of the analyzed patient/tissue factors for TRPV4. Similarly, TRPV1 was well expressed in both non-degenerated and degenerated IVD tissue, but no significant differences were found when evaluated against patient- or tissue-related factors. TRPV1 is responsive to pro-inflammatory agents (e.g. TNF-α), which are known to be higher expressed in painful IVD tissue. Therefore, the inflammatory environment associated with DDD likely increases the probability for channel opening [21]. Other members of the TRPV channel family, but TRPV1, where not detected or were expressed at the detection limit during the initial screening and were hence excluded from the further analysis in this study. The TRP vanilloid (V) subfamily constitutes of six members (V1-6) in humans, with a main role in thermo-sensation. Additionally, TRPV4 was shown to also play a role in mechano- and osmo-sensation in the IVD and cartilage [22] and TRPV1 may be gated by low pH ($\leq$ 5.9) [20]. Upon channel activation (via e.g. mechanical or osmotic stress), TRPV4 causes transient increases of intracellular calcium, which acts as a secondary messenger that activates NFAT5/TonEBP, MAP kinases and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathways [23], which are involved in cell hemostasis as well as inflammation [24]. It was demonstrated that TRPV4 expression increases with decreasing IVD osmolarity as observed during IVD degeneration [9] and may correlate with pro-

inflammatory molecules such as TNF-α [9] and interferons (IFN) A1 and B1[13] in the degenerated IVD. Interestingly, TRPV1 was shown to function as an osmosensor in the brain [25, 26], although no supporting data exists on its function in the IVD. Taken together, since TRPV1 and TRPV4 are expressed in both non-degenerated and degenerated IVD tissue, it may indicate that both channels are important for the IVD cells fundamental functions. However, future studies should confirm their exact function and further analyze the previously reported involvement of TRPV4 in IVD degeneration [9].

In contrast to TRPV4 and TRPV1, the expression of TRPC6 was broadly affected by patient-related characteristics. TRPC6 belongs to the TRP canonical (C) subfamily, together with five other channels (C1-6). The TRPC subfamily may be involved in mechanosensing [14, 22], as well as in pain and inflammation in joint tissues [10]. We were able to show that the mRNA expression of TRPC6 was significantly higher in degenerated IVDs than in non-degenerated IVDs and that its expression tended to increase with the IVD degeneration grade as previously reported [13]. In line with it, on the protein level, TRPC6 expression was consistent in the asymptomatic and symptomatic degenerated IVD samples and it was absent in non-degenerated IVD samples. Moreover, we showed that higher TRPC6 gene expression was associated with specific Modic types, moderate pain and tended to increase with the duration of disease symptoms. Due to limited sample size, we were not able to confirm these findings on the protein level. Interestingly, no zonal (AF, NP) differences were found (data not shown) in contrary to a previous publication [13]. However, this may be due to the fact that a clear separation of the zones within IVD tissue (especially in higher stages of degeneration) is difficult, as well as due to a smaller sample size. TRPC6 was previously shown to correlate with inflammatory markers such as TNF-α, IL-6, IL-8 and IL-15 in the IVD [13] and potentially plays a significant role in modulating immune response, as well as mediate inflammatory processes in other tissue types such as lung, kidney and neurons [27]. These data indicate that TRPC6 could be an interesting pharmaceutical target for pain treatment of patients with high degree of disc degeneration. Besides TRPC6, three other members of the canonical subfamily, TRPC1, TRPC3 and TRPC4, were screened in this study. TRPC1 and TRPC3 were detected at similar levels in non-degenerated and degenerated IVDs and they showed no statistically significant relation to any of investigated factors. Both channels were previously detected on the mRNA, but not on the protein level in human chondrocytes, where elevated TRPC3 mRNA expression

levels, but not TRPC1 were observed with higher passaging [25]. TRPC1 and TRPC3 are also hypothesized to have mechanosensitive function, but their exact role in the IVD remains thus far unknown[10]. For the last investigated canonical member, TRPC4, we could show differences in its expression with the degree of IVD degeneration and the type of Modic change. Although previously unexplored in the IVD, TRPC4 is known to be expressed in human chondrocytes [25] as well as in the central nervous system (CNS), smooth muscle cells, kidney and endothelium [22]. In the CNS, it contributes to the axonal regeneration after the dorsal root ganglion injury [28] and hence may be involved in the regulation of IVD innervation.

The TRPM2 and TRPM7 channels belong to the TRP melastatin (M) subfamily that consists of eight members (M1-8) in humans and is most known for its function in thermo-sensation [22]. Importantly, TRPM7 was also detected in human chondrocytes, where it showed the overall strongest mRNA expression out of all tested target [25]. Similarly as in the human chondrocytes [25], TRPM7 was overall consistently expressed in non-degenerated and degenerated human IVDs, albeit with higher expression in IVDs with higher degeneration grades. Although no other data on TRPM7 in the IVD exist, it was shown that TRPM7 may act as a mechano-sensor and mediate osmolarity-induced cell volume changes in the kidney and salivary glands [29, 30]. A non-degenerated IVD tissue is characterized by diurnal osmolarity changes (~400-500 mOsm) while reduced tissue osmolarity (~300 mOsm) is a hallmark of IVD degeneration [31]. Since an osmotic balance is necessary for an IVD to maintain its function, it could be hypothesized that TRPM7 may be involved in osmo-sensing and osmo-adaptation in the IVD.

TRPM2 is hypothesized to play an important role in pain development by acting as an oxidative stress and reactive oxygen species (ROS) sensor in immune cells [32] and regulator of pro-inflammatory cytokine release in the CNS [33]. In this study, TRPM2 was expressed at higher mRNA levels in degenerated IVDs compared to non-degenerated IVDs. Additionally, in the non-degenerated IVD samples, TRPM2 was detected only in the AF tissue, whereas in the degenerated IVDs, TRPM2 was detected both in the AF and NP tissue. These data imply that TRPM2 exists in a native human AF tissue, however its elevated levels, as well as its occurrence in both AF and NP tissue may be due to the nerve and blood vessels ingrowth, which are commonly observed during IVD degeneration [34], as well as to a possible contamination during tissue separation. On the protein level, TRPM2

was consistently well expressed in symptomatic and asymptomatic degenerated IVD samples and to the lesser extend or not at all in the non-degenerated samples, hence pointing towards degeneration-driven TRPM2 expression. Interestingly, we could show that TRPM2 mRNA expression was strongly linked to pain. We could also demonstrate that the TRPM2 mRNA expression was significantly decreased in patients, who underwent steroid treatment and was higher in patients, who described their pain as intense. However, it should be noted that the majority of patients who described their pain as disabling were also the ones who received steroid treatment. These data indicate a pivotal role of TRPM2 in pain mediation in the IVD and suggest that its modulation may create new opportunities for therapeutic strategies of the low back pain treatment.

The TRP mucolipin (ML) subfamily consists of three, thus far poorly characterized, members (ML1-3) in humans. Recent evidence suggest that TRPML channels may be involved in the lysosomal function and regulation of autophagy [22, 35], which can be also present in the IVD. Increased TRPML1 activity was observed during stress conditions and can be triggered by catabolic signaling [36]. Shen *et al.* [37] showed serum deprivation and inflammation induced autophagy of rat AF cells. In contrast, compression and ROS induced generation of autophagy in rat NP cells [38] reduced the catabolic effects of IL-1B and TNF-α [39]. However, whether and how TRPML channels are involved in autophagy in the IVD is unknown. Here, we could show for the first time that the TRPML subfamily is present in the IVD, with TRPML1 being significantly higher expressed on the gene level in the degenerated IVDs, in an inverse, grade-depended manner. On the protein level, TRPML1 was detected in symptomatic degenerated IVDs, but not in non-degenerated and asymptomatic (non-painful) IVD samples. Additionally, we could show that the expression of TRPML1 on gene and protein level was higher in patients who described their pain as intense.

The TRP polycystin (P) subfamily compromises three channel members (TRPP1 known as PKD2 (previously TRPP2), TRPP2 known as PKD2L1 (previously TRPP3) and TRPP3 known as PKD2L2 (previously TRPP5) [40]. Additional information on the TRPP channel subfamily nomenclature is provided in the Supplementary Material. Importantly, TRPP1 (PKD2) does not produce cation currents until assembled with PKD1 [41]. Although we were able to detect PKD1 and TRPP1 (PKD2) in non-degenerated and degenerated specimens at relatively high levels (yet unaffected by patient- or tissue-related

characteristics), the lack of general information on their function hinders interpretation of their relevance in the IVD. It was proposed that TRPP1 (PKD2) might either be a mechanosensitive channel or regulate mechanosensitive channels by interacting with the TRPV4 in the cilium [40, 42] and rat endothelial cells [43].

Although TRP channels constitute potential pharmaceutical targets for the treatment of disc-related back pain and IVD degeneration, their modulation has to be approached with caution as several TRP channels are also present in non-degenerated IVDs and therefore are likely to be of relevance not only in tissue pathophysiology, but also homeostasis. Therefore, the channel activity in non-degenerated and degenerated IVDs should also be considered and determined in order to design adequate treatment strategies such as pharmacological inhibition of TRP channels. In recent years, several potent and selective TRP channels agonists and antagonists have been identified (e.g. GSK1016790A activates and GSK2193874 blocks TRPV4, Waixenicin A inhibits TRPM7 etc.), however many compounds commonly used in TRP channel research also activate/inhibit a broad range of other channels (e.g. SKF96365 can inhibit TRPC subfamily, as well as TRPV4) [44-46]. Future developments of specific modulators of those TRP channels potentially involved in back pain may constitute a viable alternative to intradiscal steroid injections (with high side effects) or spinal surgery (with high complication rates). Furthermore, future studies should determine whether enhanced mRNA and protein expression in degenerated samples (e.g. for TRPC6, TRPM2 and TRPML1) is a protective mechanism or de facto contributes to the progression of IVD degeneration. Clear technical limitations of this study include a small sample size, especially of non-degenerated samples that are rare to obtain. Likewise, a bigger sample size could allow for a more comprehensive screening of selected targets on the protein level.

This study is the first to present a complete screening of all currently known TRP channels in non-degenerated and degenerated IVD tissue. In summary, our results indicate that TRP channels may play an important role in IVD degeneration, as well as in low back pain. We were able to confirm the presence and relevance of previously described TRP channels (e.g. TPC6) in degenerated IVDs, but also reveal novel TRP candidates originating e.g. from the TRPM, TRPML and TRPP subfamilies. These findings may be of importance in designing future anti-pain treatment strategies, as well as for the better understanding of the molecular mechanisms underlying disc degeneration and degenerative disc disease.

5.4 Materials and methods

Sample Collection

A total of 34 IVD samples were obtained from 27 individuals (mean age = 49.7 [age range 17-80 years]):

- Twenty individuals underwent elective spinal surgery due to IVD degeneration/herniation (= 22 degenerated IVD samples). Informed consent for sample collection was obtained from each patient and the study was approved through the local ethics committee (Ethics Committee of the Canton Lucerne/Switzerland, #1007).
- Seven individuals were organ donors without any signs of IVD degeneration and no history of back pain (= 12 non-degenerated IVD samples). Informed consent for sample collection was obtained from family members and the study was approved through the local ethics committee (A04-M53-08B). Spines were harvested within 3h of the aortic clamping.

Following macroscopic tissue evaluation, each sample was characterized as NP (degenerated n=10, non-degeneratedd n=7) and/or AF (degenerated n=10, non-degeneratedd n=5) whenever possible. Samples in which no distinction was possible were termed mix (degenerated n=2). Assessment of the disease state was performed using Pfirrmann grading (IVD degeneration) and Modic grading (endplate changes) for the degenerated samples, as well as Thompson grading for the organ donor samples. Detailed patient information is given in **Table 1** and **2**. All methods were performed in accordance with institutional guidelines and regulations.

Table 1. Patient information.

Sample No.	Condition	Spine	Level	Region	Age	Sex	Pathology#	Degeneration grade*	Modic type
1	0	Lumbar	L1/L2	NP	51	m		2	n/a
2	0	Lumbar	L1/L2	AF	51	m		2	n/a
3	0	Lumbar	L2/L3	AF	53	f		2	n/a
4	0	Lumbar	L2/L4	NP	53	f		2	n/a
5	0	Lumbar	L3/L4	NP	27	m		2	n/a
6	0	Lumbar	L3/L4	AF	27	m		2	n/a
7	0	Thoracic and lumbar	L1/2-L2/3-L3/4	NP	34	m		2	n/a
8	0	Thoracic and lumbar	L1/2-L2/3-L3/4	AF	34	m		2	n/a
9	0	Lumbar	L1/2	NP	55	f		2	n/a
10	0	Lumbar	L1/2	AF	55	f		2	n/a
11	0	Lumbar	L1-L5, all four	NP	52	m		2	n/a
12	0	Thoracic and lumbar	T12-S1, all six	NP	17	m		2	n/a
13	1	Lumbar	L5/S1	NP	70	m	DH	4	1
14	1	Lumbar	L3/4	AF	80	m	DDD	4	2
15	1	Lumbar	L5/S1	AF	62	f	DDD	5	0
16	1	Lumbar	L5/S1	NP	59	f	DDD	5	0
17	1	Lumbar	L4/5	AF	66	f	DH	2	2
18	1	Lumbar	L5/S1	AF	33	m	DH	2	1
19	1	Lumbar	L5/S1	AF	39	m	DH	3	1
20	1	Cervical	C5/6	AF	47	f	DDD	3	1
21	1	Lumbar	L4/5 L5/S1	AF	31	m	DDD	4	2
22	1	Lumbar	L5/S1	AF	59	f	DDD	5	0
23	1	Lumbar	L5/S1	AF	46	f	DH	3	1
24	1	Lumbar	L5/S1	NP	54	f	DH	2	1
25	1	Lumbar	L5/S1	NP	46	f	DH	3	1
26	1	Lumbar	L4/5	NP	36	m	DH	3	2
27	1	Lumbar	L5/S1	NP	53	m	DH	3	1
28	1	Cervical	C6/7	NP	58	m	DDD	2	1
29	1	Lumbar	L4/5	NP	74	m	DH	4	2
30	1	Lumbar	L4/5	mix	55	m	DDD	3	2
31	1	Lumbar	L5/S1	AF	55	f	DDD	4	2
32	1	Lumbar	L5/S1	NP	50	f	DH	3	2
33	1	Cervical	C6/7	mix	78	f	DDD	4	2
34	1	Lumbar	L5/S1	NP	33	m	DDD	3	1

1 = degenerated; 0 = non-degenerated; NP = nucleus pulposus; AF = annulus fibrosus; m = male; f = female; DH = disc herniation; DDD = degenerative disc disease; n/a = data not available; # = not applicable to the healthy donors one to 12; * cadaveric samples were assessed with Thompson classification and degenerated biopsies with Pfirrmann classification

Table 2. Patient information.

Sample No.	BMI	Steroids#	Smoking#	Pain score#	Duration of symptoms (grouped) #	Cause of death*
1	24.39					n/a
2	24.39					n/a
3	20.49					Anoxia Carbon Monoxide
4	20.49					Anoxia Carbon Monoxide
5	n/a					Trauma
6	n/a					Trauma
7	22.72					Stroke
8	22.72					Stroke
9	n/a					Anoxia
10	n/a					Anoxia
11	n/a					Stroke
12	24.39					Brain death
13	24	1	0	3	2	
14	26	0	1	2	3	
15	25	0	0	1	3	
16	25	0	1	1	3	
17	25	1	0	2	2	
18	28	1	1	2	2	
19	27	0	0	2	3	
20	24	0	0	3	2	
21	26	1	1	2	3	
22	25	0	1	1	3	
23	18	1	1	1	3	
24	24	0	0	2	1	
25	18	1	1	1	3	
26	40	0	1	2	2	
27	27	1	1	1	2	
28	26	0	1	2	2	
29	23	0	0	2	3	
30	27	1	1	3	3	
31	23	1	0	2	3	
32	20	1	1	3	1	
33	21	1	0	3	1	
34	23	1	0	2	3	

Steroids or smoking: 0 = no, 1 = yes; Pain score: 1 = moderate pain, 2 = intense pain, 3 = disabling pain; Pain duration: 1: 1-2 months, 2: 2-12 months, 3: >1 year; n/a = data not available; * = applicable only to the healthy donors one to 12; # = not applicable to cadaveric samples one to 12

RNA extraction from tissue

IVD tissue samples were saved in RNAlater (Thermo Fisher, Switzerland) and transported to the laboratory. Upon processing, RNAlater was aspirated and tissue samples were shock frozen in liquid nitrogen, and pulverized using custom-made grinders. The obtained tissue powder was transferred into TRIzol (1 ml per 200 mg tissue, Thermo Scientific, Switzerland) and the sample was further homogenized with a polytron three times for 30 seconds (POLYTRON® PT 10/35 GT), with cooling on ice in between. Next, samples were incubated for 5 min at RT, vortexed and centrifuged (4°C, 12000g, 15 min) to remove tissue debris. Supernatants were supplemented with chloroform (1 part chloroform to 5 parts sample), vortexed and incubated for 5 min at RT. Next, samples were centrifuged (4°C, 12000g, 10 min), the aqueous phase was mixed with 70% ethanol (1:1 ratio) and RNA was purified by the RNeasy Mini Kit (Qiagen, Switzerland), according to the manufacturer's recommendation. The quality and quantity of RNA was quantified using a Nanodrop (Thermo Fisher, Switzerland), specifically controlling the 260/280 and 260/230 ratio.

RNA extraction from isolated IVD cells

Five out of 22 degenerated IVD samples with sufficient size were not only used for direct RNA isolation as described above, but also for IVD cell isolation in order to test whether enzymatic digestion affects the TRP channel expression profile (see Supplementary Material).

cDNA synthesis and pre-amplification

Extracted RNA was reverse transcribed into a cDNA in a total volume of 60 µL, using the reverse transcription kit (Thermo Fisher, Switzerland). Subsequently, a preamplification step with the TaqMan PreAmp Master Mix (2✕) (Thermo Fisher, Switzerland) and Custom TaqMan PreAmp Pools (Thermo Fisher, Switzerland) was conducted according to the manufacturer's protocol. Briefly, 100 ng of cDNA in 5 µL total volume was mixed with 5 µL of Custom TaqMan PreAmp Pool and 10 µL of TaqMan PreAmp Master Mix (2✕), followed by a pre-amplification through 14 cycles at 60°C/90°C.

Gene expression analysis: Array

In order to identify the most prominent TRP channel candidates in the IVD, an initial set of eight IVD samples was used for the gene expression screening with TaqMan Array Fast Plates (Thermo Fisher, Switzerland). The screening sample set included four degenerated IVD samples (1x AF Pfirrmann Grade 4, 1x NP Pfirrmann Grade 4, 1x AF Pfirrmann Grade 5, 1x NP Pfirrmann Grade 5) and four non-degenerated IVD samples (2x AF, 2x NP).

The array was conducted according to the protocol provided by the manufacturer. Briefly, 180 µL of amplified cDNA (mixed with RNAse-free water) was combined with 180 µL of the TaqMan Fast Universal PCR Master Mix (2X) (Thermo Fisher, Switzerland) and added to a 96-well plate (10 µL per well), pre-coated with selected TaqMan primers, and gene expression was measured by the real-time qPCR (CFX96 Touch™ Detection System, Biorad). Each array constituted of 32 targets out of which 28 included human TRP channel targets: TRPA1, TRPC1-7, TRPM1-8, TRPML1-3, TRPP1-3, TRPV1-6, and PKD1, as well as three internal controls: 18s, YWHAZ and GUSB. Additionally, to identify the most stable reference gene, six additional internal controls (ACTB, GAPDH, RPL4, RPL13A, SDHA and TBP, see Supplementary Material) were tested on the IVD screening sample set and analyzed with the geNorm algorithm [47].

Gene expression analysis: standard qPCR

The remaining samples underwent standard qPCR (CFX96 Touch™ Detection System, Biorad, Hercules, CA, USA), measuring all selected TRP channels as well as YWHAZ in duplicates. To ensure comparability with the array data, identical TaqMan primers (**Table 3)** were used.

The qPCR and the array data were combined and the obtained Ct values were analyzed by comparative method (gene of interest relative to YWHAZ) and displayed as $2^{-\Delta Ct}$ values. To determine whether TRP channels gene expression was affected by enzymatic cell isolation, expression in cells was compared to its respective tissue sample and results are displayed as $2^{-\Delta\Delta Ct}$ (see Supplementary Material).

Table 3. TaqMan primers used for the qPCR analysis.

Gene	Gene class	Primer number
YWHAZ	Internal control	Hs01122445_g1
TRPA1	Transient receptor potential channel subfamily A member 1	Hs00175798_m1
TRPC1	Transient receptor potential channel subfamily C member 1	Hs00608195_m1
TRPC2	Transient receptor potential channel subfamily C member 2	Hs03453915_g1
TRPC3	Transient receptor potential channel subfamily C member 3	Hs00162985_m1
TRPC4	Transient receptor potential channel subfamily C member 4	Hs01077392_m1
TRPC5	Transient receptor potential channel subfamily C member 5	Hs00202960_m1
TRPC6	Transient receptor potential channel subfamily C member 6	Hs00988479_m1
TRPC7	Transient receptor potential channel subfamily C member 7	Hs00220638_m1
TRPM1	Transient receptor potential channel subfamily M member 1	Hs00931865_m1
TRPM2	Transient receptor potential channel subfamily M member 2	Hs01066091_m1
TRPM3	Transient receptor potential channel subfamily M member 3	Hs00257553_m1
TRPM4	Transient receptor potential channel subfamily M member 4	Hs00214167_m1
TRPM5	Transient receptor potential channel subfamily M member 5	Hs00175822_m1
TRPM6	Transient receptor potential channel subfamily M member 6	Hs01019356_m1
TRPM7	Transient receptor potential channel subfamily M member 7	Hs00559080_m1
TRPM8	Transient receptor potential channel subfamily M member 8	Hs01066596_m1
TRPML1	Transient receptor potential channel subfamily ML member 1	Hs01100653_m1
TRPML2	Transient receptor potential channel subfamily ML member 2	Hs00401916_m1
TRPML3	Transient receptor potential channel subfamily ML member 3	Hs00539554_m1
PKD1	Polycystin 1, Transient Receptor Potential Channel Interacting	Hs00947377_m1
TRPP1 (PKD2)	Transient receptor potential channel subfamily P member 1	Hs00960946_m1
TRPP2 (PKD2L1)	Transient receptor potential channel subfamily P member 2	Hs00175850_m1
TRPP5 (PKD2L2)	Transient receptor potential channel subfamily P member 3	Hs00950467_m1
TRPV1	Transient receptor potential channel subfamily V member 1	Hs00218912_m1
TRPV2	Transient receptor potential channel subfamily V member 2	Hs00901648_m1
TRPV3	Transient receptor potential channel subfamily V member 3	Hs00376854_m1
TRPV4	Transient receptor potential channel subfamily V member 4	Hs01099348_m1
TRPV5	Transient receptor potential channel subfamily V member 5	Hs00219765_m1
TRPV6	Transient receptor potential channel subfamily V member 6	Hs00367960_m1

YWHAZ = Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta

Immunohistochemical (IHC) analysis of TRPC6, TRPM2 and TRPML1 expression in human IVD tissue

Immunohistochemistry specific for TRPC6, TRPM2 and TRPML1 was performed on 3 µm histologic sections cut from paraffin embedded IVD tissue samples that were excised from patients undergoing spinal surgery due to back pain (n=7 for each marker). Additional asymptomatic IVD sections (n=3) were added for each marker (detailed patient information given in **Tables 4** and **5**). All sections were deparaffinized. For TRPM2, heat-mediated antigen retrieval (0.1M citrate puffer, ph 6.0) was used for 20 min (97°C, Agilent Dako PT-Link Pre-Treatment Module), while all other antibodies were "ready-to-use". The endogenous peroxidase was blocked with 0.2% sodium azide and 3% hydrogen peroxide; the non-specific protein was bound by "Protein Block Serum-Free" (Agilent Dako X0909 Lot#1014188). Thereafter, sections were incubated with rabbit polyclonal anti-TRPC6 (1:50, Alomone, #ACC-120, lot: ACC120AN0350), rabbit polyclonal anti-TRPM2 (1:50, Abcam, ab11168, lot: GR30307-24) or rabbit polyclonal anti- Mucolipin 1 (TRPML1, 1:300, Osenses, OSM00039W, lot: Rb0064-180807-WS) overnight at room temperature. Secondary detection was performed with goat anti-mouse and anti-rabbit immunoglobulins IgG (Dako, EnVisionTM+ Dual Link System-HRP) for 30min at room temperature. The amino-9-ethyl-carbazole substrate kit (Dako) was employed as a chromogen. Finally, the sections were counter-stained with Gill's hematoxylin for 3 min, and cover-slipped with an aqueous mounting media (Glicerine, Sigma-Aldrich). The evaluation was performed on the Leica DMR System with Leica DFC320 Camera (Leica Microsystems) and compared with image scans. The images were scanned using NanoZoomer S360 Digital slide scanner: C13220-01 and extracted with NDP.view2 Viewing software. All slides were examined and semi-quantitatively evaluated by two investigators for percentage of positively stained cells and the intensity of the reaction product (visible staining). The two scores (A,B) were added together for a final score with eight possible values. Scores of 0 to 2 were considered negative and scores of 3 to 8 were considered positive (**Table 7**). The complete IHC evaluation is presented in **Table 6**.

Table 4. Immunohistochemistry (IHC): patient information in relation to clinical findings.

Sample No.	Condition	Region	Age	Sex	Pathology	Degeneration grade*	Modic type	TRPC6	TRPM2	TRPML1
35	1	AF	76	m	DDD	3	2		x	x
36	1	NP	47	m	DH	3	1		x	x
37	1	mix	59	f	DH	3	1		x	x
38	1	AF	56	f	DDD	3	n/a		x	x
39	1	AF	43	f	DDD	3	2		x	x
40	1	mix	32	f	DDD	3	1	x		
41	1	mix	50	f	DDD	3	2	x		
42	1	mix	47	m	DDD	3	1	x		
43	1	mix	74	m	DDD	3	2	x		
44	1	NP	66	m	DH	3	1	x		
45	1	mix	67	m	DDD	4	2	x	x	x
46	1	mix	71	f	DDD	4	2	x	x	x
47	0	entire IVD	16	f	#	2	#	x	x	x
48	1	entire IVD	77	f	n/a	4	n/a	x	x	x
49	0	entire IVD	3 days	m	#	1	#	x	x	x

1 = degenerated; 0 = non-degenerated; NP = nucleus pulposus; AF = annulus fibrosus; m = male; f = female; DH = disc herniation; DDD = degenerative disc disease; n/a = data not available; # = not applicable; * cadaveric samples were assessed with Thompson classification and degenerated biopsies with Pfirrmann classification; x = sample on which IHC was performed with a listed antibody.

Table 5. Immunohistochemistry (IHC): patient information in relation to clinical findings.

Sample No.	BMI#	Steroids#	Smoking#	Pain score#	Duration of symptoms (grouped) #	Cause of death*	TRPC6	TRPM2	TRPML1
35	27.8	0	0	2	2			x	x
36	26.9	0	0	3	2			x	x
37	33	1	1	2	2			x	x
38	28	1	0	3	2			x	x
39	26	1	1	2	2			x	x
40	23.8	0	1	1	3		x		
41	24.4	1	1	3	3		x		
42	33	0	1	1	3		x		
43	28	1	0	2	3		x		
44	26	1	1	2	3		x		
45	25	0	0	2	2		x	x	x
46	20	0	1	2	2		x	x	x
47						craniocerebral trauma	x	x	x
48						myocardial infarction	x	x	x
49						endocarditis	x	x	x

Steroids or smoking: 0 = no, 1 = yes; Pain score: 1 = moderate pain, 2 = intense pain, 3 = disabling pain; Pain duration: 1: 1-2 months, 2: 2-12 months, 3: >1 year; * = applicable only to the entire IVD samples, donors 47 to 49; # = not applicable to the entire IVD samples, donors 47 to 49; x = sample on which IHC was performed with a listed antibody.

Table 6. Immunohistochemistry (IHC): Evaluation

Sample No.	Condition*	Region	Expression grade (IHC signal intensity)			Distribution (% positive cells)			Total score (A+B)		
			TRPC6	TRPM2	TRPML1	TRPC6	TRPM2	TRPML1	TRPC6	TRPM2	TRPML1
35	1	AF		1	1		5	1		**6**	**2**
36	1	NP		2	1		4	3		**6**	**4**
37	1	mix		1	3		4	3		**5**	**6**
38	1	AF		2	1		5	2		**7**	**3**
39	1	AF		2	2		5	3		**7**	**5**
40	1	mix	3			5			**8**		
41	1	mix	2			5			**7**		
42	1	mix	1			1			**2**		
43	1	mix	2			3			**5**		
44	1	NP	2			2			**4**		
45	1	mix	3	3	1	5	5	4	**8**	**8**	**5**
46	1	mix	3	3	3	5	5	4	**8**	**8**	**7**
47	0	entire IVD	0	0	0	0	0	0	**0**	**0**	**0**
48	1	entire IVD	2	2	1	3	3	1	**5**	**5**	**2**
49	0	entire IVD	0	1	1	0	3	1	**0**	**4**	**2**

1 = degenerated; 0 = non-degenerated; x = sample on which IHC was performed with a listed antibody; scoring system is described in **Table 5**.

Table 7. Combinative semi-quantitative scoring system by Fedchenko and Reifenrath [48]

Proportion score A	Positive cells %	Signal intensity	Intensity score B
0	0	None (-)	0
1	<1	Weak (+)	1
2	1 to 10	Intermediate (++)	2
3	11 to 33	Strong (+++)	3
4	34 to 66	**Final score (A+B): 0-8**	
5	≥67		

Statistical Analysis

Data consistency was checked and data were screened for outliers. Continuous variables were also tested for normality by using skewness, kurtosis, omnibus test. Variance homogeneity between both groups were tested by using variance ratio test and modified Levene test. In case of deviation of normality, randomization tests were computed based on Monte Carlo simulations (due to the small sample sizes). In case of normality, Student t-tests for equal or Aspin-Welch unequal variance tests were applied. All reported tests were two-sided, and p-values < 0.05 were considered as statistically significant. All statistical analyses in this report were performed by use of NCSS (NCSS 10, NCSS, LLC. Kaysville, UT) and STATISTICA 13 (Hill, T. & Lewicki, P. Statistics: Methods and Applications. StatSoft, Tulsa, OK). All figures were created using GraphPad Prism version 7.03 for Windows, GraphPad Software, La Jolla California USA.

Data Availability

The datasets generated and analyzed during this study are available from the corresponding author on a reasonable request.

Acknowledgments

The study was financially supported by the Swiss National Science Foundation (SNF PP00P2_163678/1) as well as the Spine Society of Europe (Eurospine 2016_4).
Non-degenerated tissue was provided by the McGill Scoliosis and Spine Group.
Degenerated tissue was provided by the Hirslanden Klinik St. Anna.
Asymptomatic degenerated and non-degenerated entire disc sections used for immunohistological analysis were provided by Andreas Nerlich from the Institute for Pathology at the Klinikum Bogenhausen Munich.

Authors Contributions

A.S. performed experiments, collected data, interpreted the results, prepared figures and tables, and wrote the manuscript. W.H. did the statistical analysis and assisted in results interpretation. A.K. developed the IHC protocols and performed the immunohistochemistry and data analysis. P.J. and O.N.H managed the collection of degenerated samples and provided clinical relevance. L.H. managed the collection of non-degenerated samples. H.C.

assisted in non-degenerated sample collection and sample processing. K.W.K. designed the study, wrote the manuscript and secured funding. All authors reviewed, edited and approved the final manuscript.

Competing Interests

The authors declare that they have no competing financial and non-financial interests.

Ethical approval

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

Supplementary Material

Internal control screening

Method: To identify the most stable reference gene in human non-degenerated and degenerated IVD tissue, geNorm [47] software was used. Nine reference genes were investigated: ACTB, GAPDH, GUSB, RPL4, RPL13A, SDHA, TBP, YWHAZ and 18s (**Table S2**) in human non-degenerated (2xAF, 2xNP) and degenerated (1xAF Pfirrmann Grade 4, 1xNP Pfirrmann Grade 4, 1xAF Pfirrmann Grade 5, 1xNP Pfirrmann Grade 5) IVD tissue using standard qPCR procedure. GUSB was not detected in one of the analyzed samples and was thus excluded from the geNorm analysis.

Results: geNorm identified YWHAZ, GAPDH and PRL4 as the most stable reference genes (**Fig. S1**) in the analyzed sample set. In contrast, the commonly used reference genes ACTB, and TBP showed more variation in the expression between the samples (**Fig. S1**).

Conclusion: Based on these results and due to its availability and compatibility with other studies currently being conducted in our laboratory (data not showed), YWHAZ was selected as the reference gene for this study.

TRP channel expression: tissue versus isolated cells

Method: To evaluate whether the mRNA expression of TRP channels is affected by enzymatic digestion for cell isolation, five out of 22 degenerated IVD samples with sufficient size were not only used for direct RNA isolation (as described in the main document material and methods section), but also for IVD cell isolation. After intra-operative excision, IVD sample were transported to the laboratory either in RNAlater (Thermo Fisher, Switzerland) or in DMEM/F12 (Sigma-Aldrich, Switzerland) with 3% antibiotic-antimycotic (Anti-Anti) solution (300 U/ml Penicillin; 300 µg/ml Streptomycin; 3.75 µg/ml Amphotericin B; Gibco, USA). Fresh samples were washed in sterile phosphate-buffered saline (PBS) solution and cut into fine pieces before incubation in an enzymatic digestion solution (0.3% Collagenase NB4 (Serva, Germany), 0.2% Dispase II (Roche Diagnostics, Germany), 3% Anti-Anti in PBS) for 12h at 37°C, 5% CO_2, 21% O_2. Following filtration of the tissue digest through cell strainers (70 and 100 µm), the cells were pelleted (1000 rpm, 10 minutes, 20°C with a PBS washing step in-between) and lysed in 1 mL of TRIzol (Thermo Fisher, Switzerland).

RNA was extracted by TRIzol/chloroform method, followed by affinity-based purification. Cell pellets were supplemented with 200 µL chloroform (1-part chloroform to 5 parts sample) as described above. One part of isopropanol was added (1:1 ratio) to the aqueous phase and samples were shaken 10 times, incubated for 5 min at RT and centrifuged (4°C, 12000g, 10 min). Next, the supernatant was removed and the pellet was mixed with 70% Ethanol, vortexed and centrifuged again (4°C, 12000g, 5 min). Lastly, the Ethanol was removed and samples were left for 5 min at the RT to allow the remaining Ethanol to evaporate. Finally, extracted RNA was re-suspended in 25 µL of RNAse-free water.

Results: No statistically significant differences ($p>0.05$, **Table S3**) were found in the TRP channels mRNA expression between IVD tissue and cells collected directly after isolation (**Fig. S2**).

Conclusion: We were able to show that the enzymatic digestion of the IVD tissue did not affect the mRNA expression of the selected TRP channels (and PDK1, **Fig. S2**). This finding may be important when considering future experimental set-ups, as well as accessibility to certain laboratory equipment and consumables, including liquid nitrogen containers in hospitals.

Transient Receptor Potential (TRP) polycystin (P) channel subfamily

Since it was reviewed that little evidence exist for the 11-TM subfamily (PKD1) to be forming functional ion channels, PKD1 was excluded from the TRP channel family, leading to a change in nomenclature[40]. However, it was recently proposed that PDK1 may be necessary for the PDK2 in order to produce cation currents [41], hence PKD1 should not be excluded from the analysis when evaluating the TRPP1 (PDK2) channel. Consequently, old and new nomenclature can still be find in publications, as well as in biotechnology products. Hence, caution must be taken when reviewing the literature or purchasing biological assays. To avoid the confusion, the old and new nomenclature is provided in the **Table S4**.

Tables and Figures

Table S1. Results of the TaqMan array test of 29 TRP channel targets, displayed as Ct values.

Gene	Donor 1 AF PDG 4	Donor 2 NP PDG 4	Donor 3 AF PDG 5	Donor 4 NP PDG 5	Donor 5 AF N-DEG	Donor 6 NP N-DEG	Donor 7 AF N-DEG	Donor 8 NP N-DEG	Selection
TRPA1	n.d	25.93	28.16	28.42	n.d	n.d	n.d	n.d	
TRPC1	18.84	18.05	17.46	20.61	23.30	24.49	29.20	26.24	
TRPC2	26.87	22.14	24.96	28.68	26.96	28.64	31.38	26.31	
TRPC3	26.75	19.67	21.06	23.06	n.d	n.d	n.d	n.d	
TRPC4	30.05	23.85	24.45	26.20	n.d	n.d	n.d	n.d	
TRPC5	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	
TRPC6	27.14	19.95	19.91	23.24	30.71	n.d	n.d	n.d	
TRPC7	28.98	n.d	30.00	n.d	n.d	n.d	n.d	n.d	
TRPM1	n.d	29.40	n.d	30.06	n.d	n.d	n.d	n.d	
TRPM2	26.07	20.91	22.83	24.45	n.d	n.d	n.d	n.d	
TRPM3	n.d	20.98	24.69	25.80	n.d	n.d	n.d	n.d	
TRPM4	22.58	19.38	21.05	22.68	n.d	27.85	n.d	n.d	
TRPM5	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	
TRPM6	25.61	22.26	21.44	26.36	n.d	33.19	n.d	n.d	
TRPM7	18.62	16.35	17.51	19.96	23.90	24.21	n.d	27.42	
TRPM8	30.64	26.95	n.d	n.d	n.d	n.d	n.d	n.d	
TRPML1	21.72	18.54	19.91	21.90	27.50	27.87	n.d	29.71	
TRPML2	22.08	21.96	23.27	22.80	27.10	28.43	n.d	n.d	
TRPML3	21.30	20.20	20.93	21.83	27.70	27.87	n.d	28.93	
PDK1	19.10	18.66	19.00	19.14	26.55	26.24	29.70	28.96	
TRPP1 (PKD2)	18.42	16.50	16.89	19.11	24.48	24.44	27.95	27.38	
TRPP2 (PKD2L1)	30.57	29.22	n.d	n.d	n.d	n.d	n.d	n.d	
TRPP3 (PKD2L2)	30.08	n.d	n.d	n.d	n.d	n.d	n.d	n.d	
TRPV1	22.33	20.94	21.69	23.52	27.41	29.05	31.10	30.78	
TRPV2	24.38	19.58	21.58	23.53	n.d	n.d	n.d	n.d	
TRPV3	25.81	25.03	25.91	27.01	28.96	31.33	n.d	30.64	
TRPV4	16.19	17.57	18.20	17.52	23.25	24.14	28.60	26.07	
TRPV5	29.59	26.55	28.28	28.22	n.d	30.55	n.d	n.d	
TRPV6	41.88	27.78	n.d	n.d	n.d	n.d	n.d	n.d	

AF = annulus fibrosus; NP = nucleus pulposus; PDG = Pfirrmann degeneration grade; N-DEG = non-degenerated; n.d = non-detectable; TRP = transient receptor potential channel; filled field in the Selection column indicates that the target was selected for further tests

Table S2. TaqMan primers used for the reference gene screening using qPCR analysis.

Gene	Gene class	Primer number
ACTB	Internal control	Hs01060665_g1
GAPDH	Internal control	Hs02758991_g1
GUSB	Internal control	Hs00939627_m1
RPL4	Internal control	Hs03044646_g1
RPL13A	Internal control	Hs04194366_g1
SDHA	Internal control	Hs00188166_m1
TBP	Internal control	Hs00427620_m1
YWHAZ	Internal control	Hs01122445_g1
18s	Internal control	Hs99999901_s1

ACTB = Beta Actin; GAPDH = Glyceraldehyde-3-phosphate Dehydrogenase; GUSB = Glucuronidase Beta; RPL4 = Ribosomal Protein L4; RPL13A = Ribosomal Protein L13a; SDHA = Succinate Dehydrogenase Complex Flavoprotein Subunit A; TBP = TATA-Box Binding Protein; YWHAZ = Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta

Table S3. The statistical significance values (p-values) for the tested TRP channels comparing dCt values of the mRNA expression between the values obtained from tissue versus cells samples, calculated with Monte Carlo Randomization test with a significance level at $p<0.05$.

Target	p-value
TRPC1	0.13
TRPC3	0.25
TRPC4	1
TRPC6	0.25
TRPM2	0.75
TRPM7	1
TRPML1	0.24
TRPML2	0.88
TRPP1	0.07
TRPP2	0.56
TRPV1	0.06
TRPV4	0.18

Table S4. TRPP channel subfamily nomenclature.

Gene alias	Former name	Current name
PKD1	TRPP1	PKD1
PKD2	TRPP2	TRPP1
PKD2L1	TRPP3	TRPP2
PKD2L2	TRPP5	TRPP3

PKD1 = Polycystins include putative 11-TM also called the PC1 family; PKD2 = polycystic kidney disease 2 also called polycystin 2 (PC2); PKD2L1 = Polycystic kidney disease 2-like 1; PKD2L1 = Polycystic kidney disease 2-like 2; TRPP (1,2,3,5) = Transient receptor potential channel subfamily P member 1, 2, 3, 5

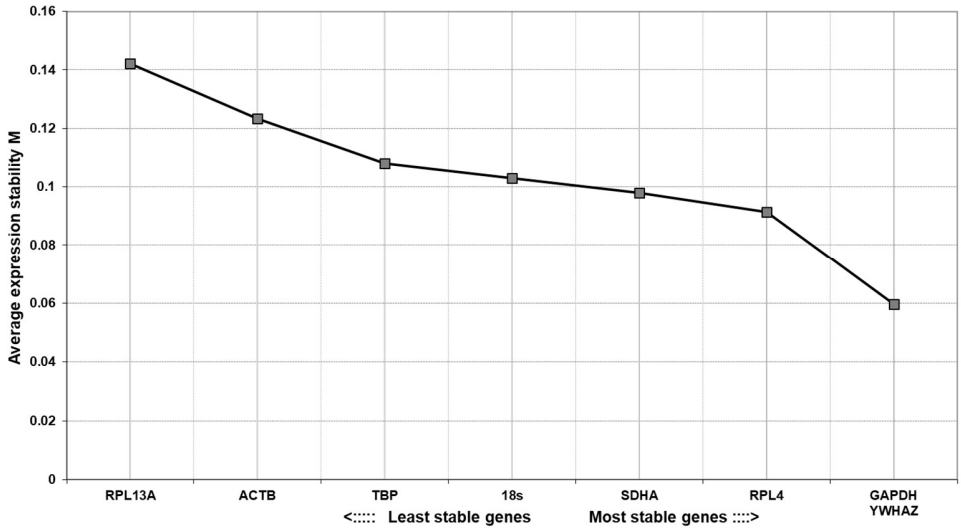


Figure S1. The average expression stability values (M) obtained using geNorm tool. The expression of ACTB, GAPDH, RPL4, RPL13A, SDHA, TBP, YWHAZ and 18s was tested on human non-degenerated (2x AF, 2xNP) and degenerated (2xAF, 2xNP) IVD tissue. The most stable genes are presented on the right and the least stable on the left.

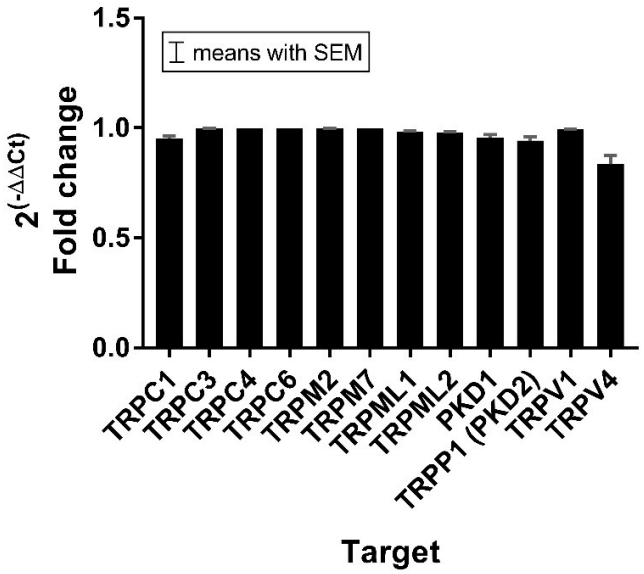


Figure S2. Comparison of the mRNA expression of selected TRP channels in the human degenerated IVD samples between the RNA samples obtained directly from the tissue (via pulverization in the liquid nitrogen) and non-passaged cells (via enzymatic digestion). Results are presented as $2^{-\Delta\Delta Ct}$ values of mRNA normalized to the tissue samples.

References

1. Neidlinger-Wilke, C., et al., *Mechanical loading of the intervertebral disc: from the macroscopic to the cellular level.* Eur Spine J, 2014. **23 Suppl 3**: p. S333-43.
2. Raj, P.P., *Intervertebral disc: anatomy-physiology-pathophysiology-treatment.* Pain Pract, 2008. **8**(1): p. 18-44.
3. Wuertz, K. and L. Haglund, *Inflammatory mediators in intervertebral disk degeneration and discogenic pain.* Global Spine J, 2013. **3**(3): p. 175-84.
4. Risbud, M.V. and I.M. Shapiro, *Role of cytokines in intervertebral disc degeneration: pain and disc content.* Nat Rev Rheumatol, 2014. **10**(1): p. 44-56.
5. Gawri, R., et al., *High mechanical strain of primary intervertebral disc cells promotes secretion of inflammatory factors associated with disc degeneration and pain.* Arthritis Res Ther, 2014. **16**(1): p. R21.
6. Lang, G., et al., *An intervertebral disc whole organ culture system to investigate proinflammatory and degenerative disc disease condition.* J Tissue Eng Regen Med, 2018. **12**(4): p. e2051-e2061.
7. Li, P., et al., *Role of the ERK1/2 pathway in osmolarity effects on nucleus pulposus cell apoptosis in a disc perfusion culture.* J Orthop Res, 2017. **35**(1): p. 86-92.
8. Johnson, Z.I., et al., *TNF-alpha promotes nuclear enrichment of the transcription factor TonEBP/NFAT5 to selectively control inflammatory but not osmoregulatory responses in nucleus pulposus cells.* J Biol Chem, 2017. **292**(42): p. 17561-17575.
9. Walter, B.A., et al., *Reduced tissue osmolarity increases TRPV4 expression and pro-inflammatory cytokines in intervertebral disc cells.* Eur Cell Mater, 2016. **32**: p. 123-36.
10. Krupkova, O., J. Zvick, and K. Wuertz-Kozak, *The role of transient receptor potential channels in joint diseases.* Eur Cell Mater, 2017. **34**: p. 180-201.
11. Baratchi, S., et al., *Shear Stress Regulates TRPV4 Channel Clustering and Translocation from Adherens Junctions to the Basal Membrane.* Sci Rep, 2017. **7**(1): p. 15942.
12. Baratchi, S., et al., *The TRPV4 Agonist GSK1016790A Regulates the Membrane Expression of TRPV4 Channels.* Front Pharmacol, 2019. **10**: p. 6.
13. Sadowska, A., et al., *Inflammaging in cervical and lumbar degenerated intervertebral discs: analysis of proinflammatory cytokine and TRP channel expression.* Eur Spine J, 2018. **27**(3): p. 564-577.
14. Franco-Obregon, A., et al., *TRPC6 in simulated microgravity of intervertebral disc cells.* Eur Spine J, 2018.

15. Palacio-Mancheno, P.E., et al., *Hyperosmolarity induces notochordal cell differentiation with aquaporin3 upregulation and reduced N-cadherin expression.* J Orthop Res, 2018. **36**(2): p. 788-798.
16. Pfirrmann, C.W., et al., *Magnetic resonance classification of lumbar intervertebral disc degeneration.* Spine (Phila Pa 1976), 2001. **26**(17): p. 1873-8.
17. Thompson, J.P., et al., *Preliminary evaluation of a scheme for grading the gross morphology of the human intervertebral disc.* Spine (Phila Pa 1976), 1990. **15**(5): p. 411-5.
18. Rannou, F., et al., *High-sensitivity C-reactive protein in chronic low back pain with vertebral end-plate Modic signal changes.* Arthritis Rheum, 2007. **57**(7): p. 1311-5.
19. Albert, H.B., et al., *Modic changes, possible causes and relation to low back pain.* Med Hypotheses, 2008. **70**(2): p. 361-8.
20. Nilius, B., *TRP channels in disease.* Biochim Biophys Acta, 2007. **1772**(8): p. 805-12.
21. Brederson, J.D., P.R. Kym, and A. Szallasi, *Targeting TRP channels for pain relief.* Eur J Pharmacol, 2013. **716**(1-3): p. 61-76.
22. Nilius, B. and G. Owsianik, *The transient receptor potential family of ion channels.* Genome Biol, 2011. **12**(3): p. 218.
23. Kerr, G.J., et al., *Decoding the intervertebral disc: Unravelling the complexities of cell phenotypes and pathways associated with degeneration and mechanotransduction.* Semin Cell Dev Biol, 2017. **62**: p. 94-103.
24. Wuertz, K., et al., *Inflammatory and catabolic signalling in intervertebral discs: the roles of NF-kappaB and MAP kinases.* Eur Cell Mater, 2012. **23**: p. 103-19; discussion 119-20.
25. Gavenis, K., et al., *Expression of ion channels of the TRP family in articular chondrocytes from osteoarthritic patients: changes between native and in vitro propagated chondrocytes.* Mol Cell Biochem, 2009. **321**(1-2): p. 135-43.
26. Liedtke, W., *Role of TRPV ion channels in sensory transduction of osmotic stimuli in mammals.* Exp Physiol, 2007. **92**(3): p. 507-12.
27. Ramirez, G.A., et al., *Ion Channels and Transporters in Inflammation: Special Focus on TRP Channels and TRPC6.* Cells, 2018. **7**(7).
28. Wu, D., et al., *TRPC4 in rat dorsal root ganglion neurons is increased after nerve injury and is necessary for neurite outgrowth.* J Biol Chem, 2008. **283**(1): p. 416-26.
29. Numata, T., T. Shimizu, and Y. Okada, *TRPM7 is a stretch- and swelling-activated cation channel involved in volume regulation in human epithelial cells.* Am J Physiol Cell Physiol, 2007. **292**(1): p. C460-7.

30. Kim, J.M., S. Choi, and K. Park, *TRPM7 Is Involved in Volume Regulation in Salivary Glands.* J Dent Res, 2017. **96**(9): p. 1044-1050.
31. Ishihara, H., et al., *Proteoglycan synthesis in the intervertebral disk nucleus: the role of extracellular osmolality.* Am J Physiol, 1997. **272**(5 Pt 1): p. C1499-506.
32. Yamamoto, S., et al., *TRPM2-mediated Ca2+influx induces chemokine production in monocytes that aggravates inflammatory neutrophil infiltration.* Nat Med, 2008. **14**(7): p. 738-47.
33. Isami, K., et al., *Involvement of TRPM2 in peripheral nerve injury-induced infiltration of peripheral immune cells into the spinal cord in mouse neuropathic pain model.* PLoS One, 2013. **8**(7): p. e66410.
34. Binch, A.L., et al., *Nerves are more abundant than blood vessels in the degenerate human intervertebral disc.* Arthritis Res Ther, 2015. **17**: p. 370.
35. Venkatachalam, K., C.O. Wong, and M.X. Zhu, *The role of TRPMLs in endolysosomal trafficking and function.* Cell Calcium, 2015. **58**(1): p. 48-56.
36. Lie, P.P.Y. and R.A. Nixon, *Lysosome trafficking and signaling in health and neurodegenerative diseases.* Neurobiol Dis, 2019. **122**: p. 94-105.
37. Shen, C., et al., *Autophagy in rat annulus fibrosus cells: evidence and possible implications.* Arthritis Res Ther, 2011. **13**(4): p. R132.
38. Ma, K.G., et al., *Autophagy is activated in compression-induced cell degeneration and is mediated by reactive oxygen species in nucleus pulposus cells exposed to compression.* Osteoarthritis Cartilage, 2013. **21**(12): p. 2030-8.
39. Xu, K., et al., *Autophagy attenuates the catabolic effect during inflammatory conditions in nucleus pulposus cells, as sustained by NF-kappaB and JNK inhibition.* Int J Mol Med, 2015. **36**(3): p. 661-8.
40. Wu, L.J., T.B. Sweet, and D.E. Clapham, *International Union of Basic and Clinical Pharmacology. LXXVI. Current progress in the mammalian TRP ion channel family.* Pharmacol Rev, 2010. **62**(3): p. 381-404.
41. Hanaoka, K., et al., *Co-assembly of polycystin-1 and -2 produces unique cation-permeable currents.* Nature, 2000. **408**(6815): p. 990-4.
42. Kottgen, M., et al., *TRPP2 and TRPV4 form a polymodal sensory channel complex.* J Cell Biol, 2008. **182**(3): p. 437-47.
43. Du, J., et al., *TRPV4, TRPC1, and TRPP2 assemble to form a flow-sensitive heteromeric channel.* FASEB J, 2014. **28**(11): p. 4677-85.
44. Jin, M., et al., *Determinants of TRPV4 activity following selective activation by small molecule agonist GSK1016790A.* PLoS One, 2011. **6**(2): p. e16713.
45. Chubanov, V., et al., *Natural and Synthetic Modulators of the TRPM7 Channel.* Cells, 2014. **3**(4): p. 1089-101.

46. Singh, A., et al., *The transient receptor potential channel antagonist SKF96365 is a potent blocker of low-voltage-activated T-type calcium channels.* Br J Pharmacol, 2010. **160**(6): p. 1464-75.
47. Vandesompele, J., et al., *Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes.* Genome Biol, 2002. **3**(7): p. RESEARCH0034.
48. Fedchenko, N. and J. Reifenrath, *Different approaches for interpretation and reporting of immunohistochemistry analysis results in the bone tissue - a review.* Diagn Pathol, 2014. **9**: p. 221.

Chapter 6

6 Osmosensing, osmosignalling and inflammation: how intervertebral disc cells respond to altered osmolarity

Osmosensing, osmosignaling and inflammation: How intervertebral disc cells respond to altered osmolarity

A. Sadowska[1*], T. Kameda[1,2*], O. Krupkova[1], K. Wuertz-Kozak[1,3,4,5]

[1]Institute for Biomechanics, DHEST, ETH Zurich, Zurich, Switzerland

[2]Department of Orthopaedic Surgery, Fukushima Medical University, School of Medicine, Fukushima, Japan

[3]Spine Center, Schön Clinic Munich Harlaching, Munich, Germany

[4]Department of Health Sciences, University of Potsdam, Potsdam, Germany

[5]Academic Teaching Hospital and Spine Research Institute, Paracelsus Private Medical University Salzburg, Salzburg, Austria

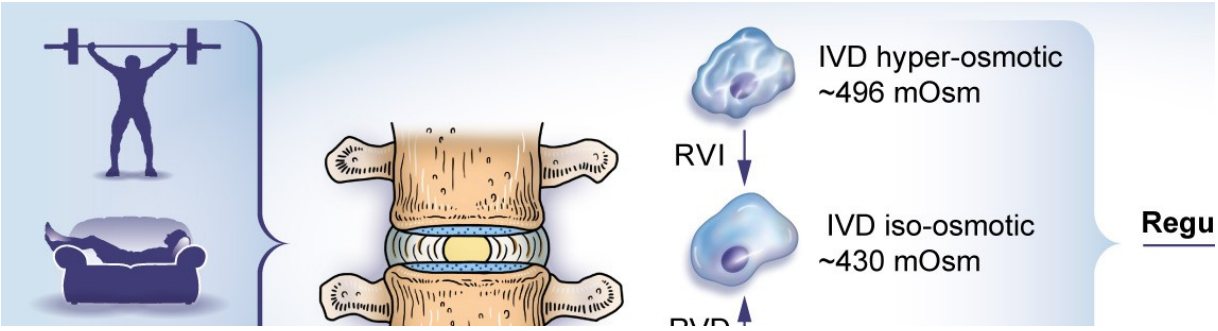
*These authors have contributed equally.

Published as:

Sadowska, A., *et al.* "Osmosensing, osmosignalling and inflammation: how intervertebral disc cells respond to altered osmolarity."

European cells & materials 36 (2018): http://doi.org/10.22203/ecm.v036a17

Graphical abstract:



Abstract:

Intervertebral disc (IVD) cells are naturally exposed to high osmolarity and complex mechanical loading, which drive microenvironmental osmotic changes. Age- and degeneration-induced degradation of the IVD's extracellular matrix causes osmotic imbalance, which, together with an altered function of cellular receptors and signaling

pathways, instigates local osmotic stress. Cellular responses to osmotic stress include osmoadaptation and activation of pro-inflammatory pathways. This review summarizes the current knowledge on how IVD cells sense local osmotic changes and translate these signals into physiological or pathophysiological responses, with a focus on inflammation. Furthermore, it discusses the expression and function of putative membrane osmosensors (e.g. solute carrier transporters, transient receptor potential channels, aquaporins and acid-sensing ion channels) and osmosignalling mediators [e.g. tonicity response element-binding protein/nuclear factor of activated T-cells 5 (TonEBP/NFAT5), nuclear factor kappa-light chain-enhancer of activated B cells (NF-κB)] in healthy and degenerated IVDs. Finally, an overview of the potential therapeutic targets for modifying osmosensing and osmosignalling in degenerated IVDs is provided.

Keywords: Intervertebral disc (IVD) degeneration, degenerative disc disease (DDD), osmolarity, hyperosmolarity, hypoosmolarity, osmotic, inflammatory, transient receptor potential (TRP) channel, aquaporin (AQP), Tonicity-responsive enhancer binding protein (TonEBP/nuclear factor of activated T-cells 5 (NFAT5))

6.1 Introduction

The intervertebral disc (IVD) is a mechanically loaded tissue with early signs of degeneration that are associated with a loss in proteoglycan content and thus changes in the osmotic environment [1, 2]. The IVD contains a small population of cells embedded in an extracellular matrix (ECM) that is predominantly composed of water (60-99%) [3, 4]. The mechanical properties of the IVD are determined by its biochemical structure, with the highly hydrated nucleus pulposus (NP) in the middle, surrounded by the annulus fibrosus (AF). The AF is rich in collagen type I towards the outer rim, where it provides greater strength, whereas the inner part of the AF is composed of a fibrocartilage that steadily fuses into a transition zone (TZ) with the NP [5]. The NP, an immune-privileged structure, consists of sparsely distributed cells surrounded by a gelatinous network that is primarily composed of collagen type II and proteoglycans (PGs) [6], illustrating the IVD's similarity to cartilage. With aging, the IVD undergoes degenerative changes, which are associated with tissue weakening, dehydration and a loss in ECM components [7, 8]. Associated changes in tissue hydration and hence in the IVD's osmotic environment influence the IVD's mechanical properties [9], possibly leading to low back pain (LBP), activity limitation,

disability [10] and consequently a high economic burden on the society [11]. The goal of this review is (1) to summarize the current knowledge on how IVD cells sense and respond to osmotic changes, (2) to provide an outlook on possible future research on disc hydration and osmolarity and (3) to highlight potential therapies related to IVD osmosignaling.

6.2 Osmoregulation

General concept of osmoregulation

The capacity to maintain an osmotic balance (=osmoregulation) and control the cell volume is important for preserving cell function. The volume of a cell depends on the water movements across its membrane, driven by osmotic gradients that develop from differences in the chemical concentrations of the intra- and extra-cellular fluids under normal physiological conditions [12]. Solutions with higher solvent concentration tend to have a lower water content, and vice versa. Hence, water will move across the membrane from the solution with the low solute (or high water) content to the one with high solute (or low water) content – a phenomenon that is defined as osmosis [12]. Osmolarity (or osmotic concentration) is a concentration of solutes in a solution expressed in osmole/L, whereas osmolality is expressed in osmole/kg and both terms are often used interchangeably [13]. A decrease or increase of the extracellular osmolarity will result in cell swelling (= inflow of water) or cell shrinkage (= outflow of water), respectively [14]. To resist cell swelling, the osmotic pressure – defined as a minimal hydrostatic pressure necessary to stop water from diffusing across two barriers – has to be developed [12].

Osmoregulation in the IVD

Proteoglycans (PGs) are crucial for maintaining hydration and osmotic pressure in the IVD, with aggrecan being the primary type [5]. Aggrecans are composed of three globular domains (G1, G2, G3) and attached GAG side chains [15]. The primary types of GAGs found in the IVD are CS and KS. The ionic balance of the IVDs extracellular matrix is regulated by the negatively charged GAGs [16]. The sulfated GAGs of aggrecan create a high negative charge, contributing to the molecule's ability to electrostatically bind water. On the tissue level, this translates into the generation of an osmotic pressure in the IVD, causing the NP to ingest water; it also contributes to the high swelling pressure and load-bearing ability of the IVD [5, 17-19] and the resistance to high compressive loads experienced, for example, during

weight lifting or forward bending activities [20]. If the applied loading exceeds the osmotic pressure, water is diffused and the osmotic pressure increases, while water is absorbed during IVD unloading, resulting in an osmotic equilibrium [21, 22]. In the lack of external loads, the hydrostatic pressure is maintained due to the structure of the AF and the endplates that constrain the swelling of the NP [23]. In *vivo* studies demonstrated that the NP's water content decreases by around 15% under loading conditions (human lumbar IVDs, unloaded vs loaded, 1500 N, 6h) [21]. However, in contrary to static or high-impact loading, daily physiological loading, such as dynamic loading or loading at moderate speed (e.g. jogging [24]), are beneficial for IVD hydration, and therefore the tissue's health [25].

For a normal osmotic function in the IVD, it is essential that the aggrecan content, charge and size remain as large as possible. However, aggrecans are enzymatically cleaved by proteinases such as matrix metalloproteinases (MMPs) and aggrecanases, whose expression increases in degenerated IVDs [26-28]. Age-related or degeneration-induced loss of aggrecan causes a drop in osmotic pressure, reducing the IVD's ability to respond to mechanical loads. In a healthy state, the extracellular osmolarity can vary from ~430 (iso-osmotic) to ~496 mOsm/L (hyper-osmotic) [29, 30] – values which are in a physiological range for IVD cells, but would be considered high for most mammalian cells [31, 32]. The IVD's osmolarity can decrease to around 300 mOsm/L (hypo-osmotic) in degenerated IVDs [9] due to a loss in PGs and thus IVD hydration and occurrence of fibrosis, however the same osmolarity would be considered physiological for cells of other tissues [33]. In this review, the terms: hyper-, hypo- and iso-osmotic are used in the context of the IVD tissue. Although an altered osmotic environment is rather a hallmark of IVD degeneration than its primary cause, the reduced tissue osmolarity can activate and/or interplay with pro-inflammatory factors and catabolic responses, and hence promote IVD inflammation (see "*Cell responses to osmotic changes: target genes and signaling pathways"*) and degenerative disc disease (DDD).

Osmolarity-related cell volume changes

At the cellular level, a change in intra- or extra-cellular osmolarity causes mammalian cell volume regulation by the solubility–diffusion water transport across the cell membrane through several water, ion and molecule transport pathways, such as pores, ion channels, and membrane carriers (see "*Membrane proteins as potential osmosensors in the IVD"*) [34, 35]. The increased cell volume induces a prompt activation of the RVD, a mechanism that

acts to recover the cell volume homeostasis. In contrast, hyper-osmolarity causes cell shrinkage, to which a cell responds by activating the RVI mechanism [36]. However, very limited information on the cell volume regulation in the IVD exists. In bovine NP cells, a decrease in extra-cellular osmolarity (from 430 to 230 mOsm/L) increases the cell volume by up to 20 %, leading to a reduced PG synthesis rate [29]. Changes in the IVD cell volume may involve depolarization and reorganization of the actin cytoskeleton and initiation of calcium transits from the intra-cellular stores [37]. Interestingly, the response to the hyper-osmotically-induced volume changes may be zone dependent (AF vs. NP) due to the differences in the mechanical composition of these cells. In comparison with AF cells, NP cells were found to be stiffer and more viscous due to differences in the cytoskeletal arrangement [38]. Additionally, vacuoles (or vesicles), which can be found in the notochordal cells within the NP tissue (canine), contain a low-osmolarity solution that is released into the cytoplasm under a condition of hyper-osmotic stress and helps to restore the osmotic balance [39]. However, this mechanism has not been confirmed in other notochordal-free species and, therefore, cannot be generalized.

Membrane proteins as potential osmosensors in the IVD

Cells sense osmotic stress through membrane proteins such as carriers (SLC) or channels (TRP and AQPs) (**Table 1a-c, Table 2, Fig. 1**), which are responsible for transporting molecules (e.g. ions, sugars etc.) across the cell membrane. Carriers physically bind to a specific solute and change their own conformation to release the solute on the other side of the cell membrane; channels form a pore, which can open to allow a specific molecule to pass by diffusion or osmosis [40].

The SLC is a group of membrane transport proteins that includes over 400 transporters in humans. The SLC transporters play an important role in homeostasis by transporting soluble molecules (such as nutrients) across the lipid membranes [41] and are involved in e.g. glucose transport (SLC5) [42], pH regulation (SLC16) [43], as well as hormone and/or drug uptake (SLC21A12) [44-46]. As shown in human NP cells, an increase in osmolarity (450 mOsm/L) upregulates the expression of the solute carriers SLC21A12 (= SLCO) and SLC5A3 but downregulates the expression of SLC16A6 [47]. Although they have a putative role in regulatory volume mechanisms in other tissue types [48], their exact role in the IVD is largely unknown.

The transient receptor potential (TRP) channels, a superfamily of cation selective transmembrane receptors, have recently emerged as potential contributors to IVD degeneration and discogenic pain [49-52]. TRP channels are multimodal ion channels regulated by a diverse range of stimuli, including mechanical and osmotic stress [50, 53]. Previous studies examining several tissue types, such as kidney [54, 55], central nervous system (CNS) [56], smooth muscles [57] and others [58, 59], point towards the TRP vanilloid (TRPV) subfamily (especially TRPV4) as a potential cellular osmo- and volume-sensor involved in the RVD mechanism [60-63]. Interestingly, Becker *et al.* [61] demonstrated that TRPV4 has a key role in the cell-volume regulation by transiently transfecting CHO cells with TRPV4: CHO cell volume decreases after hypo-osmotic (200 mOsm/L) treatment (RVD response after swelling), in contrast to untransfected CHO control cells. Hence, Becker *et al.* [61] and others [64, 65] suggest that cell swelling caused by hypo-osmotic treatment leads to the generation of tension on the cell membrane and thus activation (=opening of a channel pore [66]) of the TRPV4 channel, which mediates the influx of extra-cellular Ca^{2+} that initiates a signaling cascades, causing RVD response. TRP channels, including TRPV4, are expressed in the human, bovine and mouse IVD [49, 52, 67]. In the human IVD, hypo-osmotic conditions (200-334 mOsm/L) induce an up-regulation of TRPV4 (on the protein level), leading to an activated calcium influx [52]. In contrast, hyper-osmolarity (530 mOsm/ L) combined with cyclic loading (10 min on and off for 1.5 h/day) significantly downregulates TRPV4 expression (mouse NP cells) [67]. Moreover, the expression and/or activity of TRPV4 is modulated by the inflammation (upregulated cytokines IL-6 and IL-1B) and correlates with pro-inflammatory cytokines in the IVD and cartilage [49, 52, 65, 68]. Therefore, Walter *et al.* [52] suggest that alternations in TRPV4 mediated sensation of osmotic changes (also known as osmosensing) could aid the progression of disc degeneration. Additionally, both TRPV4 mediated osmotic and inflammatory signals may be regulated through the p38 mitogen-activated protein kinases (p38/MAPK) and extracellular signal-regulated kinase ($ERK_{1/2}$) pathways [60, 69, 70].

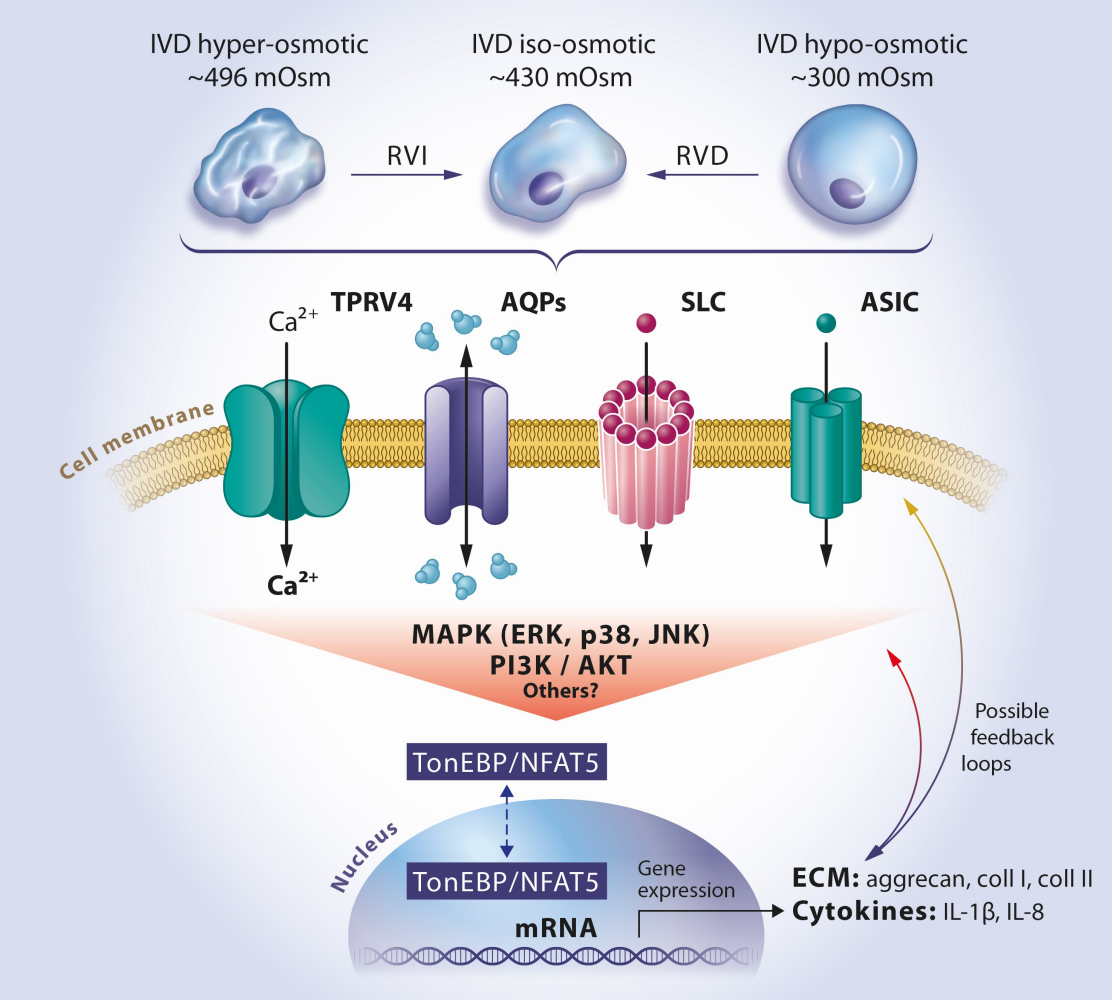


Fig. 1 Osmolarity-related changes in the IVD cells. Schematic representation of the IVD response to the osmotic stimuli. Changes in cell volume (top left: cell shrinkage under an hyper-osmotic challenge; top right: cell swelling under an hypo-osmotic challenge) trigger the activation of volume recovery mechanisms (RVI and RVD) to restore the homeostasis but can also act as stress signals, activating cell membrane receptors. TRP channels, AQPs, SLCs and ASICs are membrane proteins with a potentially crucial role in osmosensing and osmo-adaptation. Transduced signals activate the MAPKs pathway (middle). Further, osmolarity changes lead to increase in NFAT nuclear shuttling, which induces transcription of genes involved in matrix homeostasis and pro-inflammatory cytokines (bottom middle). These changes may activate a positive/negative feedback loop to the osmo-receptor and/or signalling pathways. However, the exact and complete osmosensing pathway has not been yet extensively studied in the disc.

Aquaporins (AQPs) are small transmembrane channel proteins responsible for water transport and are of relevance in osmoregulation. The presence of AQPs is confirmed in the healthy human NP [71], with decreased expression during degeneration as an adaptive mechanism demonstrated in various species: rat NP and AF [72], rabbit NP [73], human NP [74, 75]. These findings are in line with the observed downregulation of AQP-1 under reduced osmolarity (rabbit NP [73]) and the increased expression of AQP-3 under hyper-osmotic conditions (mouse NP [67]) and consequent NP maturation (but without changes in AQP-1). Furthermore, it was reported that TRPV4 and AQP-4 interact and form a channel complex (in astrocytes), which might constitute an important link in the cell volume homeostasis by integrating water transport and calcium signaling (at least in the CNS) [76, 77].

Additionally, AQPs might play a role in inflammation in the IVD and cartilage [78-80]. In human chondrocytes, AQP-1 co-localizes (on the protein level, as shown using immunofluorescence) with the catabolic factor ADAMTS-4 (an aggrecan-degrading enzyme involved in IVD and cartilage degeneration) and a downregulation of AQP1 decreases the expression of ADAMTS-4 [80, 81]. Additionally, a knockdown of AQP-9 (human chondrocytes) decreases mRNA levels of other catabolic factors [79]. On the other hand, an overexpression of AQP-3 (human degenerated NP cells) decreases the expression of ADAMTS 4 and 5, and suppresses the Wnt/β-catenin signaling, which is involved in IVD cell senescence [82-84]). These findings suggest a protective role of AQP-3 against disc degeneration [78]. Yet, in corneal epithelial cells, hyper-osmotic treatment (450, 500 and 550 mOsm/L) induces the upregulation of the AQP-5 (*via* JNKs pathway), leading to an upregulation of IL-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α and caspase-1 [85], with similar findings in cartilage [86] and the IVD [75, 87].

Table 1a. Targets regulated by hyper-osmotic treatment.

Target gene (symbol)	Expression level change	Function	Model	Reference
Guanylate binding protein 1 (GBP1)	Gene ↓	Cell-cell interaction/adhesion	human IVD tissue	[47]
Kelch motif protein (KIAA1309)	Gene ↓	Cell-cell interaction/adhesion	human IVD tissue	[47]
Small inducible cytokine A2 (SCYA2)	Gene ↓	Cell-cell interaction/adhesion	human IVD tissue	[47]
Vascular cell adhesion molecule 1 (VCAM1)	Gene ↓	Cell-cell interaction/adhesion	human IVD tissue	[47]
cyclin-dependent kinase inhibitor 1 ($p21^{WAF1}$)	Protein ↑	Cell-cycle, arrest of G0/G1	bovine NP cells	[88]
Phospho p38 (p-p38) mitogen-activated protein kinase (MAPK)	Protein ↑	Cell-cycle, arrest of G0/G1	bovine NP cells	[88]
Phospho- p53 (p-p53) mitogen-activated protein kinase (MAPK)	Protein ↑	Cell-cycle, arrest of G0/G1	bovine NP cells	[88]
Aryl hydrocarbon receptor translocator-like (ARNTL)	Gene ↑	Cell-cycle/ apoptosis	human IVD tissue	[47]
CDC28 protein kinase 2 (CKS2)	Gene ↑	Cell-cycle/ apoptosis	human IVD tissue	[47]
Growth arrest specific 1 (GAS1)	Gene ↑	Cell-cycle/ apoptosis	human IVD tissue	[47]
Mucosa assoc lymphoid tissue translocation gene (MALT1)	Gene ↑	Cell-cycle/ apoptosis	human IVD tissue	[47]
ATPase Na+/K+ transporting subunit alpha 1 (ATP1A1)	Gene ↑ Protein ↑	Cell-cycle/ DNA synthesis	bovine IVD	[89]
Caspase 8 (CASP8)	Gene ↓	Cell-cycle/apoptosis	human IVD tissue	[47]
Rho-related BTB domain containing 1 (RHOBTB1)	Gene ↓	Cell-cycle/apoptosis	human IVD tissue	[47]
TNF-induced protein (GG2-1)	Gene ↓	Cell-cycle/apoptosis	human IVD tissue	[47]
Aquaporin-3 (AQP-3)	Protein ↑	Cell membrane protein	mouse NP cells	[67]
Transient potential receptor channel 4 (TRPV4)	Protein ↓	Cell membrane protein	mouse NP cells	[67]

AF = Annulus Fibrosus, IVD = Intervertebral Disc, NP = Nucleus Pulposus, TZ = Transition Zone,
↑ = upregulation, ↓ = downregulation, Only selected targets are discussed in the text

Table 1b. Targets regulated by hyper-osmotic treatment.

Target gene (symbol)	Expression level change	Function	Model	Reference
Matrix metalloproteinase-2 (MMP2)	Gene ↑	Enzyme – matrix turnover	bovine NP cells (3D)	[90]
Metalloproteinase with thrombospondin type 1 motifs (ADAMTS1)	Gene ↑	Enzyme – matrix turnover	human IVD tissue	[47]
Brain-derived neurotrophic factor (BDNF)	Gene ↑	Growth factor	human IVD tissue	[47]
Muskelin (MKLN1)	Gene ↑	Growth factor	human IVD tissue	[47]
Zinc finger protein 238 (ANF238)	Gene ↑	Growth factor	human IVD tissue	[47]
Interleukin 6 (IL-6)	Gene ↓	Growth factors/ cytokines	human IVD tissue	[47]
Norrie disease protein/Norrin (NDP)	Gene ↓	Growth factors/ cytokines	human IVD tissue	[47]
Homologous to mouse potassium-gated channel, Isk-related subfamily (KCNE4)	Gene ↑	Ion transport	human IVD tissue	[47]
Solute carrier family 16 (monocarboxylic acid transporter) (SLC16A6)	Gene ↓	Ion transport	human IVD tissue	[47]
Solute carrier family 21 member 12 (SLC21A12)	Gene ↑	Ion transport	human IVD tissue	[47]
Solute carrier family 4 member 11 (SLC4A11)	Gene ↑ Protein ↑	Ion transport	bovine NP cells	[89]
Solute carrier family 5 member 3 (SLC5A3, SMIT1)	Gene ↑	Ion transport	human IVD tissue	[47]
Solute carrier family 5 member 3 (SLC5A3, SMIT1)	Gene ↑ Protein ↑	Ion transport	bovine NP cells	[89]
Aggrecan (ACAN)	Gene ↑	IVD's ECM components	bovine NP cells (3D), human NP & AF cells (3D)	[9, 90]
Aggrecan (ACAN)	Gene ↓, Protein↓	IVD's ECM components	porcine NP in organ culture	[91]
Biglycan (BGN)	Gene ↑	IVD's ECM components	porcine TZ cells	[92]
Biglycan (BGN)	Gene ↓	IVD's ECM components	porcine NP cells	[92]

AF = Annulus Fibrosus, IVD = Intervertebral Disc, NP = Nucleus Pulposus, TZ = Transition Zone,
↑ = upregulation, ↓ = downregulation, Only selected targets are discussed in the text

Table 1c. Targets regulated by hyper-osmotic treatment.

Target gene (symbol)	Expression level change	Function	Model	Reference
Collagen-1 (COL1A1)	Gene ↓	IVD's ECM components	human NP, AF cells (3D)	[9]
Collagen-2 (COL2A1)	Gene ↑	IVD's ECM components	bovine AF cells (3D)	[9]
Collagen-2 (COL2A1)	Gene ↓, Protein↓	IVD's ECM components	porcine NP in organ culture	[91]
Decorin (DCN)	Gene ↑	IVD's ECM components	porcine TZ cells	[92]
Decorin (DCN)	Gene ↓	IVD's ECM components	porcine NP cells	[92]
Lumican (LUM)	Gene ↓	IVD's ECM components	porcine NP cells	[92]
Ephrin-B2 (EFNB2)	Gene ↑	Signal transduction/transcription	human IVD tissue	[47]
Musculoapo-neurotic fibrosarcoma oncogene (MAF)	Gene ↑	Signal transduction/transcription	human IVD tissue	[47]
Nuclear receptor coactivator 3 (NCOA3)	Gene ↑	Signal transduction/transcription	human IVD tissue	[47]
Oncogene TC21 (RRAS2/TC21)	Gene ↑	Signal transduction/transcription	human IVD tissue	[47]
SOX9	Gene↓, Protein↓	Transcription factor	porcine NP in organ culture	[91]

AF = Annulus Fibrosus, IVD = Intervertebral Disc, NP = Nucleus Pulposus, TZ = Transition Zone, ↑ = upregulation, ↓ = downregulation, Only selected targets are discussed in the text

Table 2. Targets regulated by hypo-osmotic treatment.

Target Gene (symbol)	Expression level Change	Function	Model	Reference
Aquaporin-1 (AQP-1)	Protein ↓	Cell membrane proteins	rabbit NP cells	[73]
Transient potential receptor channel 4 (TRPV4)	Receptor Function (tyrosine phosphorylation)↑	Cell membrane proteins	HEK293	[54, 93]
Transient potential receptor channel 4 (TRPV4)	Protein ↑	Cell membrane proteins	equine chondrocytes	[60]
Transient potential receptor channel 4 (TRPV4)	Protein ↑	Cell membrane proteins	human IVD	[52]
Large-conductance Ca^{2+}-activated K^+ (BK_{Ca})	Protein ↑	Cell membrane proteins	equine chondrocytes	[60]
Matrix metalloproteinase-3 (MMP3)	Gene ↑	Enzyme – matrix turnover	bovine NP cells (3D)	[90]
Collagen-2 (COL2A1)	Gene ↑	IVD's ECM components	porcine TZ cells	[92]
Collagen-2 (COL2A1)	Gene ↓, Protein↓	IVD's ECM components	porcine NP in organ culture	[91]
Aggrecan (ACAN)	Gene ↑	IVD's ECM components	porcine TZ cells	[92]
Aggrecan (ACAN)	Gene ↓, Protein↓	IVD's ECM components	porcine NP in organ culture	[91]
Biglycan (BGN)	Gene ↑	IVD's ECM components	porcine TZ cells	[92]
Decorin (DCN)	Gene ↑	IVD's ECM components	porcine TZ cells	[92]
Lumican (LUM)	Gene ↓	IVD's ECM components	porcine NP cells	[92]
Tubulin (TUB)	Gene ↓	IVD's ECM components	porcine NP cells	[92]
Extracellular signal-regulated kinase ½ ($ERK_{1/2}$)	Protein ↑	Signaling pathway	equine chondrocytes	[60]
SOX9	Gene↓, Protein↓	Transcription factor	porcine NP in organ culture	[91]

AF = Annulus Fibrosus, IVD = Intervertebral Disc, NP = Nucleus Pulposus, TZ = Transition Zone,
↑ = upregulation, ↓ = downregulation, Only selected targets are discussed in the text

6.3 Cell responses to osmotic changes: target genes and signaling pathways

Cellular responses to changes in the osmotic environment are facilitated through multiple signaling mediators, including the nuclear factor of activated T-cells 5/tonicity response element-binding protein (TonEBP/NFAT5, also known as osmotic response element-binding protein (OREBP)), or signal-transduction pathways MAP kinases p38, ERK and JNK [60, 85, 91, 94-96]. These mediators do not only play a role in the cell regulatory volume mechanisms, but also initiate changes in other cellular processes such as cell survival, matrix turnover and inflammation (**Table 1a-c, Fig. 1**). Hence, the osmotic challenge and accompanying altered gene expression can contribute to the development and/or progression of the inflammatory responses in the IVD. However, the inflammation in the IVD is much more complex than how it is presented in the context of the IVD's osmotic environment (present review) and is reviewed elsewhere [28]. Briefly, inflammation can occur within the IVD, despite the IVD being immune-privileged (in a healthy state), as NP cells produce pro-inflammatory cytokines, while macrophages infiltrating the damaged IVD can further exacerbate the inflammation [28].

One of the most prominent signal transduction pathways that facilitates mammalian cell responses to numerous extracellular signals is the MAP kinase family. Three major members of the MAP kinase family are the extracellular signal-regulated kinases (ERK), c-Jun NH2-terminal kinases (JNK), and p38 isoforms (p38), which can be activated by multiple stimuli, such as growth factors [97, 98], inflammatory cytokines [99], as well as the osmotic stress [100]. Activation of each of these pathways controls several cellular functions, including among others cell cycle progression (ERK), cell proliferation and survival (JNK), cell growth, cell differentiation and –cell death, as well as inflammation and matrix catabolism (p38) [101-103]. In this review, each signal-transduction pathway is presented in the context of the IVD cell response to osmotic stress. However, these signaling pathways are also activated by different stimuli and can induce a range of cell responses not necessarily addressed within this review [104]. In the IVD, a hyper-osmotic treatment (500 and 600 mOsm/L) participates in the activation of the p38 pathway in bovine and rabbit NP cells [88, 95], and results in the ataxia telangiectasia-mutated (ATM)-mediated phosphorylation of p53 in response to DNA damage caused by hyper-osmotic shock [88, 105]. However,

MAPK activation and signaling is cell-type specific, with JNK activation occurring upon hypo-osmotic stimulation in IVD cells and upon hyper-osmotic stimulation in chondrocytes [106]. Furthermore, conflicting activation triggers are described for the ERK pathway, with authors reporting distinctive osmotic conditions for both activation and inhibition. These differences may arise from i) using different animal models and different definition of osmolarity levels (hypo-, iso- and hyper-osmotic values), ii) adjusting the osmolarity with various agents and iii) using different culture conditions, e.g. supplementation with growth factors (e.g. [107]). Activation of the ERK pathway under hyper-osmotic stress is observed in rabbit NP cells treated with 500-600 mOsm/L of medium adjusted with NaCl [95], rat NP cells treated with 450 mOsm/L media (adjusting agent unknown) [108] and bovine NP cells treated with 500 mOsm/L media adjusted with urea [109]. In rat NP cells, ERK phosphorylation leads to e.g. activation of TonEBP/NFAT5 [108] - an adaptation factor to high osmotic stress that protects IVD cells from undergoing apoptosis [108, 110]. In contrary, ERK inhibition can lead to the suppression of TonEBP/NFAT5 and the augmentation of cell apoptosis [NP cells in a porcine disc culture [100], rat NP cells [108], rabbit NP cells [95]). Activation of p38 and JNK induces cell apoptosis (rabbit NP cells [95]), indicating the involvement of these pathways in the degenerative shift under hyper-osmotic conditions. This is in line with the study of Haschtmann *et al.* [111], in which following a hyper-osmotic treatment (485 mOsm/L), cells (rabbit IVD in organ culture) exhibited a reversible drop in viability. Moreover in the IVD, the ERK pathway controls the expression of collagen type II [9, 92] and matrix metalloproteinase-2 (MMP2) [90], while collagen type I [9], IL-6 [47] and MMP-3 [90] are identified as targets of the p38/MAPK pathway [112-114]. However, aggrecans are regulated by both pathways: in bovine NP cells, lactoferricin-induced upregulation of aggrecan mRNA levels are decreased when p38/MAPK and ERK pathways are inhibited by SD203580 and PD98059 respectively [115]. In a study by [116], prolonged activation of the $ERK_{1/2}$ pathway increases the mRNA expression level of aggrecan in human NP cells. On the contrary, leptin-induced p38 phosphorylation upregulates aggrecanases and downregulates aggrecan on the gene and protein level in human NP cells [117]. Another signalling pathway that could be involved in the IVD's osmo-adaptation, but has not been investigated thus far, is the non-canonical PKC pathway. Increased activity of the PKC pathway is involved in the regulation of aggrecan expression, matrix synthesis and cell proliferation [116, 118, 119] in rat and human NP cells, as well as bovine

chondrocytes, and may be involved in Ca^{2+}-mediated activation of the TRPV4 channel [120, 121], which is previously linked to IVD osmosensing [52].

One of the key cellular osmoregulative mediators is TonEBP/NFAT5, which modulates the expression of genes induced by osmotic stress. In response to hyper-osmotic challenges and upon the activation of the ERK and p38-MAPK pathways, TonEBP/NFAT5 accumulates in the cell nucleus [108, 122] (**Fig. 1**). Consequently, it induces the expression of genes that are involved in the production of organic osmolytes (to counterbalance the osmotic challenge) [123], aggrecan [110] and the pro-inflammatory cytokines, IL-1β and tumor necrosis factor (TNF)-α [124-126], which have known implications in IVD degeneration [49, 127, 128]. In the IVD, hyper-osmotic stress is not the only regulator of the TonEBP/NFAT5 complex. The expression and activity of TonEBP/NFAT5, which is calcium-dependent [129, 130], can be also regulated by growth factors, such as bone morphogenetic protein 2 (BMP-2) and transforming growth factor-β (TFG-β) [97, 131]. These multimodal activation mechanisms indicate that TonEBP/NFAT5 is critical not only for osmoregulation, but also for other cellular functions (e.g. cell survival, matrix synthesis, etc.). TonEBP/NFAT5 modulates the NF-κB pathway, which is a pivotal element in the cellular response to inflammation and stress, with downstream targets including TNF-α, IL-1β, IL-6 and IL-8 [132-134]. The NF-κB pathway is implicated in many chronic conditions such as osteoarthritis [135], osteoporosis [136] and IVD degeneration [137-139]. Changes in cell volume, triggered by hyper-osmotic conditions and sensed via an osmo-sensing channel (e.g. TRP), can be perceived by a cell as a stress signal. Such a signal can initiate a pro-inflammatory cascade via the ROS-mediated activation of the NF-κB (and/ or p38/MAPK) pathway [140-143] and activate the NLRP3 inflammasome – a protein complex, downstream targets of which are caspase-1 and IL-1β [144]. In various cell types, activation of the NLRP3 inflammasome during RVD mechanisms leads to the activation of the pro-inflammatory cytokine IL-1β [145-147]. Interestingly, a positive relationship between the expression of NLRP3, IL-1β, and the IVD degeneration grade exists (NP tissue, mRNA and protein level) [144, 148]. This indicates that NLRP3 may constitute a putative target between the altered osmotic environment and the inflammation and, thus, be a possible therapeutic target for the treatment of IVD degeneration [149].

Acid-Sensing Ion Channels (ASICs) are proton activated cation channels [150] that are hypothesized to be of functional importance in IVD osmoprotection and pathophysiology

[151-153] by facilitating the adaptation of IVD cells (rodent) to an acidic and/or hyper-osmotic environment via the ERK signaling pathway [98]. The presence of ASIC is also confirmed on the mRNA and protein level in healthy and degenerated human IVD tissue [154], with higher expression levels during IVD degeneration, possibly due to changes in pH and hydrostatic pressure [154]. The viability of rat NP cells cultured in hyper-osmotic (450 mOsm/L) media decreases in a dose-dependent manner (from ~100% to ~40%) when the ASIC3 inhibitor amiloride (10–100 µM, 24h, MTT) was added [98]. However, the same study shows that increasing the osmolarity from 330 to 450 mOsm/L, without the ASIC3 inhibitor does not have a significant influence on NP cells viability [98]. In the context of the IVD and osmoregulation, an acidic pH (which is a characteristic of the degenerated IVD) downregulates the synthesis of PGs, thereby contributing to low extracellular osmolarity [155, 156]. The altered expression of ASICs can, on the one hand, indicate a causative and hence detrimental role in IVD degeneration or may, on the other hand, constitute a mechanism to better cope with the degenerative conditions [98].

Therapeutic modulation of osmosensing

Dysregulated tissue osmolarity is a hallmark of several chronic diseases, suggesting that the efficiency of the body's osmoprotective mechanisms decreases with age and/or tissue degeneration [32]. Non-physiological concentrations of intra- and extra-cellular structural molecules and signalling mediators alter the cellular responses to otherwise normal stimuli, such as physiological loading. A healthy IVD could be described by its ability to effectively adapt to diurnal osmotic changes and auto-regulate itself, without damage. From this perspective, a deviation into extreme osmotic conditions (either hyper- or hypo-osmotic), concomitant with a decreased adaptation ability, can have detrimental consequences on the IVD. Osmotic stress can be potentially counteracted by promoting osmoadaptation through stimulation of cellular defense mechanisms [16] or by combating the consequences of dysregulated osmosensing [157].

A key intracellular mediator of osmoadaptation is the transcription factor TonEBP/NFAT5, which controls the expression of genes involved in the response to hyper-osmolarity [158] and supports cell survival, especially in tissues regularly experiencing hyper-osmotic stress [108, 110, 130, 159]. Therefore, stimulating and promoting TonEBP/NFAT5 could have beneficial effects on the IVD homeostasis in a situation when the osmotic balance or the

cell's adaptation capability are disturbed. Adaptation of rat NP cells to hyperosmolarity is mediated via ERK- and p38-induced activation of TonEBP/NFAT5 [108], which regulates water balance through expression of several target genes (e.g. aquaporins [159]). In rat NP cells, dominant negative (DN)-NFAT5 significantly reduced cell viability and activated caspase 3, suggesting the pro-survival role of TonEBP/NFAT5 in hyperosmotic conditions [110]. The osmoprotective activity of TonEBP/NFAT5 could be therapeutically enhanced; however, TonEBP/NFAT5 also participates in pro-inflammatory responses in the IVD [126]. Therefore, the desired TonEBP/NFAT5-modulating compounds should ideally reduce its potential chronic pro-inflammatory effect, while enhancing its osmoprotective activity. TonEBP/NFAT5 inhibitors that selectively suppress the expression of pro-inflammatory genes without hampering TonEBP/NFAT5-induced osmoadaptation are developed and tested in a model of chronic arthritis, representing the first steps in this direction [160]. The molecular signals directing TonEBP/NFAT5 towards osmoadaptation and/or inflammation in IVD cells are currently unknown. For example, in macrophages, the putative sensors that discriminate between pro-inflammatory and osmoprotective effects of TonEBP/NFAT5 are the ROS [161]. Among other functions, TonEBP/ NFAT5 positively regulates synthesis and transport of osmolytes, gene expression of AQPs and synthesis of extracellular matrix components, all of which could be possibly therapeutically enhanced.

Organic osmolytes are solutes (e.g. sugars, polyols, amino acids) that protect biomolecules from the damage caused by changing osmotic pressure and dehydration and, thereby, provide cytoprotection and anti-inflammatory effects [162]. Natural osmolytes participate in regenerating native protein forms from unfolded states, restoring proper protein functions and, thus, possibly preventing disease development [163]. Physiological concentrations of osmolytes in the IVD and their effects in and outside IVD cells have not yet been thoroughly investigated [109]. Synthesis and uptake of osmolytes in the IVD can be possibly regulated by TonEBP/ NFAT5-COX-2-PGE2 signaling, as an osmoprotective role is demonstrated for this pathway [164, 165]. In renal cells subjected to hyper-osmolarity, COX-2 is involved in the accumulation of osmolytes [166] and COX-2 inhibition reduces cell viability [167]. Interestingly, a recent study testing TonEBP/NFAT5 in mouse hyper-osmotic IVD organ cultures shows that TonEBP/NFAT5 also provides cytoprotective effects in the IVD by inducing COX-2 [130]. In view of these findings, currently used COX-2-targeting drugs could impair IVD osmoadaptation mechanisms [168] and further contribute to the

pathophysiology of DDD. Therapeutic enhancement of osmolyte function by a COX-2-unrelated mechanism could potentially increase resistance of the IVD to osmotic stress.

Dysfunction or aberrant expression of various AQPs is likely implicated in the pathogenesis of IVD degeneration. Inducing the expression and/or activity of certain AQPs can promote an exchange of fluids in the NP, possibly reducing the progression of DDD. Both water permeability and ionic conductance of AQPs can be positively regulated by PKC [169] and cyclic nucleotides [170]. However, these molecules control numerous cellular processes and their therapeutic modulation might produce detrimental off-target effects [171]. Specific upregulation of AQP gene expression could be achieved by CRISPR gene editing, e.g. using dCas fused with VP64 domains targeted to AQP gene enhancers [172].

As a membrane receptor, TRPV4 could potentially be regulated by specific agonists or antagonists, to prevent an age-related loss of ECM and reduce inflammation in the IVD [52]. However, involvement of TRPV4 in these processes is rather complex and a tight balance in the expression/ regulation of TRPV4 is crucial in the maintenance of the musculoskeletal health. As an example, blocking TRPV4 with the antagonist GSK205 reduces chondrocyte responses to hypo-osmotic stress, including RVD and production of PGE2 (porcine cells) [65], while activating TRPV4 with 4αPDD inhibits the production of the pro-inflammatory mediator nitric oxide in rat chondrocytes [173]. The importance of a balance in TRPV4 expression/function is also shown in mouse models, where loss of TRPV4 leads to a progressive osteoarthritic joint degeneration [68], while gain of function causes various skeletal dysplasias [174, 175]. Therefore, therapeutic TRPV4 agonists or antagonists should be specific [e.g. 4α-PDD or GSK2193874, respectively [176]] (e.g. injections) and only once the benefits of modulating TRPV4 have been clearly demonstrated. Stable overexpression or knock-out of TRPV4 could be delivered into the IVD, e.g. in genetically engineered therapeutic cells.

Other therapeutic approaches could include augmentation of extracellular matrix, e.g. by upregulating glucuronosyltransferase 1, a key TonEBP/ NFAT5-dependent regulator of glycosaminoglycan synthesis [129], or by implanting biomaterials. Osmoprotective moieties, such as chondroitin sulphate, can be incorporated into injectable hydrogels to increase hydration of the synthetic matrix in tissue engineering applications [177, 178].

6.4 Conclusion and outlook

Changes in the IVD hydration and osmolarity ranging from ~ 430 (iso-osmotic) to ~ 496 mOsm/L (hyperosmotic) can be observed during daily life activities. From this perspective, the osmotic environment in the IVD is unusual, as the osmotic range which is physiological for the IVD would be considered high for other tissues (e.g. blood plasma with osmolarity of ~ 300 mOsm/L). However, a reduction in tissue osmolarity to ~ 300 mOsm/L (hypo-osmotic) is a consequence of a cascade of degenerative changes and a hallmark of IVD degeneration. In this review, an overview of the existing studies on IVD osmolarity, its potential intersection with IVD inflammation and how this knowledge could be translated into treatment strategies is presented. Increasing scientific evidence points towards a crucial role of ion channels (such as TRP channels) in the regulatory volume control mechanisms in the IVD, as well as in cartilage – a tissue with similar characteristics to the NP tissue. Simultaneously, AQPs are an emerging target involved not only in osmosensing, but also in IVD degeneration and inflammation. Importantly, TonEBP/NFAT5 (co-activated by calcium and ERK/ p38 signaling) facilitates the IVD adaptation to fluctuations of its osmotic environment. Changes in water and ion concentrations affect the homeostasis of the IVD, as indicated by dysregulation of ECM synthesis under hypo- and hyper-osmotic conditions, but many details on the underlying mechanisms are still unknown. Several questions remain to be answered, such as:

- What is the underlying mechanism of the RVD and RVI response in NP and AF cells?
- What is the mechanistic function of cell membrane carriers and ion channels in IVD osmosensing and osmoadaptation?
- Do the cell membrane carriers and ion channels interact in the IVD?
- Can the activation of PKC and NF-κB pathways be osmolarity-induced?
- What is the role of these pathways in osmoadaptation and/or osmolarity-induced inflammation?

A broader understanding of how IVD cells react to altered osmolarity, e.g. in relation to PG synthesis, cell survival/apoptosis or inflammation, is crucial when aiming to advance the current concepts of IVD pathophysiology. Once these mechanisms are better understood and possible targets are identified, suitable therapeutics that successfully and specifically modulate osmoadaptation can be developed. This new class of anti-inflammatory and

regenerative therapeutics may target the osmoprotective transcription factor TonEBP/NFAT5 or osmo-sensing membrane proteins such as TRPV4 or AQPs. Gene editing techniques (e.g. CRISPR/Cas) can be used to modulate the expression/activity of osmosensing-associated genes in locally delivered autologous therapeutic cells. For example, genes regulating the activity of osmosensors or synthesis and transport of osmolytes can be activated by dCas fused with VP64 domains or switched off by Cas-mediated knock-out/knock-down. Antiinflammatory and regenerative therapeutics may be combined with gene editing techniques, with the overall aim of maintaining proper function of the cellular osmoadaptation sensors and the ECM and to ensure efficient transport of water and solutes through loaded IVD tissue.

References

1. Roughley, P.J., M. Alini, and J. Antoniou, *The role of proteoglycans in aging, degeneration and repair of the intervertebral disc.* Biochem Soc Trans, 2002. **30**(Pt 6): p. 869-74.
2. Urban, J.P. and J.F. McMullin, *Swelling pressure of the lumbar intervertebral discs: influence of age, spinal level, composition, and degeneration.* Spine (Phila Pa 1976), 1988. **13**(2): p. 179-87.
3. Oegema, T.R., Jr., *Biochemistry of the intervertebral disc.* Clin Sports Med, 1993. **12**(3): p. 419-39.
4. Cassinelli, E.H., R.A. Hall, and J.D. Kang, *Biochemistry of intervertebral disc degeneration and the potential for gene therapy applications.* Spine J, 2001. **1**(3): p. 205-14.
5. Urban, J.P. and S. Roberts, *Degeneration of the intervertebral disc.* Arthritis Res Ther, 2003. **5**(3): p. 120-30.
6. Takada, T., et al., *Fas ligand exists on intervertebral disc cells: a potential molecular mechanism for immune privilege of the disc.* Spine (Phila Pa 1976), 2002. **27**(14): p. 1526-30.
7. Luoma, K., et al., *Low back pain in relation to lumbar disc degeneration.* Spine (Phila Pa 1976), 2000. **25**(4): p. 487-92.
8. Maher, C., M. Underwood, and R. Buchbinder, *Non-specific low back pain.* Lancet, 2017. **389**(10070): p. 736-747.
9. Wuertz, K., et al., *Influence of extracellular osmolarity and mechanical stimulation on gene expression of intervertebral disc cells.* J Orthop Res, 2007. **25**(11): p. 1513-22.
10. Walker, B.F., *The prevalence of low back pain: a systematic review of the literature from 1966 to 1998.* J Spinal Disord, 2000. **13**(3): p. 205-17.
11. Wieser, S., et al., *Cost of low back pain in Switzerland in 2005.* Eur J Health Econ, 2011. **12**(5): p. 455-67.
12. Lodish, H., et al., *Osmosis, Water channels, and the regulation of cell volume.* 2000.
13. Baltz, J.M., *Media composition: salts and osmolality*, in *Embryo Culture*. 2012, Springer. p. 61-80.
14. Hoffmann, E.K., I.H. Lambert, and S.F. Pedersen, *Physiology of cell volume regulation in vertebrates.* Physiol Rev, 2009. **89**(1): p. 193-277.
15. Sivan, S.S., E. Wachtel, and P. Roughley, *Structure, function, aging and turnover of aggrecan in the intervertebral disc.* Biochim Biophys Acta, 2014. **1840**(10): p. 3181-9.

16. Johnson, Z.I., I.M. Shapiro, and M.V. Risbud, *Extracellular osmolarity regulates matrix homeostasis in the intervertebral disc and articular cartilage: evolving role of TonEBP.* Matrix biology : journal of the International Society for Matrix Biology, 2014. **40**(2): p. 10-6.
17. Urban, J.P., et al., *Swelling pressures of proteoglycans at the concentrations found in cartilaginous tissues.* Biorheology, 1979. **16**(6): p. 447-64.
18. Urban, J.P. and A. Maroudas, *Swelling of the intervertebral disc in vitro.* Connect Tissue Res, 1981. **9**(1): p. 1-10.
19. Erwin, W.M. and K.E. Hood, *The cellular and molecular biology of the intervertebral disc: A clinician's primer.* J Can Chiropr Assoc, 2014. **58**(3): p. 246-57.
20. Shirazi-Adl, A., *Analysis of large compression loads on lumbar spine in flexion and in torsion using a novel wrapping element.* J Biomech, 2006. **39**(2): p. 267-75.
21. McMillan, D., G. Garbutt, and M. Adams, *Effect of sustained loading on the water content of intervertebral discs: implications for disc metabolism.* Annals of the rheumatic diseases, 1996. **55**(12): p. 880-887.
22. Urban, J., *The effect of physical factors on disc cell metabolism.* Musculoskeletal Soft-Tissue Aging: Impact on Mobility, 1993.
23. Newell, N., et al., *Biomechanics of the human intervertebral disc: A review of testing techniques and results.* J Mech Behav Biomed Mater, 2017. **69**: p. 420-434.
24. Belavy, D.L., et al., *Running exercise strengthens the intervertebral disc.* Sci Rep, 2017. **7**: p. 45975.
25. Belavy, D.L., et al., *Can Exercise Positively Influence the Intervertebral Disc?* Sports Med, 2016. **46**(4): p. 473-85.
26. Le Maitre, C.L., A.J. Freemont, and J.A. Hoyland, *Localization of degradative enzymes and their inhibitors in the degenerate human intervertebral disc.* J Pathol, 2004. **204**(1): p. 47-54.
27. Sztrolovics, R., et al., *Aggrecan degradation in human intervertebral disc and articular cartilage.* Biochemical Journal, 1997. **326**: p. 235-241.
28. Molinos, M., et al., *Inflammation in intervertebral disc degeneration and regeneration.* J R Soc Interface, 2015. **12**(104): p. 20141191.
29. Ishihara, H., et al., *Proteoglycan synthesis in the intervertebral disk nucleus: the role of extracellular osmolality.* American Journal of Physiology-Cell Physiology, 1997. **272**(5): p. C1499-C1506.
30. van Dijk, B., E. Potier, and K. Ito, *Culturing bovine nucleus pulposus explants by balancing medium osmolarity.* Tissue Engineering Part C: Methods, 2011. **17**(11): p. 1089-1096.

31. Appelboom, J.W., et al., *The freezing point depression of mammalian tissues in relation to the question of osmotic activity of cell fluid.* J Gen Physiol, 1956. **40**(2): p. 183-99.
32. Brocker, C., D.C. Thompson, and V. Vasiliou, *The role of hyperosmotic stress in inflammation and disease.* Biomol Concepts, 2012. **3**(4): p. 345-364.
33. Hooper, L., et al., *Diagnostic accuracy of calculated serum osmolarity to predict dehydration in older people: adding value to pathology laboratory reports.* BMJ Open, 2015. **5**(10): p. e008846.
34. Dawson, D.C., *Water Movement Through Lipid Bilayers, Pores, and Plasma Membranes: Theory and Reality.* Science, 1988. **240**(4849): p. 228-229.
35. Reuss, L., *Water transport across cell membranes.* eLS, 2012.
36. McManus, M.L., K.B. Churchwell, and K. Strange, *Regulation of cell volume in health and disease.* N Engl J Med, 1995. **333**(19): p. 1260-6.
37. Pritchard, S., G.R. Erickson, and F. Guilak, *Hyperosmotically induced volume change and calcium signaling in intervertebral disk cells: the role of the actin cytoskeleton.* Biophysical journal, 2002. **83**(5): p. 2502-10.
38. Guilak, F., et al., *Viscoelastic properties of intervertebral disc cells. Identification of two biomechanically distinct cell populations.* Spine (Phila Pa 1976), 1999. **24**(23): p. 2475-83.
39. Hunter, C.J., et al., *Osmoregulatory function of large vacuoles found in notochordal cells of the intervertebral disc running title: an osmoregulatory vacuole.* Mol Cell Biomech, 2007. **4**(4): p. 227-37.
40. Kulbacka, J., et al., *Cell Membrane Transport Mechanisms: Ion Channels and Electrical Properties of Cell Membranes.* Adv Anat Embryol Cell Biol, 2017. **227**: p. 39-58.
41. Perland, E. and R. Fredriksson, *Classification Systems of Secondary Active Transporters.* Trends Pharmacol Sci, 2017. **38**(3): p. 305-315.
42. Hediger, M.A., et al., *The ABCs of solute carriers: physiological, pathological and therapeutic implications of human membrane transport proteinsIntroduction.* Pflugers Arch, 2004. **447**(5): p. 465-8.
43. Jones, R.S. and M.E. Morris, *Monocarboxylate Transporters: Therapeutic Targets and Prognostic Factors in Disease.* Clin Pharmacol Ther, 2016. **100**(5): p. 454-463.
44. Williams, G.R., *Thyroid hormone actions in cartilage and bone.* Eur Thyroid J, 2013. **2**(1): p. 3-13.
45. Alam, K., et al., *Downregulation of Organic Anion Transporting Polypeptide (OATP) 1B1 Transport Function by Lysosomotropic Drug Chloroquine: Implication in OATP-Mediated Drug-Drug Interactions.* Mol Pharm, 2016. **13**(3): p. 839-51.

46. Zair, Z.M., et al., *Pharmacogenetics of OATP (SLC21/SLCO), OAT and OCT (SLC22) and PEPT (SLC15) transporters in the intestine, liver and kidney.* Pharmacogenomics, 2008. **9**(5): p. 597-624.
47. Boyd, L.M., et al., *Osmolarity regulates gene expression in intervertebral disc cells determined by gene array and real-time quantitative RT-PCR.* Annals of biomedical engineering, 2005. **33**(8): p. 1071-1077.
48. Arroyo, J.P., K.T. Kahle, and G. Gamba, *The SLC12 family of electroneutral cation-coupled chloride cotransporters.* Mol Aspects Med, 2013. **34**(2-3): p. 288-98.
49. Sadowska, A., et al., *Inflammaging in cervical and lumbar degenerated intervertebral discs: analysis of proinflammatory cytokine and TRP channel expression.* Eur Spine J, 2017.
50. Krupkova, O., J. Zvick, and K. Wuertz-Kozak, *The role of transient receptor potential channels in joint diseases.* Eur Cell Mater, 2017. **34**: p. 180-201.
51. Alfredo Franco-Obregón, H.K., Helen Greutert, Timon Wernas, Marcel Egli, Elena Cambria, Miho Sekiguchi, Norbert Boos, Oliver Hausmann, Stephen Ferguson, Karin Wuertz-Kozak, *The balance of transient receptor potential channel TRPC6 to TRPC1 determines ageing and mechanotransduction in intervertebral disc cells* Conference Proceeding, ORS PSRS 4th International Spine Research Symposium, Split Rock Resort, Pennsylvania (PA), USA, 2017.
52. Walter, B.A., et al., *Reduced tissue osmolarity increases TRPV4 expression and pro-inflammatory cytokines in intervertebral disc cells.* European cells & materials, 2016. **32**(7): p. 123-36.
53. Numata, T., et al., *Activation of TRP Channels in Mammalian Systems*, in *TRP Channels*, M.X. Zhu, Editor. 2011: Boca Raton (FL).
54. Vriens, J., et al., *Cell swelling, heat, and chemical agonists use distinct pathways for the activation of the cation channel TRPV4.* Proceedings of the National Academy of Sciences, 2004. **101**(1): p. 396-401.
55. Birder, L.A., et al., *Altered urinary bladder function in mice lacking the vanilloid receptor TRPV1.* Nat Neurosci, 2002. **5**(9): p. 856-60.
56. Liedtke, W. and J.M. Friedman, *Abnormal osmotic regulation in trpv4-/- mice.* Proc Natl Acad Sci U S A, 2003. **100**(23): p. 13698-703.
57. Muraki, K., et al., *TRPV2 is a component of osmotically sensitive cation channels in murine aortic myocytes.* Circ Res, 2003. **93**(9): p. 829-38.
58. Ueda, T., et al., *The TRPV4 channel is a novel regulator of intracellular Ca2+ in human esophageal epithelial cells.* American journal of physiology. Gastrointestinal and liver physiology, 2011. **301**(1): p. G138-47.

59. Yang, X.R., et al., *Upregulation of osmo-mechanosensitive TRPV4 channel facilitates chronic hypoxia-induced myogenic tone and pulmonary hypertension.* Am J Physiol Lung Cell Mol Physiol, 2012. **302**(6): p. L555-68.
60. Hdud, I.M., A. Mobasheri, and P.T. Loughna, *Effect of osmotic stress on the expression of TRPV4 and BKCa channels and possible interaction with ERK1/2 and p38 in cultured equine chondrocytes.* American journal of physiology. Cell physiology, 2014. **306**(11): p. C1050-7.
61. Becker, D., et al., *TRPV4 exhibits a functional role in cell-volume regulation.* J Cell Sci, 2005. **118**(Pt 11): p. 2435-40.
62. Liedtke, W., et al., *Vanilloid receptor-related osmotically activated channel (VR-OAC), a candidate vertebrate osmoreceptor.* Cell, 2000. **103**(3): p. 525-35.
63. Pan, Z., et al., *Dependence of regulatory volume decrease on transient receptor potential vanilloid 4 (TRPV4) expression in human corneal epithelial cells.* Cell Calcium, 2008. **44**(4): p. 374-85.
64. Arniges, M., et al., *Swelling-activated Ca2+ entry via TRPV4 channel is defective in cystic fibrosis airway epithelia.* J Biol Chem, 2004. **279**(52): p. 54062-8.
65. Phan, M.N., et al., *Functional characterization of TRPV4 as an osmotically sensitive ion channel in porcine articular chondrocytes.* Arthritis Rheum, 2009. **60**(10): p. 3028-37.
66. Liu, C. and C. Montell, *Forcing open TRP channels: Mechanical gating as a unifying activation mechanism.* Biochem Biophys Res Commun, 2015. **460**(1): p. 22-5.
67. Palacio-Mancheno, P.E., et al., *Hyperosmolarity induces notochordal cell differentiation with aquaporin3 upregulation and reduced N-cadherin expression.* J Orthop Res, 2017.
68. Clark, A.L., et al., *Chondroprotective role of the osmotically sensitive ion channel transient receptor potential vanilloid 4: age- and sex-dependent progression of osteoarthritis in Trpv4-deficient mice.* Arthritis Rheum, 2010. **62**(10): p. 2973-83.
69. Qu, Y.J., et al., *Effect of TRPV4-p38 MAPK Pathway on Neuropathic Pain in Rats with Chronic Compression of the Dorsal Root Ganglion.* Biomed Res Int, 2016. **2016**: p. 6978923.
70. Chen, Y., et al., *Temporomandibular joint pain: a critical role for Trpv4 in the trigeminal ganglion.* Pain, 2013. **154**(8): p. 1295-304.
71. Richardson, S.M., et al., *Aquaporin expression in the human intervertebral disc.* J Mol Histol, 2008. **39**(3): p. 303-9.
72. Tas, U., et al., *Aquaporin-1 and aquaporin-3 expressions in the intervertebral disc of rats with aging.* Balkan Med J, 2012. **29**(4): p. 349-53.

73. Wang, F. and Y. Zhu, *Aquaporin-1: a potential membrane channel for facilitating the adaptability of rabbit nucleus pulposus cells to an extracellular matrix environment.* J Orthop Sci, 2011. **16**(3): p. 304-12.
74. Johnson, Z.I., et al., *Aquaporin 1 and 5 expression decreases during human intervertebral disc degeneration: Novel HIF-1-mediated regulation of aquaporins in NP cells.* Oncotarget, 2015. **6**(14): p. 11945-58.
75. Hoffman, H., et al., *Aquaporin-1 Expression in Herniated Human Lumbar Intervertebral Discs.* Global Spine J, 2017. **7**(2): p. 133-140.
76. Benfenati, V., et al., *An aquaporin-4/transient receptor potential vanilloid 4 (AQP4/TRPV4) complex is essential for cell-volume control in astrocytes.* Proceedings of the National Academy of Sciences of the United States of America, 2011. **108**(6): p. 2563-8.
77. Jo, A.O., et al., *TRPV4 and AQP4 Channels Synergistically Regulate Cell Volume and Calcium Homeostasis in Retinal Muller Glia.* J Neurosci, 2015. **35**(39): p. 13525-37.
78. Xie, H., et al., *Aquaporin 3 protects against lumbar intervertebral disc degeneration via the Wnt/beta-catenin pathway.* Int J Mol Med, 2016. **37**(3): p. 859-64.
79. Takeuchi, K., et al., *Downregulation of aquaporin 9 decreases catabolic factor expression through nuclear factorkappaB signaling ins chondrocytes.* Int J Mol Med, 2018.
80. Haneda, M., et al., *Depletion of aquaporin 1 decreased ADAMTS4 expression in human chondrocytes.* Mol Med Rep, 2018. **17**(4): p. 4874-4882.
81. Sun, Z., et al., *IL-1beta promotes ADAMTS enzyme-mediated aggrecan degradation through NF-kappaB in human intervertebral disc.* J Orthop Surg Res, 2015. **10**: p. 159.
82. Hiyama, A., et al., *Enhancement of intervertebral disc cell senescence by WNT/beta-catenin signaling-induced matrix metalloproteinase expression.* Arthritis Rheum, 2010. **62**(10): p. 3036-47.
83. Hiyama, A., et al., *The relationship between the Wnt/beta-catenin and TGF-beta/BMP signals in the intervertebral disc cell.* J Cell Physiol, 2011. **226**(5): p. 1139-48.
84. Wang, M., et al., *Conditional activation of beta-catenin signaling in mice leads to severe defects in intervertebral disc tissue.* Arthritis Rheum, 2012. **64**(8): p. 2611-23.
85. Ren, Y., et al., *Hyperosmolarity-induced AQP5 upregulation promotes inflammation and cell death via JNK1/2 Activation in human corneal epithelial cells.* Sci Rep, 2017. **7**(1): p. 4727.

86. Cai, L., et al., *Overexpression of aquaporin 4 in articular chondrocytes exacerbates the severity of adjuvant-induced arthritis in rats: an in vivo and in vitro study.* J Inflamm (Lond), 2017. **14**: p. 6.
87. J, S., et al., *AQUAPORIN EXPRESSION IN THE HUMAN INTERVERTEBRAL DISC.* Orthopaedic Proceedings, 2017. **99-B**(SUPP_10): p. 23-23.
88. Mavrogonatou, E. and D. Kletsas, *High osmolality activates the G1 and G2 cell cycle checkpoints and affects the DNA integrity of nucleus pulposus intervertebral disc cells triggering an enhanced DNA repair response.* DNA Repair (Amst), 2009. **8**(8): p. 930-43.
89. Mavrogonatou, E., et al., *Deficiency in the α1 subunit of Na+/K+-ATPase enhances the anti-proliferative effect of high osmolality in nucleus pulposus intervertebral disc cells.* Journal of cellular physiology, 2015. **230**(12): p. 3037-48.
90. Neidlinger-Wilke, C., et al., *Interactions of environmental conditions and mechanical loads have influence on matrix turnover by nucleus pulposus cells.* Journal of orthopaedic research : official publication of the Orthopaedic Research Society, 2012. **30**(1): p. 112-21.
91. Li, P., et al., *Osmolarity affects matrix synthesis in the nucleus pulposus associated with the involvement of MAPK pathways: A study of ex vivo disc organ culture system.* J Orthop Res, 2016. **34**(6): p. 1092-100.
92. Chen, J., et al., *Matrix protein gene expression in intervertebral disc cells subjected to altered osmolarity.* Biochem Biophys Res Commun, 2002. **293**(3): p. 932-8.
93. Xu, H., et al., *Regulation of a transient receptor potential (TRP) channel by tyrosine phosphorylation. SRC family kinase-dependent tyrosine phosphorylation of TRPV4 on TYR-253 mediates its response to hypotonic stress.* J Biol Chem, 2003. **278**(13): p. 11520-7.
94. Sheikh-Hamad, D. and M.C. Gustin, *MAP kinases and the adaptive response to hypertonicity: functional preservation from yeast to mammals.* Am J Physiol Renal Physiol, 2004. **287**(6): p. F1102-10.
95. Dong, Z.H., et al., *The roles of MAPKs in rabbit nucleus pulposus cell apoptosis induced by high osmolality.* Eur Rev Med Pharmacol Sci, 2014. **18**(19): p. 2835-45.
96. Aramburu, J. and C. Lopez-Rodriguez, *Brx shines a light on the route from hyperosmolarity to NFAT5.* Sci Signal, 2009. **2**(65): p. pe20.
97. Hiyama, A., et al., *BMP-2 and TGF-beta stimulate expression of beta1,3-glucuronosyl transferase 1 (GlcAT-1) in nucleus pulposus cells through AP1, TonEBP, and Sp1: role of MAPKs.* J Bone Miner Res, 2010. **25**(5): p. 1179-90.

98. Uchiyama, Y., et al., *Expression of acid-sensing ion channel 3 (ASIC3) in nucleus pulposus cells of the intervertebral disc is regulated by p75NTR and ERK signaling.* J Bone Miner Res, 2007. **22**(12): p. 1996-2006.
99. Klawitter, M., et al., *Triptolide exhibits anti-inflammatory, anti-catabolic as well as anabolic effects and suppresses TLR expression and MAPK activity in IL-1beta treated human intervertebral disc cells.* Eur Spine J, 2012. **21 Suppl 6**: p. S850-9.
100. Li, P., et al., *Role of the ERK1/2 pathway in osmolarity effects on nucleus pulposus cell apoptosis in a disc perfusion culture.* J Orthop Res, 2017. **35**(1): p. 86-92.
101. Johnson, G.L. and R. Lapadat, *Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases.* Science, 2002. **298**(5600): p. 1911-2.
102. Yang, C., et al., *Differential expression of p38 MAPK alpha, beta, gamma, delta isoforms in nucleus pulposus modulates macrophage polarization in intervertebral disc degeneration.* Sci Rep, 2016. **6**: p. 22182.
103. Studer, R.K., et al., *p38 MAPK inhibition in nucleus pulposus cells: a potential target for treating intervertebral disc degeneration.* Spine (Phila Pa 1976), 2007. **32**(25): p. 2827-33.
104. Wong, W., *Focus issue: coping with cellular stress.* Sci Signal, 2009. **2**(96): p. eg14.
105. Kishi, H., et al., *Osmotic shock induces G1 arrest through p53 phosphorylation at Ser33 by activated p38MAPK without phosphorylation at Ser15 and Ser20.* J Biol Chem, 2001. **276**(42): p. 39115-22.
106. Racz, B., et al., *Hyperosmotic stress-induced apoptotic signaling pathways in chondrocytes.* Bone, 2007. **40**(6): p. 1536-43.
107. Mavrogonatou, E. and D. Kletsas, *Effect of varying osmotic conditions on the response of bovine nucleus pulposus cells to growth factors and the activation of the ERK and Akt pathways.* Journal of orthopaedic research : official publication of the Orthopaedic Research Society, 2010. **28**(10): p. 1276-82.
108. Tsai, T.T., et al., *MEK/ERK signaling controls osmoregulation of nucleus pulposus cells of the intervertebral disc by transactivation of TonEBP/OREBP.* J Bone Miner Res, 2007. **22**(7): p. 965-74.
109. Mavrogonatou, E. and D. Kletsas, *Differential response of nucleus pulposus intervertebral disc cells to high salt, sorbitol, and urea.* J Cell Physiol, 2012. **227**(3): p. 1179-87.
110. Tsai, T.T., et al., *TonEBP/OREBP is a regulator of nucleus pulposus cell function and survival in the intervertebral disc.* J Biol Chem, 2006. **281**(35): p. 25416-24.
111. Haschtmann, D., J.V. Stoyanov, and S.J. Ferguson, *Influence of diurnal hyperosmotic loading on the metabolism and matrix gene expression of a whole-*

organ intervertebral disc model. Journal of orthopaedic research : official publication of the Orthopaedic Research Society, 2006. **24**(10): p. 1957-66.
112. Sano, M., et al., *ERK and p38 MAPK, but not NF-kappaB, are critically involved in reactive oxygen species-mediated induction of IL-6 by angiotensin II in cardiac fibroblasts.* Circ Res, 2001. **89**(8): p. 661-9.
113. Park, J.J., et al., *Induction of proinflammatory cytokine production in intervertebral disc cells by macrophage-like THP-1 cells requires mitogen-activated protein kinase activity.* J Neurosurg Spine, 2016. **24**(1): p. 167-75.
114. Jung, Y.K., et al., *Calcium-phosphate Increases MMP3 and MMP13 through p38 Map Kinase and Calcineurin Signaling in Hypertrophic Chondrocyte.* Osteoarthritis and Cartilage, 2017. **25**: p. S317.
115. Kim, J.S., et al., *Lactoferricin mediates anabolic and anti-catabolic effects in the intervertebral disc.* J Cell Physiol, 2012. **227**(4): p. 1512-20.
116. Tsirimonaki, E., et al., *PKCepsilon signalling activates ERK1/2, and regulates aggrecan, ADAMTS5, and miR377 gene expression in human nucleus pulposus cells.* PLoS One, 2013. **8**(11): p. e82045.
117. Li, Z., et al., *Leptin downregulates aggrecan through the p38-ADAMST pathway in human nucleus pulposus cells.* PLoS One, 2014. **9**(10): p. e109595.
118. Arai, F., et al., *The expression and role of non-canonical (PKC) signaling in nucleus pulposus cell metabolism.* J Orthop Res, 2012. **30**(9): p. 1478-85.
119. Rottmar, M., et al., *Interference with the contractile machinery of the fibroblastic chondrocyte cytoskeleton induces re-expression of the cartilage phenotype through involvement of PI3K, PKC and MAPKs.* Exp Cell Res, 2014. **320**(2): p. 175-87.
120. Fan, H.C., X. Zhang, and P.A. McNaughton, *Activation of the TRPV4 ion channel is enhanced by phosphorylation.* J Biol Chem, 2009. **284**(41): p. 27884-91.
121. Xu, F., E. Satoh, and T. Iijima, *Protein kinase C-mediated Ca2+ entry in HEK 293 cells transiently expressing human TRPV4.* Br J Pharmacol, 2003. **140**(2): p. 413-21.
122. Ho, S.N., *Intracellular water homeostasis and the mammalian cellular osmotic stress response.* J Cell Physiol, 2006. **206**(1): p. 9-15.
123. Lee, S.D., et al., *TonEBP stimulates multiple cellular pathways for adaptation to hypertonic stress: organic osmolyte-dependent and -independent pathways.* Am J Physiol Renal Physiol, 2011. **300**(3): p. F707-15.
124. Lee, J.H., et al., *NFAT5 induction and its role in hyperosmolar stressed human limbal epithelial cells.* Invest Ophthalmol Vis Sci, 2008. **49**(5): p. 1827-35.
125. Trama, J., W.Y. Go, and S.N. Ho, *The osmoprotective function of the NFAT5 transcription factor in T cell development and activation.* J Immunol, 2002. **169**(10): p. 5477-88.

126. Johnson, Z.I., et al., *TNF-alpha promotes nuclear enrichment of the transcription factor TonEBP/NFAT5 to selectively control inflammatory but not osmoregulatory responses in nucleus pulposus cells.* J Biol Chem, 2017. **292**(42): p. 17561-17575.
127. Risbud, M.V. and I.M. Shapiro, *Role of cytokines in intervertebral disc degeneration: pain and disc content.* Nat Rev Rheumatol, 2014. **10**(1): p. 44-56.
128. Lee, J.M., et al., *Interleukin-1beta induces angiogenesis and innervation in human intervertebral disc degeneration.* J Orthop Res, 2011. **29**(2): p. 265-9.
129. Hiyama, A., et al., *Activation of TonEBP by calcium controls {beta}1,3-glucuronosyltransferase-I expression, a key regulator of glycosaminoglycan synthesis in cells of the intervertebral disc.* J Biol Chem, 2009. **284**(15): p. 9824-34.
130. Choi, H., et al., *COX-2 expression mediated by calcium-TonEBP signaling axis under hyperosmotic conditions serves osmoprotective function in nucleus pulposus cells.* J Biol Chem, 2018. **293**(23): p. 8969-8981.
131. Halterman, J.A., H.M. Kwon, and B.R. Wamhoff, *Tonicity-independent regulation of the osmosensitive transcription factor TonEBP (NFAT5).* Am J Physiol Cell Physiol, 2012. **302**(1): p. C1-8.
132. Tak, P.P. and G.S. Firestein, *NF-kappaB: a key role in inflammatory diseases.* J Clin Invest, 2001. **107**(1): p. 7-11.
133. Lopez-Rodriguez, C., et al., *Bridging the NFAT and NF-kappaB families: NFAT5 dimerization regulates cytokine gene transcription in response to osmotic stress.* Immunity, 2001. **15**(1): p. 47-58.
134. Roth, I., et al., *Osmoprotective transcription factor NFAT5/TonEBP modulates nuclear factor-kappaB activity.* Mol Biol Cell, 2010. **21**(19): p. 3459-74.
135. Makarov, S.S., *NF-kappa B in rheumatoid arthritis: a pivotal regulator of inflammation, hyperplasia, and tissue destruction.* Arthritis Res, 2001. **3**(4): p. 200-6.
136. Kim, H.J., et al., *Antioxidant alpha-lipoic acid inhibits osteoclast differentiation by reducing nuclear factor-kappaB DNA binding and prevents in vivo bone resorption induced by receptor activator of nuclear factor-kappaB ligand and tumor necrosis factor-alpha.* Free Radic Biol Med, 2006. **40**(9): p. 1483-93.
137. Zhongyi, S., et al., *Effects of nuclear factor kappa B signaling pathway in human intervertebral disc degeneration.* Spine (Phila Pa 1976), 2015. **40**(4): p. 224-32.
138. Xu, K., et al., *Autophagy attenuates the catabolic effect during inflammatory conditions in nucleus pulposus cells, as sustained by NF-kappaB and JNK inhibition.* Int J Mol Med, 2015. **36**(3): p. 661-8.
139. Nasto, L.A., et al., *ISSLS prize winner: inhibition of NF-kappaB activity ameliorates age-associated disc degeneration in a mouse model of accelerated aging.* Spine (Phila Pa 1976), 2012. **37**(21): p. 1819-25.

140. Rajamaki, K., et al., *p38delta MAPK: A Novel Regulator of NLRP3 Inflammasome Activation With Increased Expression in Coronary Atherogenesis.* Arterioscler Thromb Vasc Biol, 2016. **36**(9): p. 1937-46.
141. Abolhassani, M., et al., *Hyperosmolarity causes inflammation through the methylation of protein phosphatase 2A.* Inflamm Res, 2008. **57**(9): p. 419-29.
142. Panahi, G., et al., *High glucose induces inflammatory responses in HepG2 cells via the oxidative stress-mediated activation of NF-kappaB, and MAPK pathways in HepG2 cells.* Arch Physiol Biochem, 2018: p. 1-7.
143. Schwartz, L., et al., *Is inflammation a consequence of extracellular hyperosmolarity?* J Inflamm (Lond), 2009. **6**: p. 21.
144. Chen, Z.H., et al., *Enhanced NLRP3, caspase-1, and IL- 1beta levels in degenerate human intervertebral disc and their association with the grades of disc degeneration.* Anat Rec (Hoboken), 2015. **298**(4): p. 720-6.
145. Compan, V., et al., *Cell volume regulation modulates NLRP3 inflammasome activation.* Immunity, 2012. **37**(3): p. 487-500.
146. Compan, V. and P. Pelegrin, *Methods to Study Cell Swelling-Induced Inflammasome Activation.* Methods Mol Biol, 2018. **1714**: p. 191-197.
147. Ip, W.K. and R. Medzhitov, *Macrophages monitor tissue osmolarity and induce inflammatory response through NLRP3 and NLRC4 inflammasome activation.* Nat Commun, 2015. **6**: p. 6931.
148. Song, Y., et al., *Advanced glycation end products regulate anabolic and catabolic activities via NLRP3-inflammasome activation in human nucleus pulposus cells.* J Cell Mol Med, 2017. **21**(7): p. 1373-1387.
149. Tang, P., et al., *Honokiol alleviates the degeneration of intervertebral disc via suppressing the activation of TXNIP-NLRP3 inflammasome signal pathway.* Free Radic Biol Med, 2018. **120**: p. 368-379.
150. Yoder, N., C. Yoshioka, and E. Gouaux, *Gating mechanisms of acid-sensing ion channels.* Nature, 2018. **555**(7696): p. 397-401.
151. Uchiyama, Y., et al., *SMAD3 functions as a transcriptional repressor of acid-sensing ion channel 3 (ASIC3) in nucleus pulposus cells of the intervertebral disc.* J Bone Miner Res, 2008. **23**(10): p. 1619-28.
152. Li, X., et al., *Acid-sensing ion channel 1a-mediated calcium influx regulates apoptosis of endplate chondrocytes in intervertebral discs.* Expert Opin Ther Targets, 2014. **18**(1): p. 1-14.
153. Yuan, F.L., et al., *Involvement of acid-sensing ion channel 1a in matrix metabolism of endplate chondrocytes under extracellular acidic conditions through NF-kappaB transcriptional activity.* Cell Stress Chaperones, 2016. **21**(1): p. 97-104.

154. Cuesta, A., et al., *Acid-sensing ion channels in healthy and degenerated human intervertebral disc.* Connect Tissue Res, 2014. **55**(3): p. 197-204.
155. Wuertz, K., K. Godburn, and J.C. Iatridis, *MSC response to pH levels found in degenerating intervertebral discs.* Biochem Biophys Res Commun, 2009. **379**(4): p. 824-9.
156. Ohshima, H. and J.P. Urban, *The effect of lactate and pH on proteoglycan and protein synthesis rates in the intervertebral disc.* Spine (Phila Pa 1976), 1992. **17**(9): p. 1079-82.
157. van Dijk, B., et al., *Reduced tonicity stimulates an inflammatory response in nucleus pulposus tissue that can be limited by a COX-2-specific inhibitor.* J Orthop Res, 2015. **33**(11): p. 1724-31.
158. Lopez-Rodriguez, C., et al., *Loss of NFAT5 results in renal atrophy and lack of tonicity-responsive gene expression.* Proc Natl Acad Sci U S A, 2004. **101**(8): p. 2392-7.
159. Gajghate, S., et al., *Osmolarity and intracellular calcium regulate aquaporin2 expression through TonEBP in nucleus pulposus cells of the intervertebral disc.* J Bone Miner Res, 2009. **24**(6): p. 992-1001.
160. Han, E.J., et al., *Suppression of NFAT5-mediated Inflammation and Chronic Arthritis by Novel kappaB-binding Inhibitors.* EBioMedicine, 2017. **18**: p. 261-273.
161. Kim, N.H., et al., *Reactive oxygen species regulate context-dependent inhibition of NFAT5 target genes.* Exp Mol Med, 2013. **45**: p. e32.
162. Rabbani, G. and I. Choi, *Roles of osmolytes in protein folding and aggregation in cells and their biotechnological applications.* Int J Biol Macromol, 2018. **109**: p. 483-491.
163. Alfieri, R.R., et al., *Compatible osmolytes modulate the response of porcine endothelial cells to hypertonicity and protect them from apoptosis.* J Physiol, 2002. **540**(Pt 2): p. 499-508.
164. Kim, J.A., et al., *Hypertonicity stimulates PGE2 signaling in the renal medulla by promoting EP3 and EP4 receptor expression.* Kidney Int, 2009. **75**(3): p. 278-84.
165. Favale, N.O., et al., *Hypertonic induction of COX2 expression requires TonEBP/NFAT5 in renal epithelial cells.* Biochem Biophys Res Commun, 2009. **381**(3): p. 301-5.
166. Moeckel, G.W., et al., *COX2 activity promotes organic osmolyte accumulation and adaptation of renal medullary interstitial cells to hypertonic stress.* J Biol Chem, 2003. **278**(21): p. 19352-7.
167. Neuhofer, W., et al., *Chronic COX-2 inhibition reduces medullary HSP70 expression and induces papillary apoptosis in dehydrated rats.* Kidney Int, 2004. **65**(2): p. 431-41.

168. Bonner, J.S., et al., *Aspirin Impairs Transport of Protective Osmolytes in Renal Inner Medullary Collecting Duct Cells.* The Open Urology & Nephrology Journal, 2009. **2**(1).
169. Zhang, W., et al., *Aquaporin-1 channel function is positively regulated by protein kinase C.* J Biol Chem, 2007. **282**(29): p. 20933-40.
170. Lorenz, D., et al., *Cyclic AMP is sufficient for triggering the exocytic recruitment of aquaporin-2 in renal epithelial cells.* EMBO Rep, 2003. **4**(1): p. 88-93.
171. Cooke, M., et al., *Protein kinase C in cancer: The top five unanswered questions.* Mol Carcinog, 2017. **56**(6): p. 1531-1542.
172. Konermann, S., et al., *Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex.* Nature, 2015. **517**(7536): p. 583-8.
173. Hu, F., W. Zhu, and L. Wang, *MicroRNA-203 up-regulates nitric oxide expression in temporomandibular joint chondrocytes via targeting TRPV4.* Arch Oral Biol, 2013. **58**(2): p. 192-9.
174. Mah, W., et al., *Gain-of-function mutation in TRPV4 identified in patients with osteonecrosis of the femoral head.* J Med Genet, 2016. **53**(10): p. 705-9.
175. Weinstein, M.M., et al., *Mice expressing mutant Trpv4 recapitulate the human TRPV4 disorders.* J Bone Miner Res, 2014. **29**(8): p. 1815-1822.
176. McNulty, A.L., et al., *TRPV4 as a therapeutic target for joint diseases.* Naunyn Schmiedebergs Arch Pharmacol, 2015. **388**(4): p. 437-50.
177. Farnsworth, N.L., et al., *Ionic osmolytes and intracellular calcium regulate tissue production in chondrocytes cultured in a 3D charged hydrogel.* Matrix Biol, 2014. **40**: p. 17-26.
178. Chen, F., et al., *An Injectable Enzymatically Crosslinked Carboxymethylated Pullulan/Chondroitin Sulfate Hydrogel for Cartilage Tissue Engineering.* Sci Rep, 2016. **6**: p. 20014.

Chapter 7

7 Hypo-osmotic loading induces expression of IL-6 in nucleus pulposus cells of the intervertebral disc independent of TRPV4 and TRPM7

Hypo-osmotic loading induces expression of IL-6 in nucleus pulposus cells of the intervertebral disc independent of TRPV4 and TRPM7

Aleksandra Sadowska[1], Birsen Altinay[1], Wolfgang Hitzl[2,3,4],
Stephen J. Ferguson[1], Karin Wuertz-Kozak[1,5]

[1]Institute for Biomechanics, ETH Zurich, Zurich, Switzerland
[2]Research Office (Biostatistics), Paracelsus Medical University, Salzburg, Austria
[3]Paracelsus Medical University Salzburg, Department of Ophthalmology and Optometry Salzburg, Austria
[4]Research Program Experimental Ophthalmology and Glaucoma Research, Paracelsus Medical University, Salzburg, Austria
[5] Tissue Regeneration & Mechanobiology Lab, Department of Biomedical Engineering, Rochester Institute of Technology (RIT), Rochester, USA

Published as:
Sadowska, A., *et al.* "Hypo-Osmotic Loading Induces Expression of IL-6 in Nucleus Pulposus Cells of the Intervertebral Disc Independent of TRPV4 and TRPM7."
Frontiers in Pharmacology 11 (2020): 952. https://doi.org/10.3389/fphar.2020.00952

Abstract:

Painful intervertebral disc (IVD) degeneration is an age-related process characterized by reduced tissue osmolarity, increased catabolism of the extracellular matrix, and elevated levels of pro-inflammatory molecules. With the aging population and constantly rising treatment costs, it is of utmost importance to identify potential therapeutic targets and new pharmacological treatment strategies for low back pain. Transient receptor potential (TRP) channels are a family of Ca^{2+} permeable cell membrane receptors, which can be activated by multitude of stimuli and have recently emerged as contributors to joint disease, but were not investigated closer in the IVD. Based on the gene array screening, TRPC1, TRPM7, and TRPV4 were overall the most highly expressed TRP channels in bovine IVD cells. We demonstrated that TRPV4 gene expression was down-regulated in hypo-osmotic condition, whereas its Ca2+ flux increased. No significant differences in Ca^{2+} flux and gene

expression were observed for TRPM7 between hypo- and iso-osmotic groups. Upon hypo-osmotic stimulation, we overall identified via RNA sequencing over 3,000 up- or down-regulated targets, from which we selected aggrecan, ADAMTS9, and IL-6 and investigated whether their altered gene expression is mediated through either the TRPV4 or TRPM7 channel, using specific activators and inhibitors (GSK1016790A/GSK2193874 for TRPV4 and Naltriben/NS8593 for TRPM7). GSK1016790A induced the expression of IL-6 under iso-osmotic condition, alike to hypo-osmotic stimulation alone, indicating that this effect might be TRPV4-mediated. However, using the TRPV4 blocker GSK2193874 failed to prevent the increase of IL-6 under hypo-osmotic condition. A treatment with TRPM7-activator did not cause significant changes in the gene expression of tested targets. In conclusion, while TRPV4 and TRPM7 are likely involved in osmosensing in the IVD, neither of them mediates hypo-osmotically-induced gene expression changes of aggrecan, ADAMTS9, and IL-6.

Keywords: intervertebral disc (IVD), osmolarity, osmosensing, transient receptor potential (TRP) channels, inflammation, degenerative disc disease (DDD), membrane receptor, low back pain

7.1 Introduction

The IVD is a mechanically loaded structure composed of two main tissues – the centrally located and highly hydrated nucleus pulposus (NP), which is surrounded by circular lamellar rings known as annulus fibrosus (AF) [1]. In a healthy state, the NP contains a small populations of cells embedded in a loose matrix of collagen (COL) type II and proteoglycans (PGs), which enable the NP's high water content (60-99%) and subsequent osmotic pressure [1, 2]. In contrast, the AF is mostly composed of COL type I, which gives the tissue the ability to retain tensile stresses and hydrostatic pressure generated from NP [1]. Age- and/or degeneration-induced changes in proteoglycan content lead to a drop in osmotic concentration from ~400 mOsm/L in a healthy state to ~300 mOsm/L in a degenerated IVD [3]. Other common occurrences observed with IVD degeneration include, among others, a shift in extracellular matrix (ECM) metabolism towards catabolism and up-regulated expression of matrix metalloproteinase (MMPs) or disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) [4]. Correspondingly, biochemical and structural changes to the IVD affect its mechanical function and can promote inflammation.

Inflammation in the IVD can be characterized by an increased expression of pro-inflammatory molecules such as interleukin (IL)-6, IL-1β and tumor necrosis factor alpha (TNF-α), and contributes to the development of degenerative disc disease (DDD) and low back pain (LBP) [5]. Discogenic LBP not only reduces the patient's quality of life, but also creates a high economic burden on the individuals and the society [6]. Current treatment strategies are mostly limited to oral pain medication, physiotherapy, lumbar epidural steroid injections and surgeries (or a combination thereof), all of which are rather reactionary than preventive measures and reported efficacy varies between publications [7, 8]. Hence, there is a clear need to not only develop new treatment strategies, but also to identify specific therapeutic targets, which mediate and/or promote homeostatic or inflammatory processes in the IVD.

Transient Potential Receptor (TRP) channels are a superfamily of multimodal cation membrane receptors and have recently emerged as potential contributors to IVD and joint diseases as well as to discogenic pain. They can be activated by multiple stimuli, including mechanical and osmotic stress (e.g. TRP canonical (C) and TRP vanilloid (V) subfamilies) and function as cellular sensors [9]. TRP channels have previously been shown to be well expressed in the human IVD; importantly the gene and protein expression of some TRP channels has been reported to be degeneration-, pain intensity- and/or pain chronicity-dependent [10]. A past study on IVD degeneration reported increased TRPV4 gene expression with a decrease in osmolarity (~400 mOsm/L vs. ~300 mOsm/L and below) and suggested that TRPV4 signaling may mediate increased expression of IL-1β and IL-6 [11]. TRPM3 and TRPM7 channels, which so far were sparsely investigated in the IVD, are implicated in sensing of osmotic changes and mediation of osmolarity-induced cell volume changes in human renal cells and salivary glands [12, 13]. Furthermore, hypo-osmotic stretch was also shown to mechanically activate TRPC5 and TRPC6 channels in the central and peripheral nervous system and in renal cells [14, 15]. Hence, TRP channels constitute a promising target for the investigation of IVD degeneration and accompanying reduced tissue osmolarity. Thus far, it is unclear which TRP channels may function as osmosensors in the IVD and whether they mediate catabolic and inflammatory changes in the response to hypo-osmotic stress. Therefore, the goal of this study was to:

(1) Identify the most prominently expressed TRP channels in bovine caudal NP and AF cells by gene array screening.

(2) Investigate how changes in osmolarity affect the expression and activity of the identified TRP channels.

(3) Identify pro-inflammatory and ECM targets with altered gene expression, due to short- and long-term exposure to reduced osmolarity, and to determine whether these changes are TRP channel-mediated (= main objective).

7.2 Materials and methods

Bovine nucleus pulposus cell isolation and culture

Due to the limited accessibility of healthy human IVD tissue, healthy bovine caudal discs were used in this study. Bovine caudal discs are considered to be a suitable model for the study of the human lumbar disc (especially that of a young adult), due to their biological and biomechanical similarity to the human IVD [16]. All experiments were conducted on n = 3-7 biological replicates, as indicated in each results section.

Bovine tails from 18- to 24-month-old male and female animals were obtained from a local slaughterhouse. Bovine nucleus pulposus (NP) and annulus fibrosus (AF) cells were isolated as previously described [3]. Within 1-2 h after the slaughter, caudal IVDs were dissected under sterile conditions, where NP, AF and the transition zone (TZ) were separated from each other using either a 8, 6 or 3 mm biopsy tool and a blade. For each animal, the top eight IVD sections were used. Collected AF or NP tissue was pooled together from each animal, whereas remaining TZ tissue was discarded. The tissue was cut into fine pieces and digested overnight at 37°C, 5% CO_2 in a solution composed of 3 mg/mL Collagenase NB 4 (#S1745401, Nordmark Biochemicals, Germany), 2 mg/mL Dispase II (#2845300, Roche Diagnostics USA) and 3% antibiotic-antimycotic (A/A, #15240-062, Gibco Life Technologies, Switzerland) dissolved in 100 mL of sterile phosphate buffered saline (PBS, #09-8912-100, Medicago Sweden). On the next day, the tissue digest was filtered using cell strainers (70 µm, # 542070, Greiner Bio-One, Switerland) and centrifuged at 1000 rpm for 20 min at the room temperature (RT), with three washing steps (1x PBS, 2x cell culture media) in between. Cells from different donors were not pooled together, but used separately as biological replicates.

For the TRP screening experiment, cells were collected either directly after isolation or sub-cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, #11320033 Gibco, Switzerland; 300 mOsm, “hypo-osmotic”) supplemented with 10% fetal calf serum (FCS, #F7524, Sigma-Aldrich, Switzerland) and 1% A/A until passage (P) 2 and collected for the analysis afterwards.

For the remaining sub-culturing, cells were seeded in DMEM/F12 adjusted to ~400 mOsm (“iso-osmotic” media) using sucrose (#57903, Sigma-Aldrich, Switzerland) and supplemented with 0.1% Ampicillin (#A6352.0025, PanReac AppliChem Switzerland) and 10% FCS. Osmolarity was measured using a freezing-point osmometer (Osmomat 030, Gonotec, Germany). Serum free hypo- and iso-osmotic media was used for cell treatment experiments. Additionally, to ensure consistency throughout treatments, the osmolarity of PBS used for cell washing during cell splitting or in assays, was correspondingly adjusted to match the treatment osmolarity.

Treatments

Bovine NP cells (P2) were seeded in 6-well plates (0.2×10^6 cells/well; gene expression analysis/ELISA), 24-well plates (0.65×10^5 cells/well, for MTT assay), or 96-well plates (0.1×10^5 cells/well, for [Ca2+] imaging) and cultured for 24 h. On the following day, cells were serum-starved for up to 3 h and treated (**Table 1**) for assay-dependent durations in serum free, osmotically adjusted media with channel specific agonist, antagonist or DMSO (negative control): gene expression analysis/ELISA = 24 h; [Ca2+] imaging = 15-30 min; MTT assay = 15 min to 24 h. Afterwards, cells were directly used for MTT assay/qPCR. For ELISA, cell culture media was collected before and after the treatment.

Table 1. Chemical compounds and concentrations.

Compound	Catalog number, Manufacturer	Function	MTT concentration [µM]	[Ca2+] imaging concentration [µM]	Cell treatment concentration [µM]
GSK1016790A	#17289, Cayman Chemical Company, CH	TRPV4 agonist	0.005, 0.02, 0.025, 0.04, 0.05	0.002, 0.02, 0.04	0.05
GSK2193874	# 1336960134, Sigma-Aldrich, CH	TRPV4 antagonist	0.1, 0.5, 1, 10	0.25, 0.5	0.5
Naltriben methanesulfonate hydrate	#N156, Sigma-Aldrich, CH	TRPM7 agonist	5, 15, 20, 25, 50, 200, 300, 400, 500, 600	50, 100, 200	25
NS8593 hydrochloride	#N2538, Sigma-Aldrich, CH	TRPM7 antagonist	150, 200, 250, 300, 350, 400	80, 160	-

RNA Sequencing & Data Analysis

Twelve RNA samples were submitted to the Functional Genomics Center in Zurich (FGCZ) for RNA sequencing. The quality of the RNA was determined with a Fragment Analyzer standard sensitivity RNA measurement (SS RNA kit (15 nt), Agilent, Waldbronn, Germany). The measured concentrations (> 75 ng/µl) and RIN (>9.9) values qualified for a Poly-A enrichment strategy in order to generate the sequencing libraries applying the TruSeq mRNA Stranded Library Prep Kit (Illumina, Inc, California, USA). After Poly-A selection using Oligo-dT beads the mRNA was reverse-transcribed into cDNA. The cDNA was fragmented, end-repaired and polyadenylated before ligation of TruSeq UD Indices (IDT, Coralville, Iowa, USA). The quality and quantity of the amplified sequencing libraries were validated using a Fragment Analyzer SS NGS Fragment Kit (1–6000 bp) (Agilent, Waldbronn, Germany). The equimolar pool of 12 samples was spiked into a NovaSeq6000

run targeting 200M reads on a S1 FlowCell (Novaseq S1 Reagent Kit, 100 cycles, Illumina, Inc, California, USA). The Bcl files were demultiplexed using Illumina`s bcltofastq software allowing for one mismatch in each barcode.

The RNA-seq data analysis consisted of the following steps: The raw reads were first cleaned by removing adapter sequences, trimming low quality ends, and filtering reads with low quality (phred quality <20) using Trimmomatic (Version 0.36) [17].The read alignment was done with STAR (v2.7.0e) [18]. As reference we used the Ensembl genome build UMD_v3.1 with the gene annotations downloaded on 2018-05-30 from Ensembl (release 92). The STAR alignment options were "--outFilterType BySJout --outFilterMatchNmin 30 --outFilterMismatchNmax 10 --outFilterMismatchNoverLmax 0.05 --alignSJDBoverhangMin 1 --alignSJoverhangMin 8 --alignIntronMax 100000 --alignMatesGapMax 100000 --outFilterMultimapNmax 50". The quantification of transcript level expression was carried out using Kallisto (Version 0.44) [19]. To detect differentially expressed genes we applied a count based negative binomial model implemented in the software package EdgeR (R version: 3.6.0, EdgeR version: 3.26.8) [20]. The differential expression was assessed using an exact test adapted for over-dispersed data. Genes showing altered expression with adjusted (Benjamini and Hochberg method) p-value < 0.05 were considered differentially expressed.

Gene expression analysis

RNA was extracted using the RNeasy Mini Kit (#74106, Qiagen, Switzerland) following the manufacturer's protocol. One microgram of RNA was used to synthesize cDNA in a total volume of 30 µl, using a reverse transcription kit (#4374966, Applied Biosystems, USA).

The expression of TRP channels in bovine NP and AF cells was screened using custom TaqMan Array Fast Plates (#4413261, Thermo Fisher, Switzerland) following the manufacturer's recommendations. In the first step, obtained cDNA was amplified using TaqMan PreAmp Master Mix (2✕) (#4391128, Thermo Fisher, Switzerland) and Custom TaqMan PreAmp Pools (Thermo Fisher, Switzerland). Then, amplified cDNA (mixed with RNAse-free water) was combined 1:1 with the TaqMan Fast Universal PCR Master Mix (2X) (#4352042, Thermo Fisher, Switzerland) and added to target pre-coated (TRP

channels, **Table 2**) 96-well plates (10 µL per well). The gene expression was measured using real-time qPCR (CFX96 Touch™ Detection System, Biorad).

For the remaining gene expression analysis, TaqMan primers (**Table 2**, Thermo Fisher, Switzerland) were used according to the manufacturer's protocol. In short, 5 µl of TaqMan Fast Universal PCR Master Mix (2X) was mixed with 0.5 µl of TaqMan primers and 10 ng cDNA (combined with RNAse-free water for 4.5 µl total volume), and quantified using the real-time qPCR. Data were analyzed either as $2^{-\Delta Ct}$ values relative to the housekeeping gene (YWHAZ) or as fold change $2^{-\Delta\Delta Ct}$ values normalized to YWHAZ and to a control. YWHAZ was chosen as the housekeeping gene based on its stability in preliminary testing.

Table 2. TaqMan primers list.

Gene symbol	**Gene name**	**TaqMan Primer Assay ID**
ACAN	Aggrecan	Bt03212186_m1
ADAMTs4	Adam Metallopeptidase With Thrombospondin Type 1 Motif 4	Bt03224697_m1
ADAMTs9	Adam Metallopeptidase With Thrombospondin Type 1 Motif 9	Bt04295942_m1
COL2	Collagen Type Ii Alpha 1 Chain	Bt03251861_m1
CHOP (a.k.a. DDIT3)	Dna Damage Inducible Transcript 3	Bt03251320_g1
HAS2	Hyaluronic Acid Synthase 2	Bt03212695_g1
HAS3	Hyaluronic Acid Synthase 3	Bt04298491_m1
GRP78 (a.k.a HSPA5)	Heat Shock Protein Family A (Hsp70) Member 5	Bt03244880_m1
COX2 (a.k.a. PTGS2)	Prostaglandin-Endoperoxide Synthase	Bt03214492_m1
IL-6	Interleukin 6	Bt03211904_m1
MMP3	Matrix Metallopeptidase 3	Bt04259497_m1
TNFRSF21	Tumor Necrosis Factor Receptor Superfamily Member 21	Bt03250597_m1
TRPC1	Transient Receptor Potential Cation Channel, Subfamily C, Member 1	Bt03214647_m1
TRPC2	Transient Receptor Potential Cation Channel, Subfamily C, Member 2	Bt03817472_m1

TRPC3	Transient Receptor Potential Cation Channel, Subfamily C, Member 3	Bt03258742_m1
TRPC4	Transient Receptor Potential Cation Channel, Subfamily C, Member 4	Bt03214662_m1
TRPC5	Transient Receptor Potential Cation Channel, Subfamily C, Member 5	Bt04301428_m1
TRPC6	Transient Receptor Potential Cation Channel, Subfamily C, Member 6	Bt04301412_m1
TRPC7	Transient Receptor Potential Cation Channel, Subfamily C, Member 7	Bt04297646_m1
TRPM3	Transient Receptor Potential Cation Channel, Subfamily M, Member 3	Bt03243121_m1
TRPM7	Transient Receptor Potential Cation Channel, Subfamily M, Member 7	Bt04290223_m1
TRPM8	Transient Receptor Potential Cation Channel, Subfamily M, Member 8	Bt04288753_m1
TRPV2	Transient Receptor Potential Cation Channel, Subfamily V, Member 2	Bt03210789_m1
TRPV3	Transient Receptor Potential Cation Channel, Subfamily V, Member 3	Bt03262740_m1
TRPV4	Transient Receptor Potential Cation Channel, Subfamily V, Member 4	Bt03649002_m1
TRPV6	Transient Receptor Potential Cation Channel, Subfamily V, Member 6	Bt04290617_m1
YWHAZ	Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein, Zeta Polypeptide	Bt01122444_g1

[Ca2+] imaging

To investigate the activity of a TRP channel in hypo- and iso-osmotic environments, Fura-2 QBT™ Calcium Kit (#R8197, Molecular Devices, UK) was used following the manufacturer's protocol to detect intracellular calcium changes. In short, bovine NP cells (n = 3, P2, 0.1 × 10^5 cells/well) were seeded in a 96-well plate in iso-osmotic media supplemented with 10% FCS and 0.1% Ampicillin. On the next day, media was changed to osmotically adjusted (300 or 400 mOsm) phenol-red free media (DMEM/F-12, #11039021, Gibco, Switzerland) supplemented with 0.1% Ampicillin. After around 2-3 h, an equal volume of Fura-2 Loading Buffer was added to each well, followed by an incubation for 1 h at 37°C, 5% CO_2. Next, cells were treated with either TRPV4 antagonist, TRPM7 antagonist or DMSO (negative control, 0.01 or 1 %) for 15 min at the RT (**Table 1**). Each compound was dissolved in DMSO at 10 mM stock concentration and stored in aliquots at -20°C and was freshly diluted into various working concentrations right before the use (**Table 1**). Calcium response was measured using a microplate reader (Tecan, Infinite M200 PRO). First, a baseline over 7 cycles was recorded at an excitation wavelength of 340 nm for the bound calcium and 380 nm for the unbound calcium. At the 8th cycle, cells were treated with different concentrations of agonists (**Table 1**), and the measurement was continued until the 30th cycle, which corresponds to approximately 20 min of the total measurement time. Calcium response was analyzed as a ratio of the bound calcium to the unbound calcium (340 nm/380nm), normalized to the baseline.

MTT assay

In order to test whether applied treatments influenced cell metabolic activity, MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, #M5655, Sigma-Aldrich, Switzerland) was used. Cells were cultured and treated as described beforehand (section **Treatments** and **Table 1**). As a negative control, cells were treated with 70% methanol (#M5655, Sigma-Aldrich, Switzerland) for 30 min at 37°C, 5% CO_2. Following treatments, the supernatants were aspirated, cells were washed with PBS and incubated with freshly prepared MTT solution (0.5 mg/mL, dissolved in media) for 1 h at 37°C, 5% CO_2. Thereupon, MTT was discarded and 200 µL DMSO was added to each well and incubated on a shaker for 5 min at RT. Subsequently, the lysates were transferred to 96-well plates in duplicates and the absorbance of formazan was measured at 565 nm using a microplate

reader (Tecan, Infinite M200 PRO). Metabolic activity was calculated relative to the untreated control (set at 100% cell viability).

Immunocytochemistry (ICC)

Bovine NP cells in P2 were seeded into the chambered cover glass (Nunc Lab-Tek, #155380 or #154461, Thermo Fisher, Switzerland). Media was replaced to hypo- or iso-osmotic, FCS free media and cells were incubated for 24 h. Afterwards, cells were briefly washed 3x with PBS, fixed with ice cold 100% methanol (10 min at -20°C, #34885, Sigma-Aldrich, Switzerland), and blocked with 5% normal goat serum (#005-000-121, Jackson ImmunoResearch, PA, USA) in PBS for 1 h at the RT. Next, cells were incubated with the primary antibody (anti-TRPM7, #ACC-047 or anti-TRPV4, #ACC-034, Alomone, Israel, 1:500 in 1% goat serum in PBS) in the dark at 4°C overnight. On the next day, cells were first washed with PBS (3 x 10 min on a rocker) and then incubated with the secondary antibody (Cy2 anti-rabbit IgG, #111225144, Jackson ImmunoResearch, PA, USA; 1:200 in 1% normal goat serum) for 1 h at the RT. Next, cells were again washed with PBS (3 x 10 min on a rocker) and 1–2 drops of the Antifade Mounting Medium with DAPI (VECTASHIELD, #H-1200, Switzerland) were added shortly before imaging. Cells were imaged with a fluorescence microscope (20x, Olympus IX51 or 100x, Delta Vision System). For each primary antibody, the same imaging parameters (exposure time and magnification) were used. As a negative control, cells were incubated without primary antibody.

Enzyme-Linked Immunosorbent Assay (ELISA)

To quantify the release of IL-6 from treated IVD cells, the cell culture media was collected before and after treatment, and analyzed with IL-6 ELISA kit following the manufacturer's protocol (IL-6, #ESS0029, Thermo Fisher, Switzerland). Briefly, 96-well plates were coated with coating antibody (1:100) overnight. On the next day, wells were blocked (ELISA Blocking Buffer, #N502, Thermo Fisher, Switzerland) for 1 h at RT and afterwards loaded with samples or protein standard, and incubated for 1 h at the RT. Next, wells were washed (ELISA Wash Buffer 1X, #N503, Thermo Fisher, Switzerland), incubated with the detection antibody (1:100) for 1 h at RT, followed by washing and 1 h incubation with streptavidin-horseradish peroxidase (HRP) at RT. Next, wells were washed again and substrate solution was added to each well. After 20 min of incubation in the dark, stop solution was added to the each well, the absorbance was directly measured (Tecan, Infinite

M200 PRO) at 450 nm (with subtracted 550 nm absorbance) and IL-6 concentrations were calculated based on the standard curve.

Statistical Analysis

All data were checked for consistency and screened for outliers. For the gene array, dependent bootstrap t-test based on 7000 Monte Carlo samples was used due to the small sample sizes. In addition, classical dependent t-tests and nonparametric tests (Wilcoxon Signed test) and Quantile Sign test was used. For the calcium assay, continuous variables were also tested for normality. Generalized estimation equation models based on Gamma distributions were used to analyze data. The robust estimator for the covariance matrix was used and a full factorial model was set up. Finally, LSD tests were used to compare means pairwise. For the MTT and gene data, One-way ANOVA followed by Tukey's or Dunnett's multiple comparisons test was used to test means among different groups. All reported tests were two-sided, p-values ≤ 0.05 were considered statistically significant and all error bars present SEM. All statistical analyses in this report were performed by use of NCSS (NCSS 10, NCSS, LLC. Kaysville, UTand PASW 24 (IBM SPSS Statistics for Windows, Version 21.0., Armonk, NY) or GraphPad Prism version 8.2.0 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

7.3 Results

TRP channels mRNA expression in bovine NP and AF cells

The mRNA expression of 14 TRP channels (TRPC1-C7, TRPM3, TRPM7, TRPM8, TRPV2-V2 and TRPV6) was tested in bovine NP and AF cells obtained directly from tissue digest or passaged cells collected at P2. In the initial test, a set of three donors was used with Taqman Array Plates. **Table 3** presents the detectability of all tested TRP channels. Five out of 14 TRP targets, namely TRPC1, TRPC3, TRPC4, TRPM7 and TRPV4, were selected for further gene analysis on additional two donors. This decision was made based on either highest overall expression (e.g. TRPC1, TRPM7 and TRPV4), novelty (TRPC1, TRPC3, TRPC4) or research relevance in the IVD or other tissue types. The detectability of expression and relative mRNA expression levels from all five donors are presented in **Table 4** and **Figure 1**, respectively. The gene expression tended to be generally higher in non-passaged cells as compared to passaged cells (**Figure 1A and B**). This difference was

statistically significant ($p<0.05$) for TRPV4 channel in NP cells (**Figure 1A**) as well as TRPC3, TRPM7 and TRPV4 channels in AF cells **(Figure 1B)**. For the reason of overall high expression and the availability of a channel specific agonists and antagonists, TRPM7 and TRPV4 were chosen out of the five pre-selected TRP channels for the subsequent experiments on the NP cells. Due to their relevance to osmotic changes in the IVD, only NP cells were included in the further experiments.

Table 3. Primary screening: number of donors (min. n=0; max. n=3), in which the mRNA expression of all available TRP channel was detectable using TaqMan gene array.

Target	**NP digest**	**NP cells P2**	**AF digest**	**AF cells P2**
TRPC1	3/3	3/3	3/3	3/3
TRPC2	1/3	1/3	2/3	-
TRPC3	3/3	3/3	2/3	3/3
TRPC4	1/3	3/3	1/3	3/3
TRPC5	2/3	-	1/3	-
TRPC6	1/3	-	3/3	1/3
TRPC7	-	1/3	-	2/3
TRPM3	3/3	3/3	2/3	3/3
TRPM7	3/3	3/3	3/3	3/3
TRPM8	1/3	3/3	-	3/3
TRPV2	3/3	3/3	3/3	3/3
TRPV3	1/3	-	1/3	1/3
TRPV4	3/3	3/3	3/3	3/3
TRPV6	3/3	3/3	3/3	3/3

"-": *not expressed in any of the tested samples.*

Table 4. Secondary screening: number of donors (min. n=0; max. n=5), in which the mRNA expression of all selected TRP channels was detectable using TaqMan gene array and qPCR.

Target	**NP digest**	**NP cells P2**	**AF digest**	**AF cells P2**
TRPC1	5/5	5/5	5/5	5/5
TRPC3	5/5	5/5	4/5	3/5
TRPC4	3/5	5/5	3/5	5/5
TRPM7	5/5	5/5	5/5	5/5
TRPV4	5/5	5/5	5/5	5/5

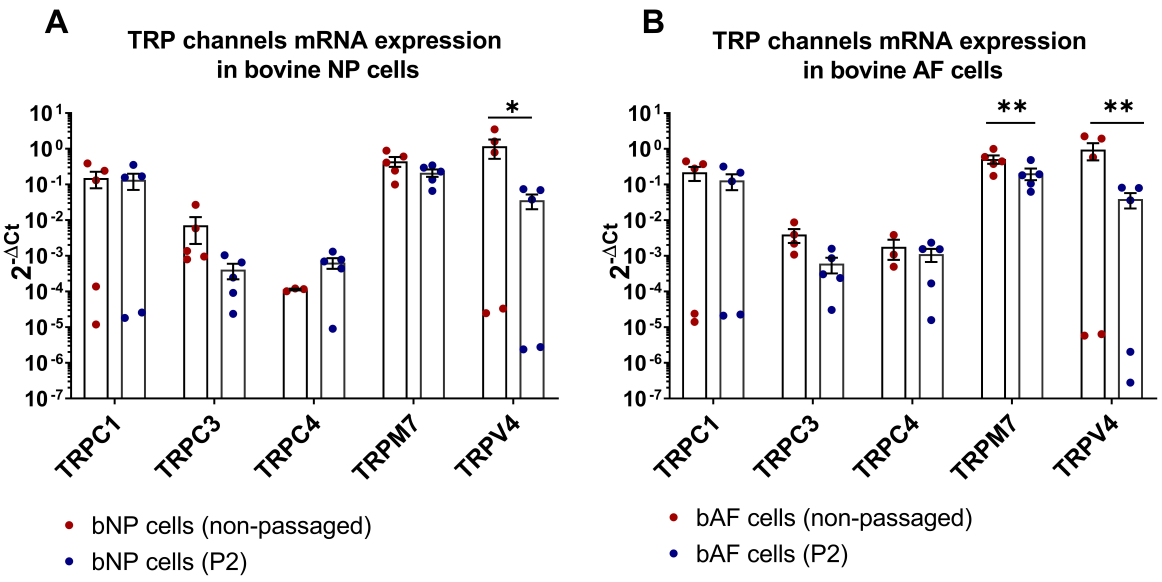


Figure 1. The mRNA expression of TRP channels in (**A**) bovine nucleus pulposus (bNP) and (**B**) annulus fibrosus (bAF) cells obtained directly after cell isolation (non-passaged) or cells passaged until passage 2 (P2). Graphs show $2^{-\Delta Ct}$ values relative to YWHAZ (mean ± SEM, n = 5). Asterisks indicate statistical significance (*p < 0.05, **p < 0.01) between non-passaged and passaged cells.

Effects of hypo-osmotic stimulation on TRPV4 and TRPM7 mRNA and protein expression

In the next step, TRPV4 and TRPM7 expression on the gene and protein level in both hypo- and iso-osmotic conditions was tested in bovine NP cells. For gene expression, cells were collected after either 24 h or five days of osmotic treatment. TRPV4 was slightly, but significantly, down-regulated in hypo-osmotic condition as compared to the iso-osmotic group at both collection points (fold change 24 h: mean 0.79, min. 0.56, max. 1.07, p = 0.03 and fold change day 5: mean 0.79, min. 0.56, max. 1, p = 0.04 **Figure 2A left**). The expression of TRPM7 was unchanged at both collection points *versus* iso-osmotic groups (fold change 24 h: mean 0.9, min. 0.69, max. 1.14, p = 0.45 and fold change day 5: mean 0.85, min. 0.49, max. 1.1., p = 0.21 **Figure 2A right**). Correspondingly, on the protein level, the two channels were abundantly and steadily expressed in both osmotic conditions (**Figure 2B**: TRPV4 (left top and bottom) and TRPM7 (right top and bottom) after 24 h

of culture. While both channels were present in the cell membrane, TRPM7 additionally showed a clear localization around the nuclear envelope.

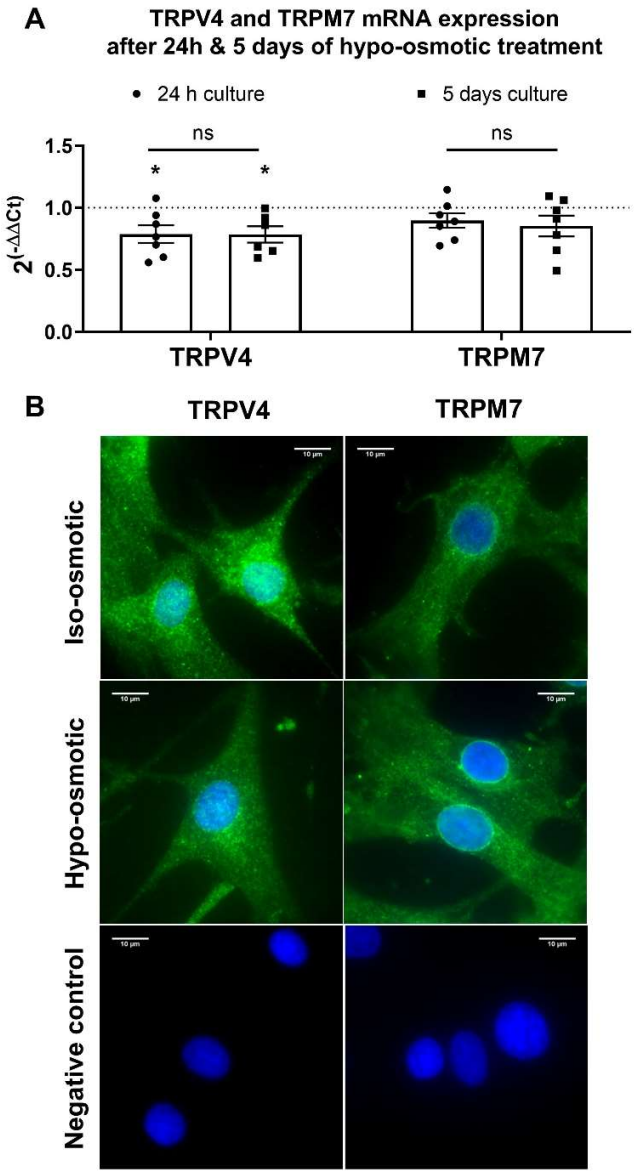


Figure 2. The mRNA expression of (**A**) TRPV4 (**left**) and TRPM7 (**right**) channels in bovine nucleus pulposus (NP) cells. Graphs show $2^{-\Delta\Delta Ct}$ values normalized to the iso-osmotic control (mean ± SEM, n = 6-7). Asterisks indicate statistical significance (*$p < 0.05$) between iso- and hypo-osmotic conditions, bars compare the difference between 24 h and 5-day long treatments (ns, no statistical difference defined as $p > 0.05$). (**B**) Protein expression of TRPV4 (**left top and middle**) and TRPM7 (**right top and bottom**) after 24 h of culture in either iso- (**top**) or hypo-osmotic media (**middle**). For the negative control (**bottom left and right**) cells were incubated without primary antibody. TRPV4/M7 are stained green, nuclei were counterstained with DAPI (blue). Scale bar is 10 µm, n = 3.

Osmotic regulation of TRPV4 and TRPM7 channels activity

Channel-specific activators and blockers of TRPV4 and TRPM7 (**Table 1**) were employed to test whether their activity differs between iso- and hypo-osmotic conditions. To ensure non-toxicity of channel activators and blockers, their dose-dependent effect on the metabolic activity of bovine NP cells was tested using MTT assay (**Figure 3**).

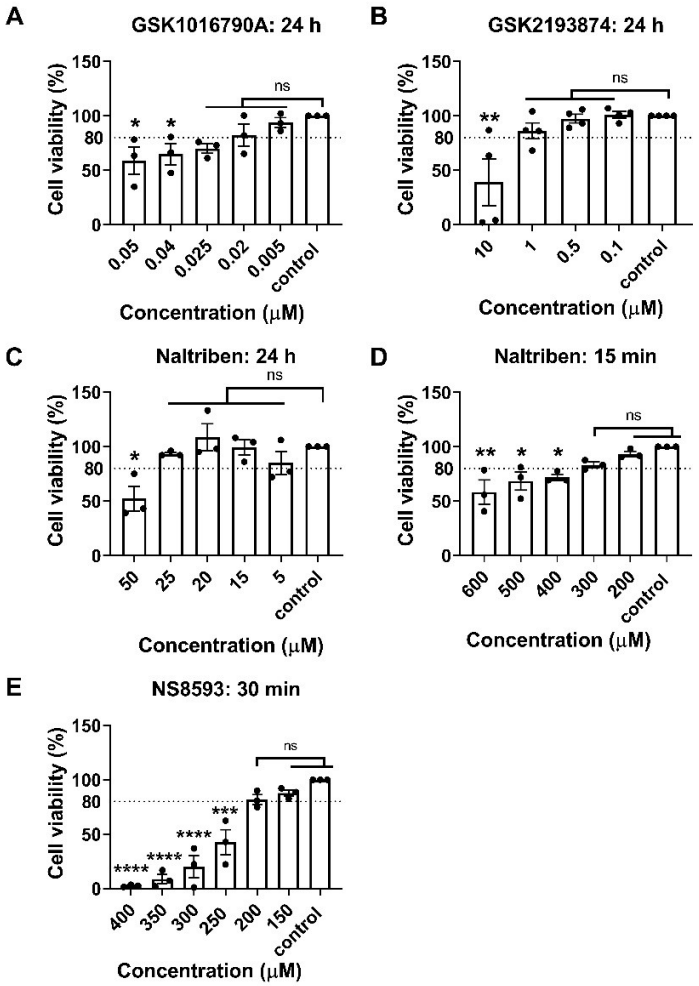


Figure 3. MTT results of bovine NP cells after different incubation periods with (**A**) GSK1016790A (TRPV4 activator), (**B**) GSK2193874 (TRPV4 blocker), (**C, D**) Naltriben methanesulfonate hydrate (TRPM7 activator), and (**E**) NS8593 hydrochloride (TRPM7 blocker). Graphs show percentage of viable cells (mean ± SEM, bNP P2, n = 3–4). Asterisks indicates statistical significance compared to the untreated control (*p < 0.05, *p < 0.01, ***p < 0.001, ****p < 0.0001, ns, no statistical difference defined at p > 0.05).

Next, the activity of a channel was measured using a calcium flux assay (n=3). Based on the MTT results, three highest, non-toxic doses of an activator were applied to untreated bovine NP cells to establish the effective concentration of an agonist necessary to activate TRPV4 or TRPM7 channel in bovine NP cells (**Supplementary Figure 1 and 2**). In the final step, a selected concentration of an activator was applied to cells cultured in either iso- or hypo-osmotic media and the response was measured for up to 15 min from the activator injection time (**Figure 4A and C**). To ensure the specificity of an activator, respective channel blockers were applied 15 min before the measurement (**Figure 4B and D**). TRPV4 mediated calcium flux was higher in cells cultured in hypo-osmotic media as compared to iso-osmotic media (**Figure 4A**, p = 0.1), and the effect became significantly different (p = 0.002) with the increased dose of the GSK1016790A (40 nM, **Supplementary Figure 3).** There was no statistical difference in the response to TRPM7

activator between iso- or hypo-osmotic treated cells, and the calcium flux was similarly high in both conditions (**Figure 4C**, $p > 0.05$).

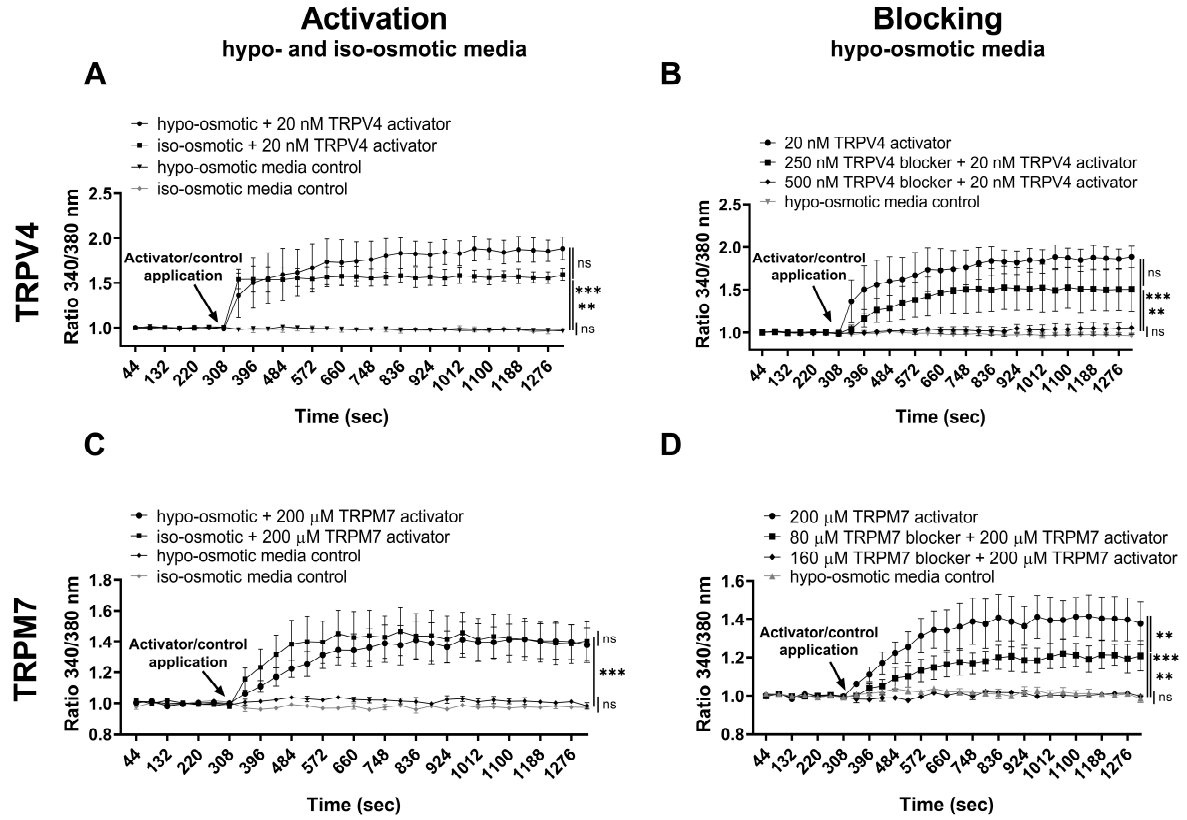


Figure 4. Ca^{2+} flux in NP cells following the application of (**A**) 20 nM GSK1016790A (TRPV4 activator) alone, (**B**) 250 or 500 nM GSK2193874 (TRPV4 blocker) and/or 20 nM GSK1016790A (TRPV4 activator), (**C**) 200 µM Naltriben (TRPM7 activator), and (**D**) 80 or 160 µM NS8593 hydrochloride (TRPM7 blocker) and/or 200 µM Naltriben (TRPM7 activator). Graphs present the ratio of the bound calcium to the unbound calcium (340 nm/380 nm) normalized to the baseline over the measurement time (mean ± SEM, bNP P2, n = 3). After the baseline measurement, empty (control) or compound-supplemented (treatment) media was added (indicated by an arrow on a graph) and the measurement was continued for up to around 15 min more. Asterisks indicate statistical significance (**$p < 0.01$, ***$p < 0.001$, ns, no statistical difference defined as $p > 0.05$) as measured on the last measurement cycle.

Effects of hypo-osmotic stimulation on genome-wide expression changes in NP cells

Bovine NP cells were cultured in osmotically adjusted media for either 24 h or 5 days and were thereafter collected for the RNA sequencing or qPCR analysis. Overall, there were 38 differently up- or down-regulated targets after 24 h of hypo- *versus* iso-osmotic stimulation, and 3062 differently up- or down-regulated targets after 5 days of hypo- *versus* iso-osmotic culture, which met the criterion of false discover rate (FDR) < 0.05 and p-value < 0.05. Out of those regulated at the 24 h time point, 12 targets were up-regulated at least 2 fold and 22 targets were down-regulated by at least 0.5 fold. In the 5 days group, 188 out of 3062 targets were up-regulated by at least 2 fold and 419 targets were down-regulated by at least 0.5 fold. **Supplementary Table 1** and **Supplementary Table 2** present the results of gene set enrichment analysis (GSEA) organized by a function of up- or down-regulated genes for 24 h and 5 days of osmotic culture, respectively. **Table 5** and **Table 6** present selected significantly up- and down-regulated targets for 24 h and 5 days long hypo- *versus* iso-osmotic culture, respectively (organized by fold change). Based on these results, 12 targets (ACAN, ADAMTS9, IL-6, ADAMTS4, COL2, COX2, MMP3, TNFRSF21, CHOP10, HAS2, HAS3 and GRP78) were selected for qPCR testing due to differential gene regulation, novelty or relevance to inflammation and IVD degeneration or discogenic pain.

Aggrecan was significantly and steadily down-regulated in both 24 h and 5 days hypo-osmotic groups (24 h fold change: mean 0.53, min. 0.24, max. 1.00, $p = 0.002$; 5 day fold change: mean 0.53, min. 0.29, max. 1.00, $p = 0.002$; **Figure 5A**) as compared to iso-osmotic groups. ADAMTS9 was slightly up-regulated after 24 h of culture (24 h fold change: mean 1.72, min. 0.94, max. 2.24; **Figure 5B left**) and although the effect was statistically insignificant after 24 h ($p = 0.15$), its up-regulation strongly increase over time and became significant (5 day fold change: mean 2.6, min. 1.45, max. 4.13, $p = 0.001$; **Figure 5B right**). Hypo-osmotic treatment consistently upregulated IL-6 mRNA expression after 24 h (24 h fold change: mean 2.69, min. 1.89, max. 3.94, $p = 0.002$; **Figure 5C left**) and 5 days of culture (5 day fold change: mean 2.69, min. 0.90, max. 4.44, $p = 0.002$; **Figure 5C right**). However, due to the limited range of the ELISA kit, IL-6 protein release in the media could not be detected (data not showed). **Figure 5D** presents the gene expression of the remaining targets: ADAMTS4, COL2, COX2, MMP3, TNFRSF21, CHOP10, HAS2, HAS3 and GRP78. Although identified as potentially interesting targets on the small gene array

data set, a more comprehensive analysis showed that hypo-osmotic treatment had little to no effect on the gene expression of ADAMTS4, COL2, TNFRSF21, CHOP10, HAS3 and GRP78. Interestingly, the gene expression of MMP3 decreased over time back to the iso-osmotic levels and the expression of COX2 and HAS2 was increased, especially at the later time point.

Finally, aggrecan, ADAMTS9 and IL-6 were chosen for further testing to check whether TRPV4 or TRPM7 channels may mediate their altered expression.

Table 5. RNA sequencing: top up- and down-regulated genes in bovine NP cells with FDR < 0.05 and p-value < 0.05 for 24 h of hypo-osmotic *versus* iso-osmotic treatment.

Top up-regulated targets		**Top down-regulated targets**	
Gene name	**Fold Change**	**Gene name**	**Fold Change**
BNIP3	4.30	**NOV**	0.47
CCL5	3.72	**SLC44A1**	0.47
SLC2A3	3.03	**ERICH5**	0.46
IL6	2.83	**NUPR1**	0.44
PTGS2	2.68	**ARHGAP24**	0.43
DDIT4	2.63	**SESN3**	0.42
SLC16A3	2.47	**NYAP1**	0.42
NDUFA4L2	2.31	**MPIG6B**	0.39
ECI1	2.28	**GLP2R**	0.39
IL11	2.23	**CPM**	0.38
PFKL	2.13	**MTUS1**	0.38
TGFBI	2.04	**KCNK5**	0.38
GAPDH	1.98	**HMGCS1**	0.37
TPI1	1.91	**DDAH2**	0.36
		PALM3	0.35
		TNFRSF11B	0.34
		GPR183	0.34
		LRRN1	0.31
		PIGZ	0.28
		EREG	0.27
		SLC6A12	0.12
		SLC4A11	0.07

Table 6. RNA sequencing: selected up- and down-regulated genes in bovine NP cells with FDR < 0.05 and p-value < 0.05 for 5 days of hypo-osmotic *versus* iso-osmotic treatment.

Top up-regulated targets		**Top down-regulated targets**	
Gene name	**Fold Change**	**Gene name**	**Fold Change**
TRIB3	6.01	**IL4R**	0.50
IL11	5.18	**TLR4**	0.47
TNFSF15	4.53	**MMP2**	0.46
TNFRSF21	4.30	**SLC4A3**	0.44
NGF	4.02	**CD14**	0.41
SLC7A11	3.94	**SLC44A1**	0.41
HAS2	3.79	**SLC6A6**	0.39
SLC7A5	3.74	**MAP4K3**	0.38
IL6	3.61	**SLC37A2**	0.37
ADAMTS9	3.55	**ACAN**	0.36
DDIT4	3.13	**CD24**	0.35
PTGS2	3.00	**SLC13A4**	0.34
COL1A1	2.92	**SLC24A3**	0.29
HSPA5	2.91	**SLC46A3**	0.27
SLC1A4	2.76	**AQP1**	0.26
COL1A2	2.74	**ADAMTS12**	0.22
VEGFC	2.66	**CXCL5**	0.21
SLC1A5	2.56	**TNFRSF11B**	0.21
SLC3A2	2.49	**SLC39A8**	0.20
VGF	2.15	**SLC4A11**	0.02

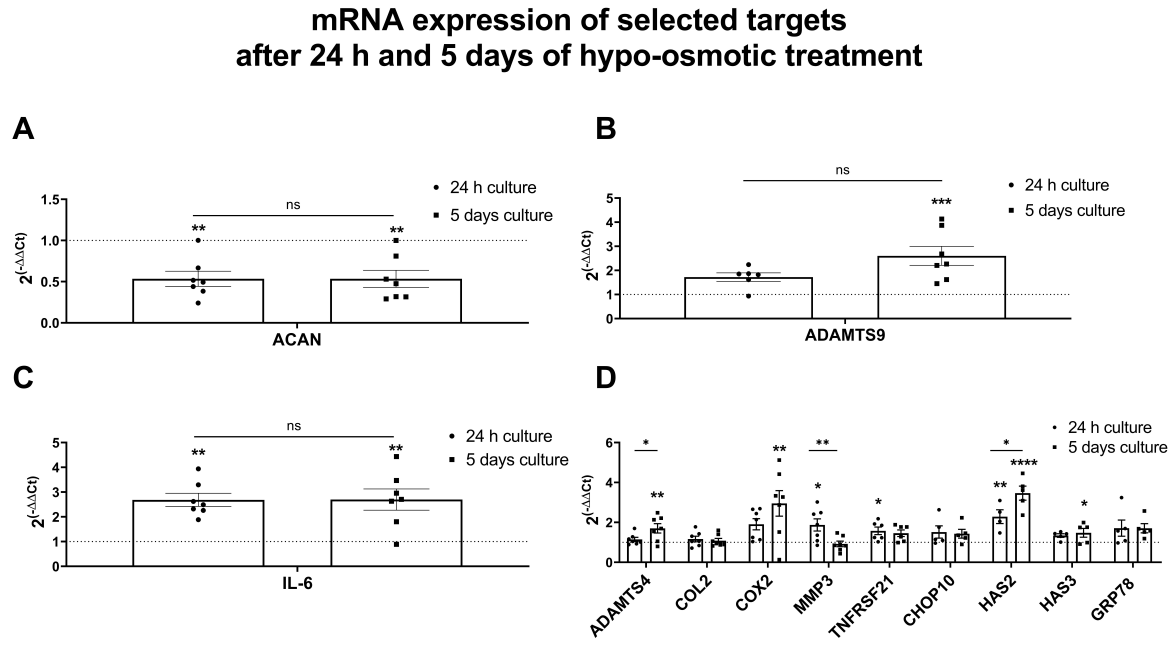


Figure 5. The mRNA expression of ECM and pro-inflammatory targets selected based on the RNA sequencing, novelty or relevance to the IVD degeneration in bovine NP cells: (**A**) aggrecan (ACAN), (**B**) ADAMTS9, (**C**) IL-6, and (**D**) ADAMTS4, COX2, HAS2, MMP3, COL2, TNFRSF21, CHOP10, HAS3, and GRP78. Graphs show $2^{-\Delta\Delta_{Ct}}$ values normalized to the iso-osmotic control (mean ± SEM, bNP P2, n = 4–7). Asterisks indicate statistical significance (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001) between hypo-osmotic and iso-osmotic groups, bars compare the difference between 24 h and 5-day long groups (ns, no statistical difference defined at p > 0.05).

Effects of TRPV4 and TRPM7 activators on the gene expression of ACAN, ADAMTS9, IL-6, TRPV4 and TRPM7

In the following step, the effect of TRPV4 and TRPM7 compounds on the gene expression of aggrecan (ACAN), ADAMTS9, IL-6, TRPM7 and TRPV4 was tested. Bovine NP cells were cultured in the iso-osmotic, hypo-osmotic or iso-osmotic media supplemented with a channel specific activator (TRPV4: 5 nM GSK1016790A or TRPM7: 25 µM Naltriben) over 24 h. The iso-osmotic activator-supplemented group was used to investigate 1) the influence of an activator on the gene expression of the tested channel, and 2) whether a TRP channel in question mediates up- or down-regulation of tested biomarker as observed during hypo-osmotic treatment alone.

Addition of 5 nM TRPV4 activator to the iso-osmotic group up-regulated the expression of TRPV4 by around 2 fold (fold change: mean 2.06, min. 1.44, max. 2.89) in a statistically significant manner ($p = 0.012$) as compared to hypo-osmotic treatment alone (fold change: mean 0.91, min. 0.68, max. = 1.1) and iso-osmotic control ($p = 0.02$) (**Figure 6A**). Iso-osmotic treatment with 25 µM TRPM7 activator slightly up-regulated TRPM7 expression (fold change: mean 1.15, min. 1.06, max. 1.24), with a statistical difference ($p = 0.01$) to the hypo-osmotic group (fold change: mean 0.86, min. 0.78, max. 1.08), but without a significant difference to the iso-osmotic group ($p = 0.15$) (**Figure 6B**).

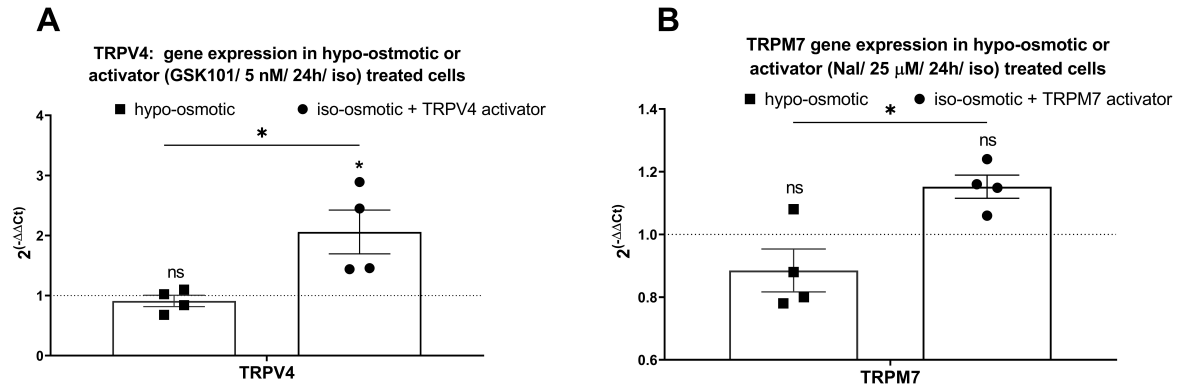


Figure 6. The mRNA expression of (**A**) TRPV4 in NP cells treated with 5 nM GSK1016790A, and (**B**) TRPM7 in NP cells treated with 25 µM Naltriben after 24 h. Graphs show $2^{-\Delta\Delta Ct}$ normalized to the iso-osmotic control (mean ± SEM, bNP P2, n = 4). Asterisks indicate statistical significance (*$p < 0.05$, no statistical difference defined at $p > 0.05$) between hypo-osmotic group, iso-osmotic group or iso-osmotic activator-supplemented group.

While aggrecan was down-regulated and ADAMTS9 up-regulated under hypo-osmotic treatment (see also **Figure 5**), addition of GSK1016790A (TRPV4 activator) or Naltriben (TRPM7 activator) to iso-osmotic media did not yield a similar gene expression profile. GSK1016790A significantly up-regulated aggrecan expression (fold change: mean ~2.18, min. 1.42, max. 3.16, $p = 0.02$; **Figure 7A right**), in contrast to hypo-osmotic treatment, which caused a significant down-regulation of aggrecan (fold change: mean 0.40, min. 0.33, max. 0.47, $p = 0.0004$; **Figure 7A left**), whereas Naltriben had no effect on aggrecan expression (fold change: mean 1.1, min. 0.84, max. 1.34, $p = 0.8$; **Figure 7B right**). Similarly, neither GSK1016790A nor Naltriben had an effect on ADAMTS9 expression (GSK101 group fold

change: mean 0.94, min. 0.52, max. 1.12, p = 0.91, **Figure 7C right** and Naltriben group fold change: mean 1.60, min. 1.10, max. 2.22, p = 0.17; **Figure 7D right**) in contrary to the significant up-regulation observed in the hypo-osmotic group (fold change: mean 3.71, min. 3.70, max. 3.80, p = 0.0002; **Figures 7C and 7D left**). Hypo-osmotic treatment up-regulated IL-6 expression (fold change: mean 1.8, min. 1.60, max. 2.1, p = 0.01; **Figure 7E left**). While, TRPM7 activator gently down-regulated IL-6 expression (fold change: 0.56, min. 0.20, max. 1.00; **Figure 7E right**) as compared to the iso-osmotic group, but the effect was statistically insignificant (p = 0.1).

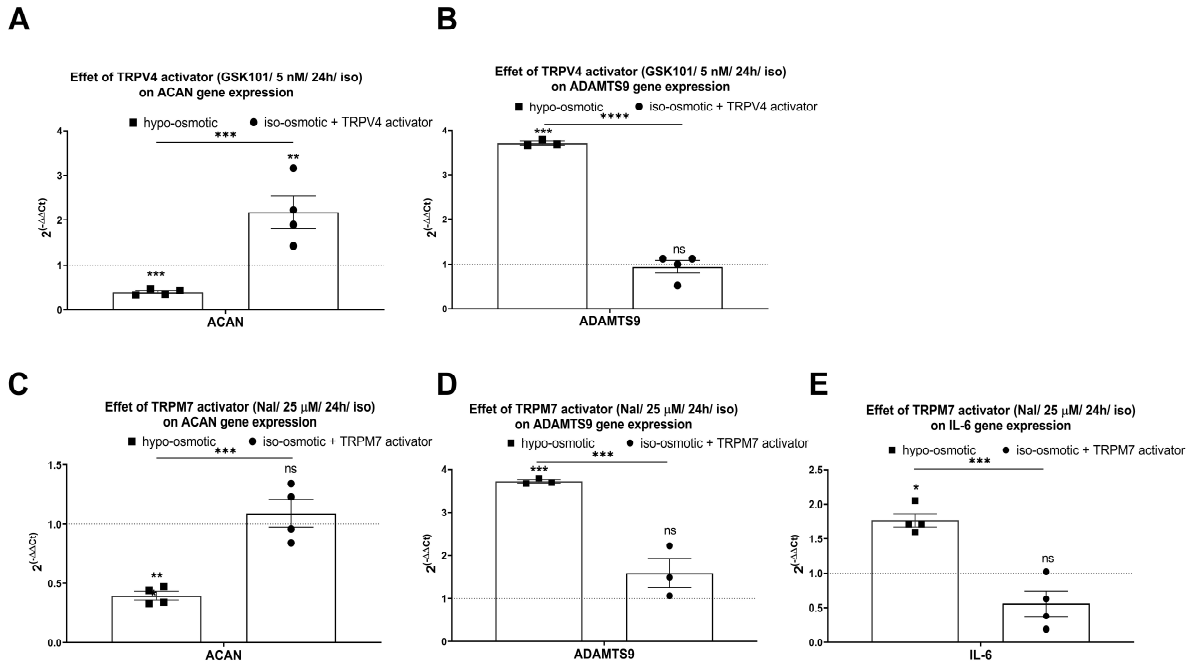


Figure 7. The mRNA expression of (**A**) aggrecan (ACAN) and (**B**) ADAMTS9 in NP cells after 24 h of treatment with TRPV4 activator (5 nM GSK1016790A); (**C**) aggrecan (ACAN), (**D**) ADAMTS9, and (**E**) IL-6 in NP cells after 24 h of treatment with TRPM7 activator (25 µM Naltriben). Graphs show $2^{-\Delta\Delta_{Ct}}$ values normalized to the iso-osmotic control (mean ± SEM, bNP P2, n = 3–4). Asterisks indicate statistical significance (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns, no statistical difference defined at p > 0.05) between hypo-osmotic group, iso-osmotic group or iso-osmotic activator-supplemented group.

Interestingly, iso-osmotic GSK1016790A-supplementation seemed to up-regulate the expression of IL-6 (fold change: mean 1.62, min. 1.20, max. 2.30, p = 0.11; **Figure 8A**

right) in a manner similar to its expression measured in hypo-osmotic group (fold change: mean 1.8, min. 1.60, max. 2.1, p = 0.01; **Figure 8A and Figure 8B left**). To further test if this phenomena was mediated by TRPV4, TRPV4 blocker (500 nM GSK2193874) was added to hypo-osmotic media and cells were collected 24 h later. However, GSK2193874 did not prevent the upregulation of IL-6 in hypo-osmotic treatment (fold change: mean 1.61, min. 0.94, max. 2.1, p = 1; **Figure 8B right),** indicating that the hypo-osmotic induction of IL-6 is not (or only partially) mediated by TRPV4.

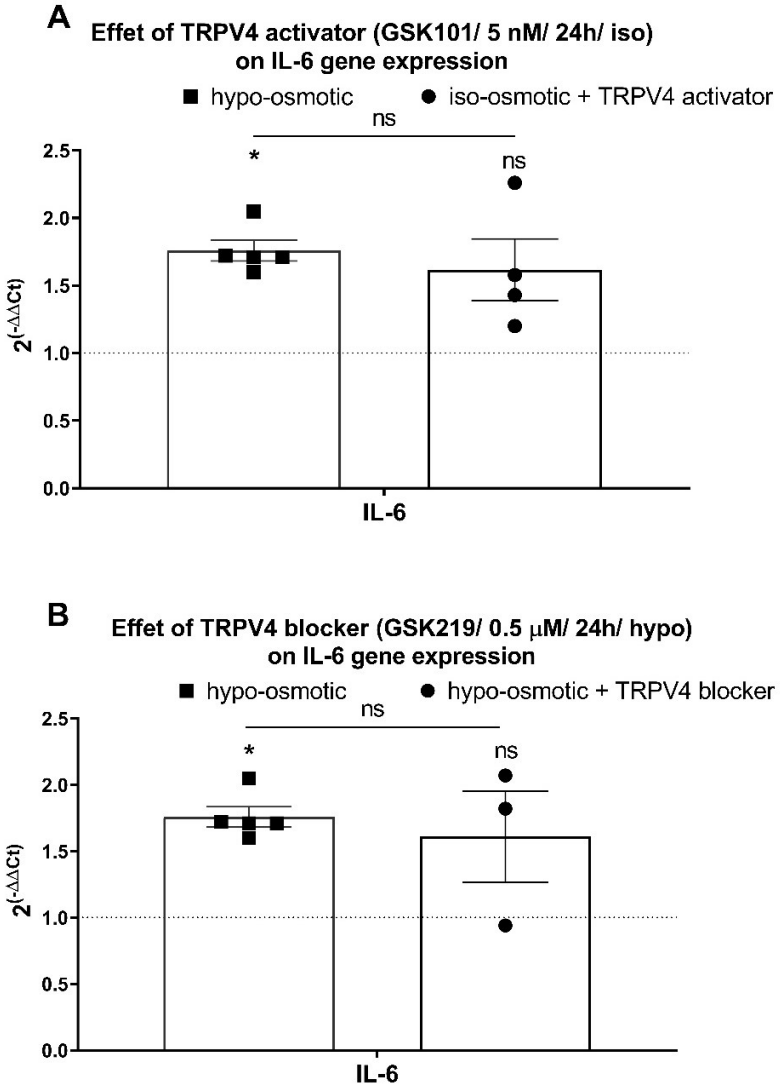


Figure 8. The mRNA expression of IL-6 in NP cells after 24 h of treatment with (**A**) TRPV4 activator (5 nM GSK1016790A) or (**B**) TRPV4 blocker (500 nM GSK2193874). Graphs show $2^{-\Delta\Delta Ct}$ values normalized to the iso-osmotic control (mean ± SEM, bNP P2, n = 3–4). Asterisks indicate statistical significance (*p < 0.05, ns, no statistical difference defined at p > 0.05) between hypo-osmotic group, iso-osmotic group, or iso-osmotic activator-supplemented group.

7.4 Discussion

Discogenic back pain has a high prevalence in the western world and there is an increasing need for improved diagnostic and treatment strategies [6, 21]. TRP channels, which constitute a superfamily of ion channels and have been shown to be involved in tissue homeostasis and disease in many organs, might also be a promising target for the treatment of low back pain [9, 10, 22]. To our knowledge, this is the first study that presents a complete gene screening of TRP channels in bovine IVD cells, demonstrates the effects of osmotic stress on bovine NP cells over short (24 h) and long (5 days) culture times using RNA sequencing, as well as shows that TRPV4 or TRPM7 channels do not seem to mediate hypo-osmolarity induced changes in aggrecan, ADAMTS9 or IL-6 expression.

According to our results and similarly to the findings reported earlier on human IVD tissue [10], the three most highly expressed TRP channels were TRPC1, TRPV4 and TRPM7. TRPC1 belongs to the canonical (C) subfamily and is broadly expressed in mammalian tissues. TRPC1 is hypothesized to have a mechanosensitive function, can be stretch activated and it can interact with other TRP channel members, such as TRPV or TRPP [23]. Decreased TRPC1 protein expression was linked to a greater increase in liver cells volume [24], indicating that TRPC1 may be a potential candidate for the investigation of osmosensing and volume regulation. We could demonstrate that TRPC1 was consistently well expressed in bovine NP and AF cells, and its expression was unaffected by passaging, which is consistent with earlier results on human non-degenerated NP and AF tissue [10] as well as on human articular chondrocytes [25]. However, due to the lack of TRPC1 channel specific activators and blockers, this channel was excluded from further research in the present study.

Osmosensing properties of the TRPV4 channel were investigated in numerous studies including tissues such as kidney, DRG neurons, epithelium, cartilage as well as IVD [11, 26-33]. Although limited data on TRPV4 in the IVD exists, it is believed to be the key mechano- and osmosensor protein in cartilage [34-38]. It was previously demonstrated that in chondrocytes TRPV4 may interact with integrin $\alpha 1\beta 1$ in response to hypo-osmotic stress (wildtype and integrin $\alpha 1$-null mice) [35], participate in cell volume regulation (porcine articular chondrocytes) [36], enhance ECM accumulation (porcine articular chondrocytes) [34] and its expression may be mediated by $ERK_{1/2}$ signaling (equine articular chondrocytes)

[39]. However, NP cells, despite being morphologically similar to articular chondrocytes, have distinctively different ECM composition and biomechanical properties, which should be considered when comparing both cell types [40-42]. In our study, TRPV4 was overall the most highly expressed TRP channel in freshly isolated NP and AF cells, but its expression significantly decreased with passaging. TRPV4 was previously shown to be equally well expressed in human non-degenerated and degenerated IVD tissue and its expression did not depend on tissue or patient specific characteristics, therefore implying of its fundamental role in the IVD homeostasis. A previous study reported increased TRPV4 protein expression in bovine NP cells after hypo-osmotic stimulation [11], however we were not able to reproduce these results. Another study demonstrated that TRPV4 gene expression seemed to be down-regulated, but without reaching statistical significance, under hypo-osmotic (130 mOsm/l) burst (1.5 h/day) and cyclic (10 min on and off for 1.5 h/day) conditions as compared to static loading (24h/day) in mice NP organ culture model [43]. In line with the latter [43], but in contrary to the earlier study [11], we showed that TRPV4 gene expression did not significantly differ between 24 h and 5 days of hypo-osmotic treatment and was significantly down-regulated as compared to iso-osmotic treatment. However, we have also noticed donor-to-donor variations in the TRPV4 (and TRPM7) gene expression. The contradictory findings between this and the past study [11] may be partially due to different detection techniques used, namely western blot versus RNA sequencing, qPCR and ICC (our study). In fact, we also intended to test TRPV4 protein expression using western blot to enhance comparability between studies, but found that antibodies available at the time of the analysis were, in our hands, non-specific for bovine cells as discerned in preliminary tests (data not shown).

Although TRPV4 expression is certainly of interest, changes in channel activity are ultimately of higher relevance in the context of sensing environmental changes and calcium signaling. Therefore, in the next step we employed GSK1016790A [44, 45] and GSK2193874 [46, 47], which are currently amongst the most selective TRPV4 channel activators and blockers, respectively. GSK1016790A was shown to induce Ca^{2+} influx through TRPV4 channel with estimated EC_{50} values of 2.1 nM (HEK293-hTRPV4 cells), 11 nM (guinea-pig urothelial cells) and 18 nM (HEK293- mTRPV4 cells) [45, 48], while GSK2193874 inhibits Ca^{2+} influx mediated by TRPV4 channels with IC_{50} values of 40-50 nM (HEK293-hTRPV4 cells) and 2 nM (HEK293-rTRPV4 cells) [46, 47]. In recent publications, it was

demonstrated that stimulation with GSK1016790A causes a down-regulation of TRPV4 channels from the plasma membrane within 20 -30 min post-treatment (10 nM in HeLa-TRPV4 cells [44] and 100 nM in HUVECs [49]) and translocation of the TRPV4 channel to the recycling endosomes [49]. We first tested whether there are any differences in TRPV4 channel activity properties between hypo- and iso-osmotic conditions and secondly whether the TRPV4 channel mediates gene expression changes of targets differently regulated by hypo-osmotic stress. When GSK1016790A was applied to both hypo- and iso-osmotic treated cells, a higher calcium flux was measured in the hypo-osmotic group, indicating that TRPV4 activity in bovine NP cells may be higher at reduced osmolarity. The mechanism of TRPV4 activation is still being discussed and several modes of action have been proposed. TRPV4 can be spontaneously activated at physiological osmolarity levels and can respond to changes in the local environment (increase its activity at reduced osmolarity and *vice versa*). In this context, TRPV4 activation can be triggered by cell volume changes, but presumably not directly due to stretch, but rather due to the activation of phospholipase A2 (PLA2) [26, 38, 50]. However, it is worth mentioning that differentiating between mechano- and osmo-function can be challenging, since osmotic cell swelling is inherently associated with membrane stretch. Interestingly, it was shown that in salivary glands, the activity of TRPV4 may be dependent on AQP5 [51], which therefore points towards a possibly complex osmosensing network.

The second most abundantly expressed TRP channel in bovine NP cells was TRPM7. TRPM7 belongs to the TRP melastatin (M) subfamily that is most recognized for its role in thermo-sensation, but also seems to be implicated in cell survival/death, cytokine release and response to oxidative stress [52, 53]. In certain tissue types that are known to experience daily osmotic changes, such as kidney, TRPM7 was identified as a mechano- and osmosensor that is involved in the regulation of cell volume changes [54, 55]. TRPM7 was so far only sparsely investigated in the IVD: TRPM7 was previously detected in non-degenerated and degenerated IVD tissue, where its expression tended to increase with the IVD degeneration grade [10]. In this study, we could demonstrate that TRPM7 was well expressed in both, bovine NP and AF cells, showed less variation than TRPV4 in its expression between passaging and its expression was unaffected by the hypo-osmotic treatment. This is partially in line with an earlier study on human articular chondrocytes, where TRPM7 was highly expressed in non-passaged cells, but contrary to our study, its expression greatly diminished

with passaging [25]. Naltriben, with an EC_{50} ~20 µM (HEK 293-mTRPM7 [56]), ~24 µM (rat ventricular myocytes [57]) and ~45 µM (ameloblast cell line LS8 cells [58]), and NS8593, with an IC_{50} ~1.6 µM (HEK 293 cells [59]) and 2 µM (rat ventricular myocytes [60]), are compounds that respectively activate and block TRPM7 channel. Naltriben was initially characterized as an antagonist of δ-opioid receptors [61] and may compete with the inhibitory effect of NS8598, but does not stimulate other TRP channels including TRPM2, TRPM3, TRPM8 and TRPV1 [56]. NS8593 is a potent TRPM7 inhibitor that exhibits complete and reversible block of TRPM7 currents, but may also target small conductance potassium channels, which are not related to TRPM7 channel [59, 62]. Inhibitory action of NS8593 on native TRPM7-like currents was demonstrated among others in HEK 293 cells, freshly isolated smooth muscle cells, ventricular myocytes as well as in primary podocytes [59]. It was shown that 50 µM Naltriben can effectively activate TRPM7 channel (without measureable off-target responses) and this response can be blocked with 20 µM NS8593 in HEK 293 cells overexpressing TRPM7 [56]. Moreover, 100 µM Naltriben activated and 100 µM NS8593 blocked Ca^{2+} fluctuations in mice chondrocytes from femoral cartilage plate [63]. In contrast, in the presented study we have used Naltriben concentrations ranging from 25 µM to 200 µM depending on the duration of the treatment and assay type. We have furthermore observed that an application of 50 µM Naltriben was not able to induce Ca^{2+} flux in primary bovine NP cells, while 100 µM had only minor effect as compared to the baseline (**Suppl. Fig. 2**). Furthermore, we have not observed differences in Ca^{2+} flux between the hypo- and iso-osmotic conditions, but the scientific evidence for TRPM7 sensitivity to osmotic gradient is inconclusive and suggests that observed differences may depend on the cell type. For example, it was shown that TRPM7 is involved in regulatory volume decrease (RVD) after osmotic cell swelling and TRPM7 silencing reduced the rate of RVD in human HeLa and human embryonic kidney HEK293T cells [54], hence indicating TRPM7's sensitivity to osmotic changes. Yet, TRPM7 current was unaffected by hypotonic solution or cell swelling in rat brain microglia [64]. Overall, our data indicate that TRPM7 gene/protein expression as well as activity is independent from hypo-osmotic stress in bovine NP cells.

Under hypo-osmotic stimulation, NP cells exhibited a significant increase in mRNA levels of pro-inflammatory and catabolic factors, such as IL-6 and IL-11, Small-Inducible Cytokine A5 (CCL5), nerve growth factor (NGF), ADAMTS4 and ADAMTS9 and factors inducing

cell apoptosis via nuclear factor kappa-B (NF-κB), e.g. Tumor Necrosis Factor Receptor Superfamily Member 21 (TNFRSF21) or Neuronal Cell Death Inducible Putative Kinase (TRIB3). In contrast, a down-regulation was observed for several membrane proteins involved in transporting soluble molecules (e.g. members of solute carrier family (SCL) and water channels (e.g. aquaporin (AQP) especially AQP1), as well as, aggrecan (ACAN). IL-6, ADAMTS9 and aggrecan were selected to test whether their altered gene expression observed under hypo-osmotic stimulation may be mediated by the TRPV4 or TRPM7 channel. IL-6 is a widely investigated cytokine in the field of painful disc degeneration. Increased IL-6 expression was observed in herniated and degenerated IVDs [65]. Moreover, IL-6 can be secreted by IVD cells and induce TNF-α expression, which is associated with neuropathic pain [66, 67]. Hence, targeting IL-6 may have beneficial effects for pain treatment. Aggrecan is the primary proteoglycan of NP cells, is important for the normal osmotic function of the IVD as it provides the ability to bind water, and is therefore contributing to the tissue's hydration, integrity and its biomechanical function (e.g. ability to withstand load). In contrast, ADAMTS9 is an aggrecanases, which together with other members of the ADAMTS family, mediates aggrecan turnover and degradation in the IVD. It was established that ADAMTS9 is expressed in non-degenerated and degenerated IVD tissue, with an increased protein expression in the latter [68]. Moreover, ADAMTS9 was shown to be induced by IL-1β and TNF-α in human chondrocytes [69]. We have demonstrated that hypo-osmotic treatment alone increases the expression of IL-6 and ADAMTS9 and decreases the expression of aggrecan in healthy bovine NP cells.

A past study has demonstrated that activation of TRPV4 with GSK1016790A may induce ECM and immune system regulatory gene expression changes (e.g. down-regulation of ADAMTS5 and NOS2) similarly to dynamic loading alone and increases ECM accumulation akin to a long-term (≥2 weeks) osmotic loading in porcine articular chondrocytes [34]. However, in the same study TRPV4 inhibition with GSK205 alone (that is without the dynamic loading) had no effect on the gene expression of tested targets [34]. Yet in another study, TRPV4 inhibition with GSK2193874 reduced cytokine production (TNFα, IL-1α and IL-6) in septic mice [70]. Here we have shown that activation of TRPV4 with GSK1016790A in the iso-osmotic condition up-regulated aggrecan (but inversely to the hypo-osmotic treatment), had no effect on ADAMTS9 expression and induced IL-6 in a manner similar to hypo-osmotic treatment alone. However, inhibition of TRPV4 activity in bovine NP cells

was not able to reverse the hypo-osmotically-induced IL-6 expression, although past studies on bovine NP cells hypothesized that IL-6 is a downstream target of TRPV4 [11]. Therefore, we speculate that the hypo-osmotically-induced gene changes of IL-6, aggrecan and ADAMTS9 are not mediated by TRPV4 in the IVD. Future studies should examine the interplay between inflammation and TRPV4 and TRPM7 sensitization and activity in the IVD. The observed IL-6 upregulation may be augmented in part by prostanoid PGE2 activation of PAR-2 signaling [71], which was shown to cause TRPV4 sensitization in mice [72] and rats [73] in studies on inflammatory and neuropathic pain as well as mechanical hyperalgesia [74]. Moreover, our data suggests that TRPM7 activation does not influence aggrecan, ADAMTS9 or IL-6 expression alike to hypo-osmotic treatment. Interestingly, it was shown that IL-6 might inhibit TRPM7 currents via JAK2-STAT3 signaling pathways in rat cortical neurons [75]. Furthermore, it was indicated that TRPM7 inhibition (with a non-specific channel inhibitor 2-APB) decreased IL-6 release in allergen-sensitized rat BMMC [76]. Thus, there is a possible relationship between IL-6 and TRPM7, whereas it might not be associated with osmotic loading alone and may be tissue/cell type dependent. Future studies will aim to establish whether the interplay of TRP channels with other membrane proteins may contribute to the altered expression of these and other targets under hypo-osmotic stimulation. A limitation of the current experimental design was the use of bovine IVD tissue instead of healthy human IVD tissue, which may in the future be beneficial for translating current findings. Moreover, in contrary to bovine tails, human spine experiences day-to-day mechanical loading from body weight and daily activities, which not only can act as a stimuli for IVD cells and TRP channels, but also lead to daily osmotic shifts (e.g. increased osmotic pressure during daily life activities and reduced osmotic pressure during rest periods) [77-79]. Hence, dynamic mechanical loading as well as diurnal osmotic loading could be incorporated in the future studies. Additionally, a lack of channel specific activators and blockers (e.g. for TRPC1) and possible off-target effects for Naltriben and NS8593 limited the scope of this project.

7.5 Conclusion

In summary, we demonstrated that several TRP channels are expressed in the bovine IVD cells. Hypo-osmotic treatment led to a higher calcium flux through TRPV4. We have presented genome-wide expression changes caused by reduced osmolarity in bovine NP cells and have shown through pharmacological activation and/or inhibition of TRPV4 and

TRPM7 that these channels likely do not mediate hypo-osmotically-induced gene expression changes of aggrecan, ADAMTS9 and IL-6 in bovine NP cells.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

AS designed and executed cell experiments, analyzed and interpreted data, prepared the graphs and drafted the manuscript. BA performed the calcium imaging experiments. WH conducted statistical analysis on the gene array and calcium data and proof-read the manuscript. SF participated in the study design and critically reviewed the manuscript. KW-K contributed to the conception of the study, provided expertise, secured the funding and helped drafting the manuscript.

Acknowledgments

We would like to acknowledge Functional Genomics Center Zurich (FGCZ), especially Susanne Kreutzer and Lennart Opitz for their support and expertise in processing RNA sequencing data.

Funding

The study was financially supported by the Swiss Neuro Foundation (Bern/Switzerland), the Swiss National Science Foundation (SNF PP00P2_163678/1) as well as the Spine Society of Europe (Eurospine 2016_4).

Data Availability Statement

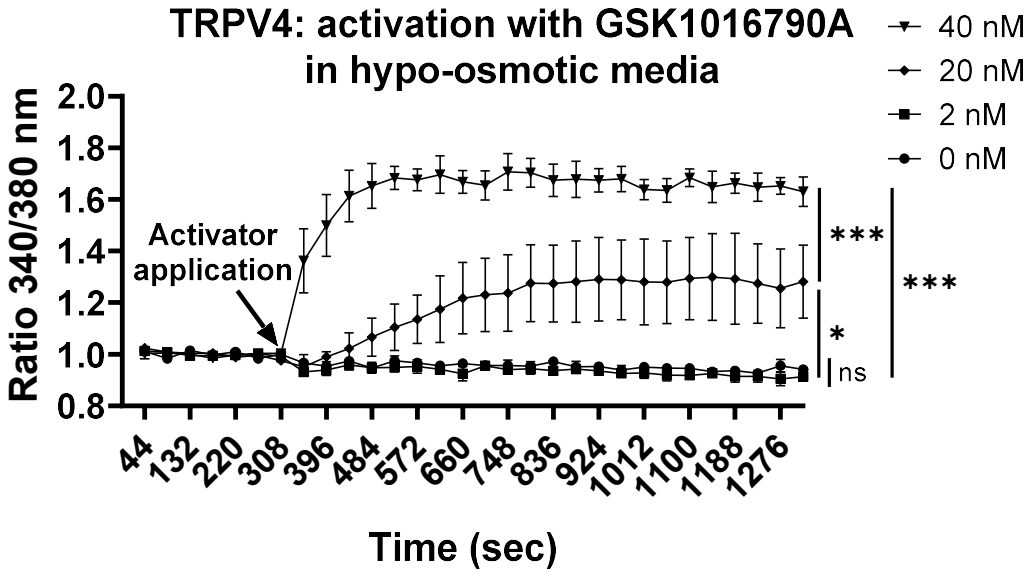
The RNA sequencing datasets for this study can be found in the European Nucleotide Archive (ENA) repository with the accession ID PRJEB37486.

Supplementary Material

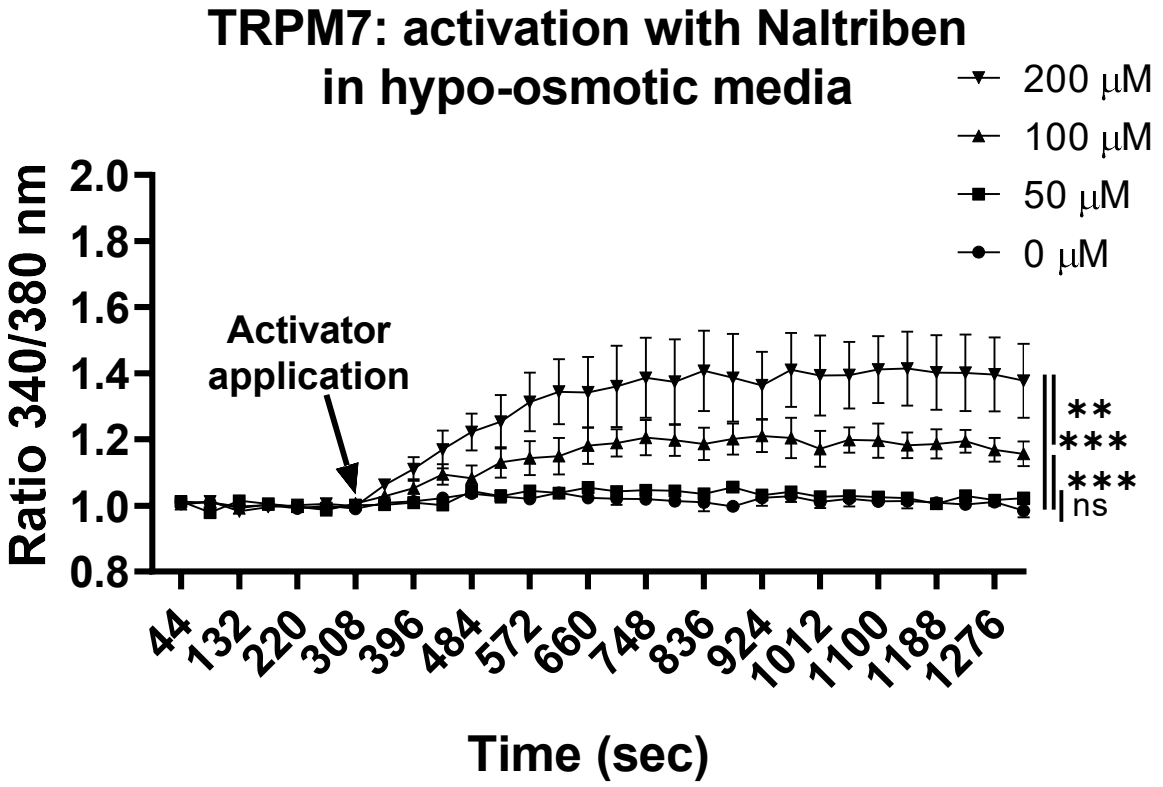
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.00952/full#supplementary-material

Supplementary table 1 Summary of GSEA results with p-value FDR < 0.05 and p-value < 0.05 for the 24 h treatment group of hypo-osmotic *versus* iso-osmotic stimulation in bovine NP cells.

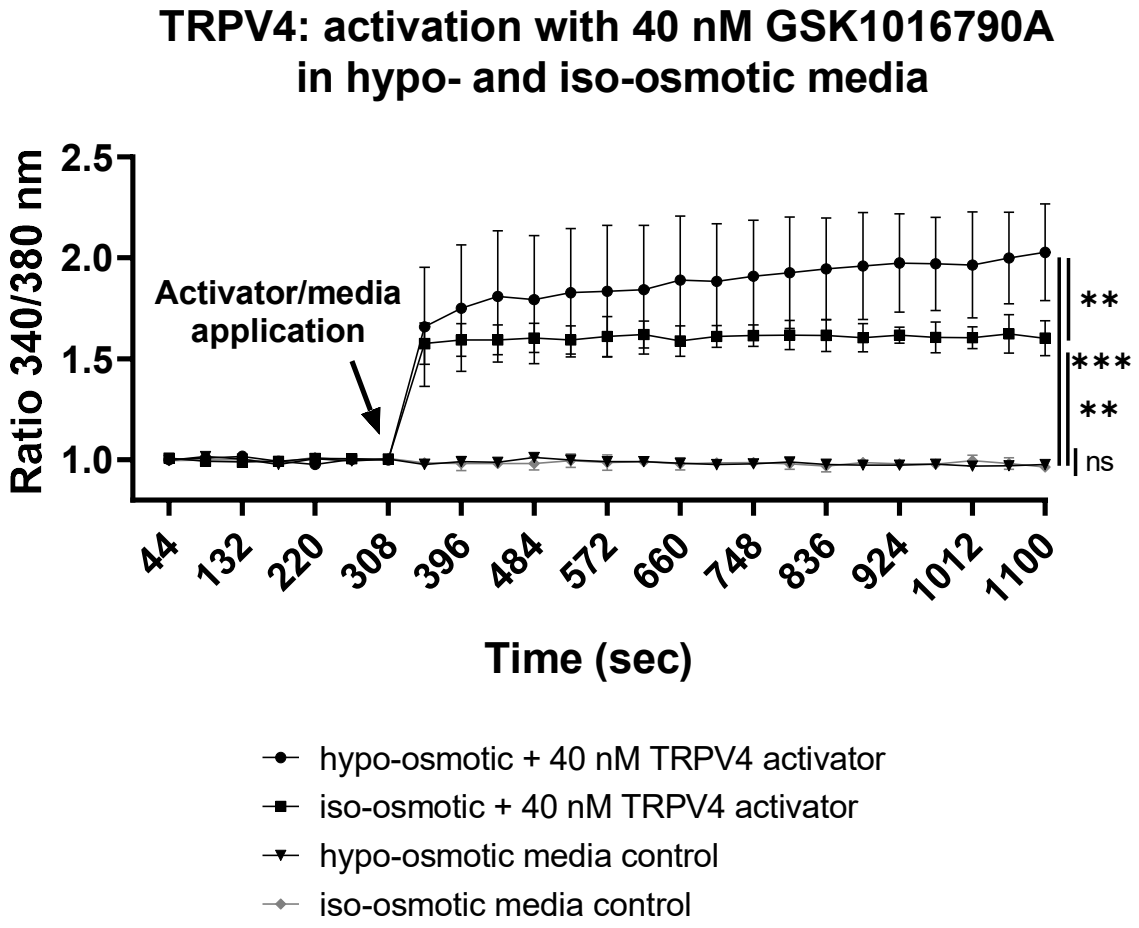
Supplementary table 2 Summary of GSEA results with p-value FDR < 0.05 and p-value < 0.05 for the 5 days long treatment group of hypo-osmotic *versus* iso-osmotic stimulation in bovine NP cells.



Supplementary Figure 1. Dose response activation of TRPV4 with GSK1016790A in hypo-osmotic condition. Graphs present the ratio of the bound calcium to the unbound calcium (340 nm/380 nm) normalized to the baseline over the measurement time (mean ± SEM, bNP P2, n = 3). After the baseline measurement, empty or compound-supplemented media was added (indicated by an arrow on a graph) and the measurement was continued for up to around 15 min more. Asterisks indicates statistical significance (* $p < 0.05$, ** p 0.01, *** p 0.001, *ns*: no statistical difference defined as $p > 0.05$) as measured on the last measurement cycle.



Supplementary Figure 2. Dose response activation of TRPM7 with Naltriben methanesulfonate in hypo-osmotic condition. Graphs present the ratio of the bound calcium to the unbound calcium (340 nm/380 nm) normalized to the baseline over the measurement time (mean ± SEM, bNP P2, n = 3). After the baseline measurement, empty or compound-supplemented media was added (indicated by an arrow on a graph) and the measurement was continued for up to around 15 min more. Asterisks indicates statistical significance (* $p < 0.05$, ** p 0.01, *** p 0.001, *ns*: no statistical difference defined as $p > 0.05$) as measured on the last measurement cycle.



Supplementary Figure 3. Ca^{2+} flux in NP cells following the application of 40 nM GSK1016790A (TRPV4 activator) in hypo- and iso-osmotic condition. Graphs present the ratio of the bound calcium to the unbound calcium (340 nm/380 nm) normalized to the baseline over the measurement time (mean ± SEM, bNP P2, n = 3). After the baseline measurement, empty (control) or compound-supplemented (treatment) media was added (indicated by an arrow on a graph) and the measurement was continued for up to around 15 min more. Asterisks indicates statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, *ns*: no statistical difference defined as $p > 0.05$) as measured on the last measurement cycle.

References

1. Cassinelli, E.H., R.A. Hall, and J.D. Kang, *Biochemistry of intervertebral disc degeneration and the potential for gene therapy applications.* Spine J, 2001. **1**(3): p. 205-14.
2. Roughley, P.J., M. Alini, and J. Antoniou, *The role of proteoglycans in aging, degeneration and repair of the intervertebral disc.* Biochem Soc Trans, 2002. **30**(Pt 6): p. 869-74.
3. Wuertz, K., et al., *Influence of extracellular osmolarity and mechanical stimulation on gene expression of intervertebral disc cells.* J Orthop Res, 2007. **25**(11): p. 1513-22.
4. Le Maitre, C.L., A.J. Freemont, and J.A. Hoyland, *Localization of degradative enzymes and their inhibitors in the degenerate human intervertebral disc.* J Pathol, 2004. **204**(1): p. 47-54.
5. Molinos, M., et al., *Inflammation in intervertebral disc degeneration and regeneration.* J R Soc Interface, 2015. **12**(104): p. 20141191.
6. Wieser, S., et al., *Cost of low back pain in Switzerland in 2005.* Eur J Health Econ, 2011. **12**(5): p. 455-67.
7. Ulger, O., et al., *Effectiveness of physiotherapy and minimal invasive technics on functional status and quality of life in geriatric patients with low back pain.* J Exerc Rehabil, 2018. **14**(6): p. 1048-1052.
8. Koc, Z., et al., *Effectiveness of physical therapy and epidural steroid injections in lumbar spinal stenosis.* Spine (Phila Pa 1976), 2009. **34**(10): p. 985-9.
9. Krupkova, O., J. Zvick, and K. Wuertz-Kozak, *The role of transient receptor potential channels in joint diseases.* Eur Cell Mater, 2017. **34**: p. 180-201.
10. Sadowska, A., et al., *Differential regulation of TRP channel gene and protein expression by intervertebral disc degeneration and back pain.* Sci Rep, 2019. **9**(1): p. 18889.
11. Walter, B.A., et al., *Reduced tissue osmolarity increases TRPV4 expression and pro-inflammatory cytokines in intervertebral disc cells.* European cells & materials, 2016. **32**(7): p. 123-36.
12. Grimm, C., et al., *Molecular and functional characterization of the melastatin-related cation channel TRPM3.* J Biol Chem, 2003. **278**(24): p. 21493-501.
13. Harteneck, C. and B. Reiter, *TRP channels activated by extracellular hypo-osmoticity in epithelia.* Biochem Soc Trans, 2007. **35**(Pt 1): p. 91-5.
14. Gomis, A., et al., *Hypoosmotic- and pressure-induced membrane stretch activate TRPC5 channels.* J Physiol, 2008. **586**(23): p. 5633-49.

15. Wilson, C. and S.E. Dryer, *A mutation in TRPC6 channels abolishes their activation by hypoosmotic stretch but does not affect activation by diacylglycerol or G protein signaling cascades.* Am J Physiol Renal Physiol, 2014. **306**(9): p. F1018-25.
16. Demers, C.N., J. Antoniou, and F. Mwale, *Value and limitations of using the bovine tail as a model for the human lumbar spine.* Spine (Phila Pa 1976), 2004. **29**(24): p. 2793-9.
17. Bolger, A.M., M. Lohse, and B. Usadel, *Trimmomatic: a flexible trimmer for Illumina sequence data.* Bioinformatics, 2014. **30**(15): p. 2114-20.
18. Dobin, A., et al., *STAR: ultrafast universal RNA-seq aligner.* Bioinformatics, 2013. **29**(1): p. 15-21.
19. Bray, N.L., et al., *Near-optimal probabilistic RNA-seq quantification.* Nat Biotechnol, 2016. **34**(5): p. 525-7.
20. Robinson, M.D., D.J. McCarthy, and G.K. Smyth, *edgeR: a Bioconductor package for differential expression analysis of digital gene expression data.* Bioinformatics, 2010. **26**(1): p. 139-40.
21. Geurts, J.W., et al., *The Impact of Chronic Discogenic Low Back Pain: Costs and Patients' Burden.* Pain Res Manag, 2018. **2018**: p. 4696180.
22. Sadowska, A., et al., *Inflammaging in cervical and lumbar degenerated intervertebral discs: analysis of proinflammatory cytokine and TRP channel expression.* Eur Spine J, 2017.
23. Du, J., et al., *TRPV4, TRPC1, and TRPP2 assemble to form a flow-sensitive heteromeric channel.* FASEB J, 2014. **28**(11): p. 4677-85.
24. Chen, J. and G.J. Barritt, *Evidence that TRPC1 (transient receptor potential canonical 1) forms a Ca(2+)-permeable channel linked to the regulation of cell volume in liver cells obtained using small interfering RNA targeted against TRPC1.* Biochem J, 2003. **373**(Pt 2): p. 327-36.
25. Gavenis, K., et al., *Expression of ion channels of the TRP family in articular chondrocytes from osteoarthritic patients: changes between native and in vitro propagated chondrocytes.* Mol Cell Biochem, 2009. **321**(1-2): p. 135-43.
26. Plant, T.D. and R. Strotmann, *TRPV4*, in *Transient Receptor Potential (TRP) Channels*, V. Flockerzi and B. Nilius, Editors. 2007, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 189-205.
27. Liedtke, W., *TRPV4 as osmosensor: a transgenic approach.* Pflugers Arch, 2005. **451**(1): p. 176-80.
28. Arniges, M., et al., *Swelling-activated Ca2+ entry via TRPV4 channel is defective in cystic fibrosis airway epithelia.* J Biol Chem, 2004. **279**(52): p. 54062-8.

29. Andrade, Y.N., et al., *TRPV4 channel is involved in the coupling of fluid viscosity changes to epithelial ciliary activity.* J Cell Biol, 2005. **168**(6): p. 869-74.
30. Mizuno, A., et al., *Impaired osmotic sensation in mice lacking TRPV4.* Am J Physiol Cell Physiol, 2003. **285**(1): p. C96-101.
31. Cohen, D.M., *TRPV4 and the mammalian kidney.* Pflugers Arch, 2005. **451**(1): p. 168-75.
32. Alessandri-Haber, N., et al., *Hypotonicity induces TRPV4-mediated nociception in rat.* Neuron, 2003. **39**(3): p. 497-511.
33. Liedtke, W. and J.M. Friedman, *Abnormal osmotic regulation in trpv4-/- mice.* Proc Natl Acad Sci U S A, 2003. **100**(23): p. 13698-703.
34. O'Conor, C.J., et al., *TRPV4-mediated mechanotransduction regulates the metabolic response of chondrocytes to dynamic loading.* Proc Natl Acad Sci U S A, 2014. **111**(4): p. 1316-21.
35. Jablonski, C.L., et al., *Integrin alpha1beta1 participates in chondrocyte transduction of osmotic stress.* Biochem Biophys Res Commun, 2014. **445**(1): p. 184-90.
36. Phan, M.N., et al., *Functional characterization of TRPV4 as an osmotically sensitive ion channel in porcine articular chondrocytes.* Arthritis Rheum, 2009. **60**(10): p. 3028-37.
37. Clark, A.L., et al., *Chondroprotective role of the osmotically sensitive ion channel transient receptor potential vanilloid 4: age- and sex-dependent progression of osteoarthritis in Trpv4-deficient mice.* Arthritis Rheum, 2010. **62**(10): p. 2973-83.
38. Servin-Vences, M.R., et al., *Direct measurement of TRPV4 and PIEZO1 activity reveals multiple mechanotransduction pathways in chondrocytes.* Elife, 2017. **6**.
39. Hdud, I.M., A. Mobasheri, and P.T. Loughna, *Effect of osmotic stress on the expression of TRPV4 and BKCa channels and possible interaction with ERK1/2 and p38 in cultured equine chondrocytes.* American journal of physiology. Cell physiology, 2014. **306**(11): p. C1050-7.
40. Cui, Y., et al., *Differential gene expression profiling of metalloproteinases and their inhibitors: a comparison between bovine intervertebral disc nucleus pulposus cells and articular chondrocytes.* Spine (Phila Pa 1976), 2010. **35**(11): p. 1101-8.
41. Lee, C.R., et al., *A phenotypic comparison of intervertebral disc and articular cartilage cells in the rat.* Eur Spine J, 2007. **16**(12): p. 2174-85.
42. Aota, Y., et al., *Comparison of cellular response in bovine intervertebral disc cells and articular chondrocytes: effects .of lipopolysaccharide on proteoglycan metabolism.* Cell Tissue Res, 2006. **326**(3): p. 787-93.

43. Palacio-Mancheno, P.E., et al., *Hyperosmolarity induces notochordal cell differentiation with aquaporin3 upregulation and reduced N-cadherin expression.* J Orthop Res, 2017.
44. Jin, M., et al., *Determinants of TRPV4 activity following selective activation by small molecule agonist GSK1016790A.* PLoS One, 2011. **6**(2): p. e16713.
45. Thorneloe, K.S., et al., *N-((1S)-1-{[4-((2S)-2-{[(2,4-dichlorophenyl)sulfonyl]amino}-3-hydroxypropanoyl)-1 -piperazinyl]carbonyl}-3-methylbutyl)-1-benzothiophene-2-carboxamide (GSK1016790A), a novel and potent transient receptor potential vanilloid 4 channel agonist induces urinary bladder contraction and hyperactivity: Part I.* J Pharmacol Exp Ther, 2008. **326**(2): p. 432-42.
46. Cheung, M., et al., *Discovery of GSK2193874: An Orally Active, Potent, and Selective Blocker of Transient Receptor Potential Vanilloid 4.* ACS Med Chem Lett, 2017. **8**(5): p. 549-554.
47. Thorneloe, K.S., et al., *An orally active TRPV4 channel blocker prevents and resolves pulmonary edema induced by heart failure.* Sci Transl Med, 2012. **4**(159): p. 159ra148.
48. Xu, X., et al., *Functional TRPV4 channels and an absence of capsaicin-evoked currents in freshly-isolated, guinea-pig urothelial cells.* Channels (Austin), 2009. **3**(3): p. 156-60.
49. Baratchi, S., et al., *The TRPV4 Agonist GSK1016790A Regulates the Membrane Expression of TRPV4 Channels.* Front Pharmacol, 2019. **10**: p. 6.
50. Pedersen, S.F. and B. Nilius, *Transient receptor potential channels in mechanosensing and cell volume regulation.* Methods Enzymol, 2007. **428**: p. 183-207.
51. Liu, X., et al., *A role for AQP5 in activation of TRPV4 by hypotonicity: concerted involvement of AQP5 and TRPV4 in regulation of cell volume recovery.* J Biol Chem, 2006. **281**(22): p. 15485-95.
52. Massullo, P., et al. *TRPM channels, calcium and redox sensors during innate immune responses.* in *Seminars in cell & developmental biology.* 2006. Elsevier.
53. McNulty, S. and E. Fonfria, *The role of TRPM channels in cell death.* Pflugers Arch, 2005. **451**(1): p. 235-42.
54. Numata, T., T. Shimizu, and Y. Okada, *TRPM7 is a stretch- and swelling-activated cation channel involved in volume regulation in human epithelial cells.* Am J Physiol Cell Physiol, 2007. **292**(1): p. C460-7.
55. Kim, J.M., S. Choi, and K. Park, *TRPM7 Is Involved in Volume Regulation in Salivary Glands.* J Dent Res, 2017. **96**(9): p. 1044-1050.

56. Hofmann, T., et al., *Activation of TRPM7 channels by small molecules under physiological conditions.* Pflugers Arch, 2014. **466**(12): p. 2177-89.
57. Tashiro, M., H. Inoue, and M. Konishi, *Modulation of Mg(2+) influx and cytoplasmic free Mg(2+) concentration in rat ventricular myocytes.* J Physiol Sci, 2019. **69**(1): p. 97-102.
58. Souza Bomfim, G.H., et al., *TRPM7 activation potentiates SOCE in enamel cells but requires ORAI.* Cell Calcium, 2020. **87**: p. 102187.
59. Chubanov, V., et al., *Natural and synthetic modulators of SK (K(ca)2) potassium channels inhibit magnesium-dependent activity of the kinase-coupled cation channel TRPM7.* Br J Pharmacol, 2012. **166**(4): p. 1357-76.
60. Tashiro, M., H. Inoue, and M. Konishi, *Physiological pathway of magnesium influx in rat ventricular myocytes.* Biophys J, 2014. **107**(9): p. 2049-58.
61. June, H.L., et al., *The delta 2-opioid receptor antagonist naltriben reduces motivated responding for ethanol.* Psychopharmacology (Berl), 1999. **147**(1): p. 81-9.
62. Jenkins, D.P., et al., *Negative gating modulation by (R)-N-(benzimidazol-2-yl)-1,2,3,4-tetrahydro-1-naphthylamine (NS8593) depends on residues in the inner pore vestibule: pharmacological evidence of deep-pore gating of K(Ca)2 channels.* Mol Pharmacol, 2011. **79**(6): p. 899-909.
63. Qian, N., et al., *TRPM7 channels mediate spontaneous Ca(2+) fluctuations in growth plate chondrocytes that promote bone development.* Sci Signal, 2019. **12**(576).
64. Jiang, X., E.W. Newell, and L.C. Schlichter, *Regulation of a TRPM7-like current in rat brain microglia.* J Biol Chem, 2003. **278**(44): p. 42867-76.
65. Lee, S., et al., *Comparison of growth factor and cytokine expression in patients with degenerated disc disease and herniated nucleus pulposus.* Clin Biochem, 2009. **42**(15): p. 1504-11.
66. Risbud, M.V. and I.M. Shapiro, *Role of cytokines in intervertebral disc degeneration: pain and disc content.* Nat Rev Rheumatol, 2014. **10**(1): p. 44-56.
67. Weber, K.T., et al., *Serum levels of the proinflammatory cytokine interleukin-6 vary based on diagnoses in individuals with lumbar intervertebral disc diseases.* Arthritis Res Ther, 2016. **18**: p. 3.
68. Pockert, A.J., et al., *Modified expression of the ADAMTS enzymes and tissue inhibitor of metalloproteinases 3 during human intervertebral disc degeneration.* Arthritis Rheum, 2009. **60**(2): p. 482-91.
69. Demircan, K., et al., *ADAMTS-9 is synergistically induced by interleukin-1beta and tumor necrosis factor alpha in OUMS-27 chondrosarcoma cells and in human chondrocytes.* Arthritis Rheum, 2005. **52**(5): p. 1451-60.

70. Dalsgaard, T., et al., *Pharmacological inhibitors of TRPV4 channels reduce cytokine production, restore endothelial function and increase survival in septic mice.* Sci Rep, 2016. **6**: p. 33841.
71. Poole, D.P., et al., *Protease-activated receptor 2 (PAR2) protein and transient receptor potential vanilloid 4 (TRPV4) protein coupling is required for sustained inflammatory signaling.* J Biol Chem, 2013. **288**(8): p. 5790-802.
72. Alessandri-Haber, N., et al., *TRPV4 mediates pain-related behavior induced by mild hypertonic stimuli in the presence of inflammatory mediator.* Pain, 2005. **118**(1-2): p. 70-9.
73. Alessandri-Haber, N., et al., *Transient receptor potential vanilloid 4 is essential in chemotherapy-induced neuropathic pain in the rat.* J Neurosci, 2004. **24**(18): p. 4444-52.
74. Grant, A.D., et al., *Protease-activated receptor 2 sensitizes the transient receptor potential vanilloid 4 ion channel to cause mechanical hyperalgesia in mice.* J Physiol, 2007. **578**(Pt 3): p. 715-33.
75. Liu, A., et al., *Regulation of TRPM7 Function by IL-6 through the JAK2-STAT3 Signaling Pathway.* PLoS One, 2016. **11**(3): p. e0152120.
76. Huang, L., et al., *Inhibition of TRPM7 channels reduces degranulation and release of cytokines in rat bone marrow-derived mast cells.* Int J Mol Sci, 2014. **15**(7): p. 11817-31.
77. Chan, S.C., S.J. Ferguson, and B. Gantenbein-Ritter, *The effects of dynamic loading on the intervertebral disc.* Eur Spine J, 2011. **20**(11): p. 1796-812.
78. Schmidt, H., et al., *Response analysis of the lumbar spine during regular daily activities--a finite element analysis.* J Biomech, 2010. **43**(10): p. 1849-56.
79. Sadowska, A., et al., *Osmosensing, osmosignalling and inflammation: how intervertebral disc cells respond to altered osmolarity.* Eur Cell Mater, 2018. **36**: p. 231-250.

Appendix II

This section includes additional information about the RNA sequencing presented in the chapter 7 and earlier unpublished data on the effect of hyper-osmolarity on TRP channels in bovine NP cells developed during the work on the manuscript: *Hypo-osmotic loading induces expression of IL-6 in nucleus pulposus cells of the intervertebral disc independent of TRPV4 and TRPM7* presented in the chapter 7.

RNA sequencing

RNA integrity is an important factor for a reliable gene measurement outcome. RNA Integrity Number (RIN) is a standardized grading system of RNA integrity and can range from 1 (totally degraded) to 10 (intact) [1]. It was shown that a higher RIN value corresponds with a higher gene expression and *vice versa* [1]. Typically, values about eight are considered suitable for the RNA sequencing [2]. Following figures present the RIN values of used samples *versus* read count (**Figure A1**) and rRNA content (**Figure A2**). In total, 12 samples were used from three different donors at two different collection times (24 h and 5 days) and two media conditions (hypo- and iso-osmotic). Nine out of 12 samples scored RIN = 10 and the remaining three samples scored RIN = 9.9 (**Table A1**). The term "reads" is used to describe the depth of a sequencing. Usually between 5 to 200 million reads or more are being used in RNA sequencing, where a higher amount of reads may quantify less abundant genes [3]. However, a higher amount of reads can also contribute to the detection of off-targets and noise [3]. Additionally, the final decision on the total number of reads may be based on experimental goals such as if the screening is targeted, global or in-depth [3]. In this study, 20 million reads per sample were used (**Table A1** and **Figure A3**), following the recommendation of the Functional Genomics Center Zurich.

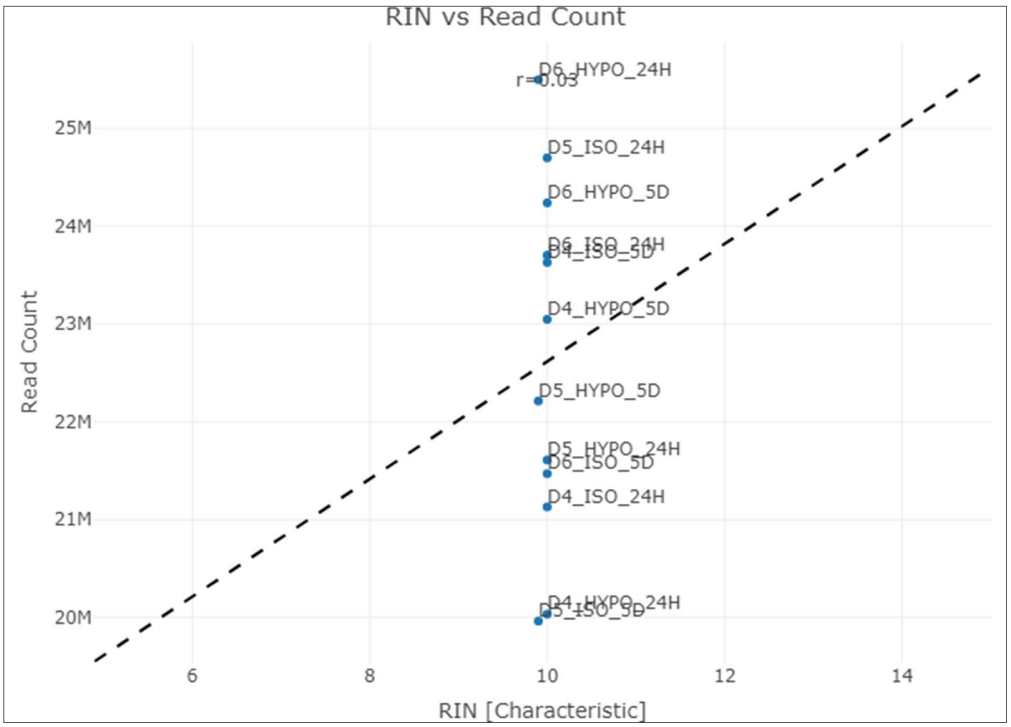


Figure A1. RIN count *versus* read count.

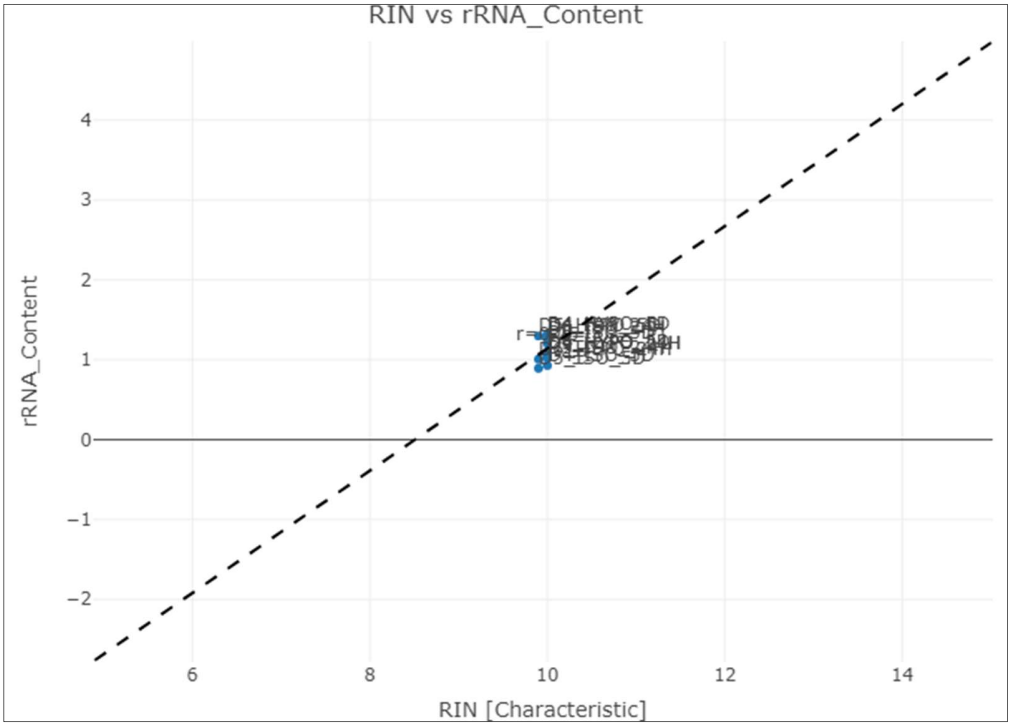


Figure A2. RIN count *versus* rRNA content.

Table A1. Additional information regarding the RNA seq run and sample quality used in project id p3140.

Sample no	Species	Sample Conc [ng/µL]	Sample Id [B-Fabric]	LibConc_100_800bp	RIN	Library PrepKit	Enrichment Method	Input Amount	Read Count
1	Bos taurus	605.5	bfs_19 7150	46.59	9.9	TruSeq RNA stranded	PolyA, TruSeq RNA Kit	500	22214111
2	Bos taurus	76.8	bfs_19 7152	75.05	9.9	TruSeq RNA stranded	PolyA, TruSeq RNA Kit	500	25498925
3	Bos taurus	448.9	bfs_19 7151	124.7	9.9	TruSeq RNA stranded	PolyA, TruSeq RNA Kit	500	19963980
4	Bos taurus	154.1	bfs_19 7148	37.55	10	TruSeq RNA stranded	PolyA, TruSeq RNA Kit	500	21610914
5	Bos taurus	141.2	bfs_19 7149	125.4	10	TruSeq RNA stranded	PolyA, TruSeq RNA Kit	500	24699500
6	Bos taurus	290.8	bfs_19 7146	81.31	10	TruSeq RNA stranded	PolyA, TruSeq RNA Kit	500	20034986
7	Bos taurus	242.3	bfs_19 7147	87.22	10	TruSeq RNA stranded	PolyA, TruSeq RNA Kit	500	21131584
8	Bos taurus	112.5	bfs_19 7153	102.6	10	TruSeq RNA stranded	PolyA, TruSeq RNA Kit	500	23704662
9	Bos taurus	1493	bfs_19 7144	42.38	10	TruSeq RNA stranded	PolyA, TruSeq RNA Kit	500	23048914
10	Bos taurus	309.7	bfs_19 7154	123.5	10	TruSeq RNA stranded	PolyA, TruSeq RNA Kit	500	24239928
11	Bos taurus	633	bfs_19 7145	117.9	10	TruSeq RNA stranded	PolyA, TruSeq RNA Kit	500	23630349
12	Bos taurus	333.2	bfs_19 7155	123.1	10	TruSeq RNA stranded	PolyA, TruSeq RNA Kit	500	21471223

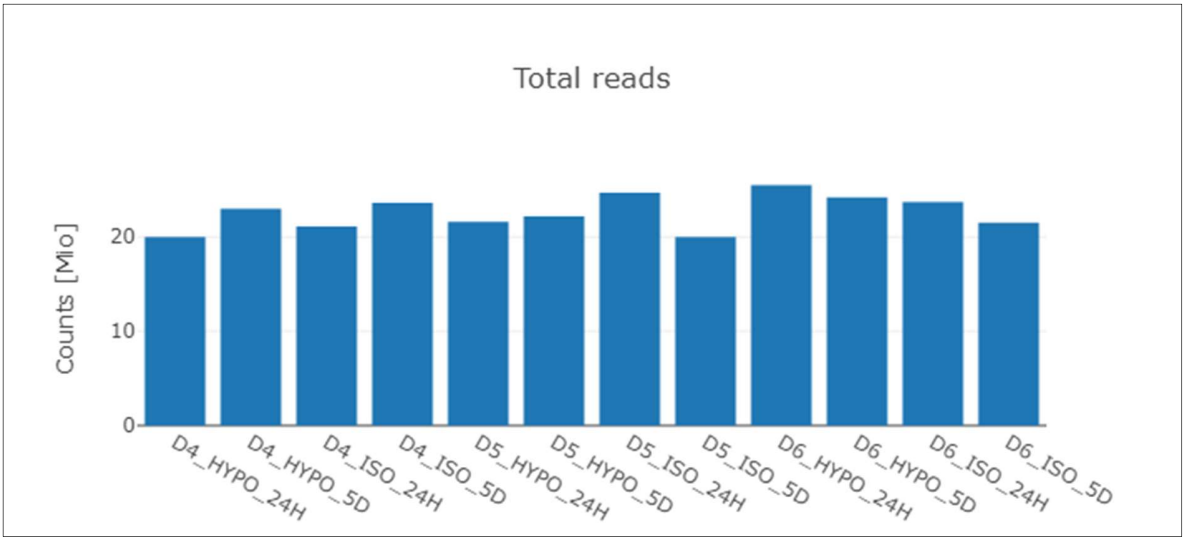


Figure A3. Number of reads per sample.

Hyper-osmolarity in the IVD and its influence of the TRPV4/M7 channels

Motivation

Hypo- (~300 mOsm) and iso-osmotic (~400 mOsm) conditions were described earlier in chapter 7. This section includes hyper-osmotic (~500 mOsm) conditions, which were tested at the early stage of this project, but discontinued due to its limited relevance to discogenic pain. A healthy IVD experiences diurnal osmotic changes and hyper-osmolarity can be experienced during daily tasks, for example lifting or load bearing, whereas a rest period and relaxation (e.g. sleep) is characterized by iso-osmotic levels [4, 5]. The influence of hyper-osmolarity on the IVD cells is described in chapter 6.

Materials and methods

Please refer to the chapter 7.

Results

Under hyper-osmotic stimulation, TRPV4 mediated Ca^{2+} influx was significantly lower compared to iso- and hypo-osmotic groups (**Figure A4**). For TRPM7 activation, there was overall no difference in response to 200 µM Naltriben (TRPM7 activator) between the three

media condition **(Figure A5),** but interestingly the difference became significant when the dose of TRPM7 activator was lowered to 50 µM **(Figure A6)**. However it must be noted that at the lower concentration dose, the exhibited Ca^{2+} influx was correspondingly much lower and only slightly above the baseline. On the gene level, IL-6 was significantly downregulated in bNP cells after five days of hyper-osmotic culture (**Figure A7**, Fold change day 1: mean: ~0.90, min. ~ 0.26, max. ~1.28, p-value: 0.91; Fold change day 5: mean ~ 0.22, min. ~ 0.14, max. ~ 0.31, p-value: 0.03, n= 3, compared to respective iso-osmotic ctrl.). Moreover, COL1 was downregulated at day five in bNP cells, but without a statistically significant difference (**Figure A8**, Fold change day 1: mean: ~ 2.1, min. ~ 0.62, max. ~ 3.0, p-value: 0.24; Fold change day 5: mean ~0.35, min. ~0.15, and max. ~0.50, p-value: 0.63, n= 3, compared to respective iso-osmotic ctrl.).

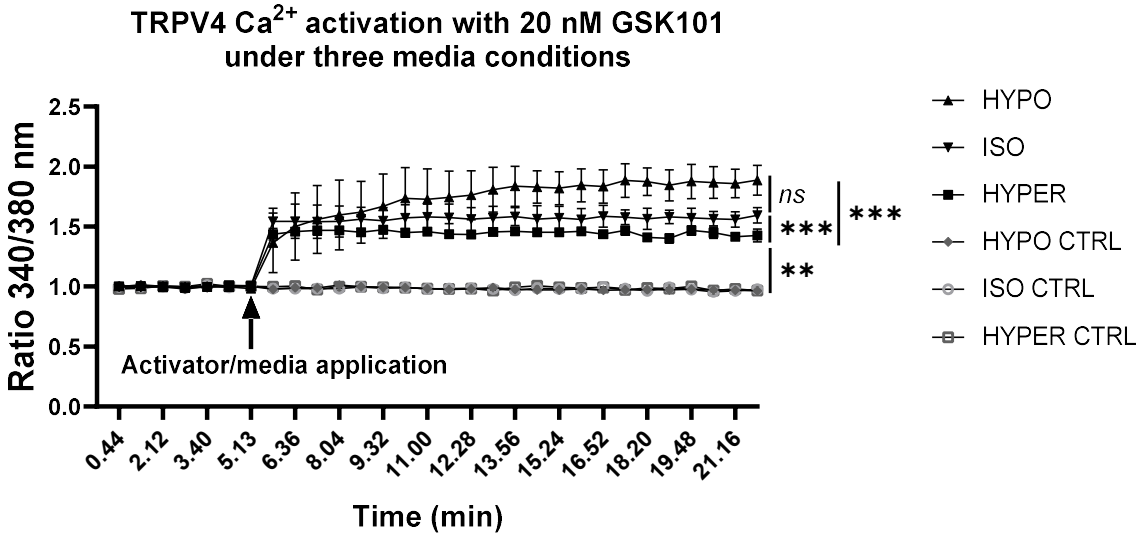


Figure A4. Ca^{2+} flux in NP cells following the application of 20 nM GSK1016790A (TRPV4 activator) to the cells cultured in hypo –, iso – or hyper-osmotic media. Graphs present the ratio of the bound calcium to the unbound calcium (340 nm/380 nm) normalized to the baseline over the measurement time (mean ± SEM, bNP P2, n = 3). After the baseline measurement, empty (control) or compound-supplemented (treatment) media was added (indicated by an arrow on a graph) and the measurement was continued for up to around 15 min more. Asterisks indicates statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, *ns*: no statistical difference defined as $p > 0.05$) as measured on the last measurement cycle.

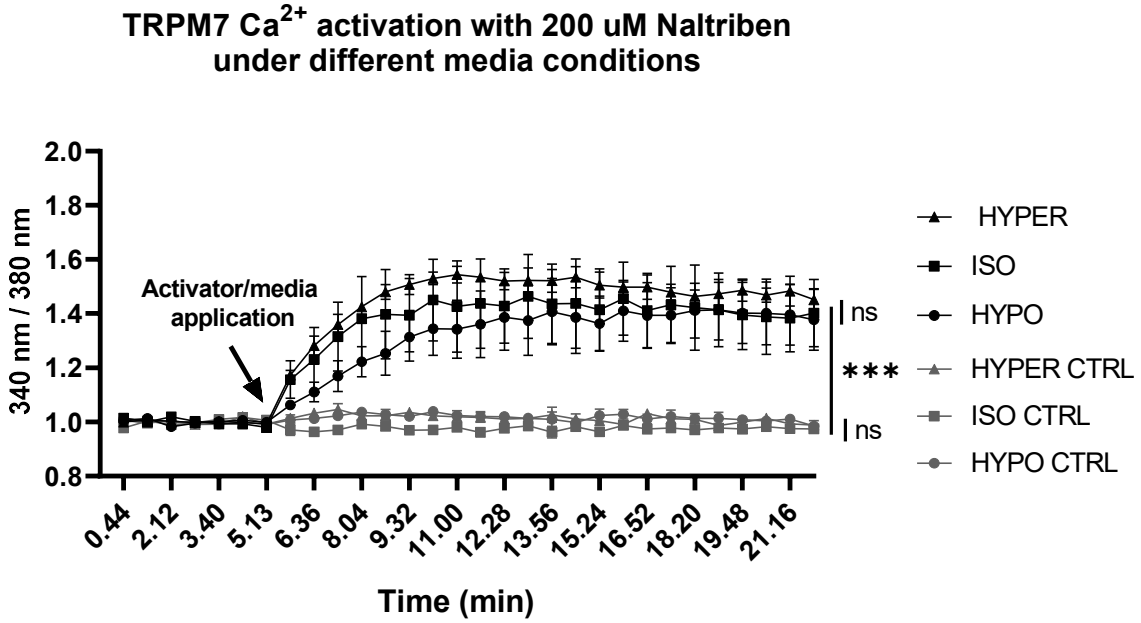


Figure A5. Ca^{2+} flux in NP cells following the application of 200 µM Naltriben (TRPM7 activator) to the cells cultured in hypo –, iso – or hyper-osmotic media. Graphs present the ratio of the bound calcium to the unbound calcium (340 nm/380 nm) normalized to the baseline over the measurement time (mean ± SEM, bNP P2, n = 3). After the baseline measurement, empty (control) or compound-supplemented (treatment) media was added (indicated by an arrow on a graph) and the measurement was continued for up to around 15 min more. Asterisks indicates statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, *ns*: no statistical difference defined as $p > 0.05$) as measured on the last measurement cycle.

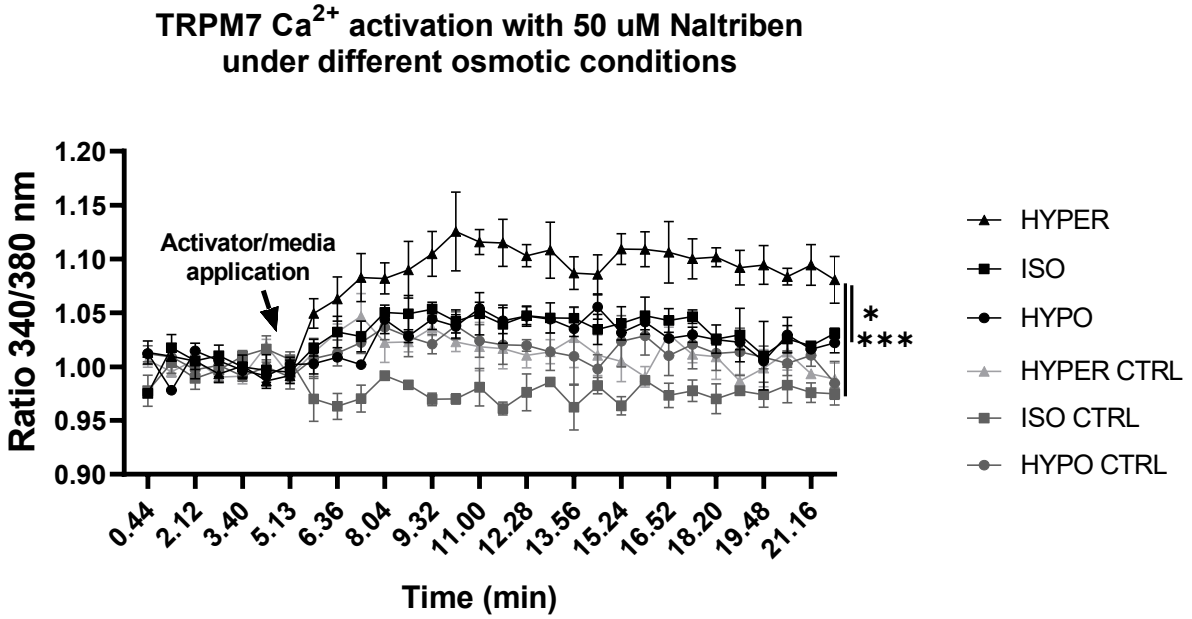


Figure A6. Ca^{2+} flux in bovine NP cells following the application of 50 µM Naltriben (TRPM7 activator) to the cells cultured in hypo –, iso – or hyper-osmotic media. Graphs present the ratio of the bound calcium to the unbound calcium (340 nm/380 nm) normalized to the baseline over the measurement time (mean ± SEM, bNP P2, n = 3). After the baseline measurement, empty (control) or compound-supplemented (treatment) media was added (indicated by an arrow on a graph) and the measurement was continued for up to around 15 min more. Asterisks indicates statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, *ns*: no statistical difference defined as $p > 0.05$) as measured on the last measurement cycle. P-values, which did not fit the graph are presented in the **Table A2**.

Table A2. P-values corresponding to Ca^{2+} influx in bovine NP cells between the osmotic conditions presented on the **Figure A6**. Hyper-, hypo- and iso-osmotic media was treated with 50 µM Naltriben (TRPM7 activator). P-values correspond to the measurement at the last data point (=last cycle).

Osmotic condition 1	Osmotic condition 2	p-value
Hyper	Iso	0.021
Hyper	Hypo	0.018
Hyper ctrl.	Hyper	<0.0001
Hyper ctrl.	Iso ctrl.	0.24
Hyper ctrl.	Hypo ctrl.	0.88
Hypo	Iso	0.084
Hypo	Hypo ctrl.	0.11
Hypo ctrl.	Iso ctrl.	0.64
Iso	Iso ctrl.	<0.0001

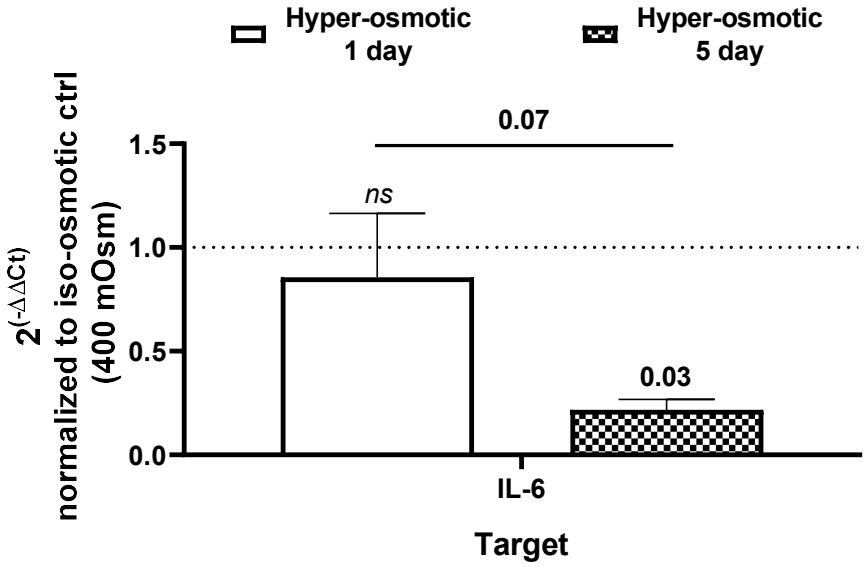


Figure A7. The mRNA expression of IL-6 after 1 day (**left**) and 5 days (**right**) of hyper-osmotic treatment as compared to respective iso-osmotic controls. Statistics: One-way ANOVA followed by Tukey's multiple comparisons test was used to test means among different groups. All reported tests are two-sided, p-values < 0.05 were considered statistically significant and all error bars present SEM. bNP P2 n=3, ns: not statistically significant at $p > 0.05$

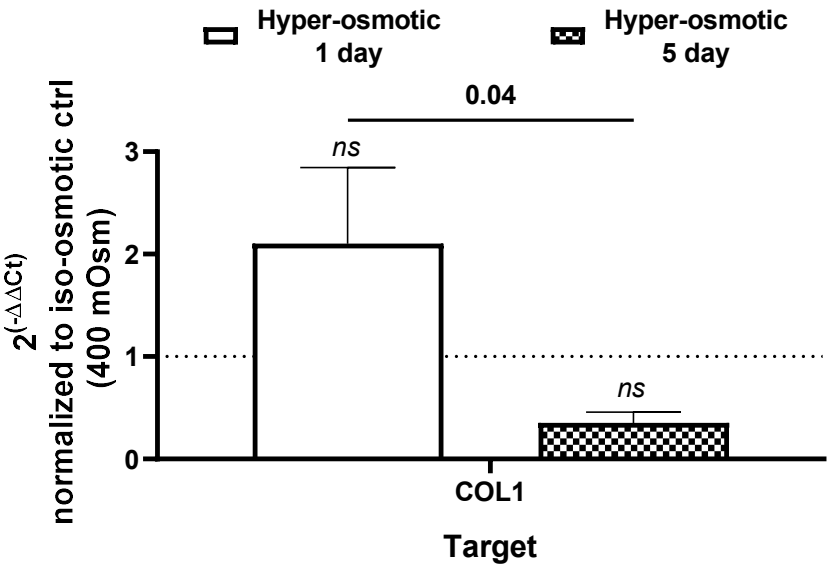


Figure A8. The mRNA expression of COL1 after 1 day (**left**) and 5 days (**right**) of hyper-osmotic treatment as compared to respective iso-osmotic controls. Statistics: One-way ANOVA followed by Tukey's multiple comparisons test was used to test means among different groups. All reported tests are two-sided, p-values < 0.05 were considered statistically significant and all error bars present SEM. bNP P2 n=3, ns: not statistically significant at $p > 0.05$

Discussion

Hyper-osmotic stress is of interest in the study of IVD cell homeostasis and regeneration [6-8]. TRPM7 was previously shown to sense osmotic gradients in kidney cells (HEK293 cells), however it was also demonstrated that hyper-osmolarity has an inhibiting effect on TRPM7 [9]. Our data indicate that TRPM7 is likely not inhibited by hyper-osmotic stress in the IVD and may be sensitive to it. Nevertheless, whether TRPM7 mediates hyper-osmotic stimuli in IVD cells and if the results presented here are physiologically relevant should be investigated in the future. TRPV4 was previously shown to mediate hypo-osmotic stimuli in a variety of tissues [10] and only limited information on its affinity to hyper-osmotic stress exist. Our data suggest that although TRPV4 can be activated at any osmotic state, the mediated Ca^{2+} influx is significantly lower at higher osmolarity. A past study has demonstrated that hyper-osmolarity in combination with TRPV4 activator (4αPDD) may have a beneficial effects on the properties of tissue-engineered neocartilage, e.g. by increasing

its tensile stiffness and collagen content [11]. In contrast, hyper-osmotic treatment (without 4αPDD) was shown to reduce collagen content on the protein level [11]. Correspondingly, COL1 was down-regulated in human degenerated NP cells cultured in alginate beads after five days of hyper-osmotic stimuli [5]. In line with these results, we could show that 5 day long hyper-osmotic culture led to a moderate decrease in COL1 on the gene level. Yet, in the another study, there was no significant differences in the mRNA levels of COL1 between hypo-, iso- and hyper-osmotic conditions in porcine IVD cells cultured in alginate beads [12]. However, it must be noted that in contrast to the herein presented results, the former studies employed either 3D culture, a different cell type (chondrocytes), animal model (porcine/human) or assay types (histology); hence, an entirely accurate comparison is not possible. Moreover, we could also demonstrate that a five-day long hyper-osmotic stimulation significantly decreased IL-6 expression on the mRNA level. This is in line with a previously reported result, where IL-6 was downregulated on the gene level in human degenerated IVD cells cultured in alginate beads exposed to hyper-osmotic media for 4 hours [13]. While it seems that hyper-osmolarity may have an inhibitory effect on IL-6 in the IVD, it was shown that in the other tissues hyper-osmolarity may promote IL-6 and inflammation *via* TonEBP/NFAT5 [14]. However, these conflicting reports may be also an effect of the differences between tissues/cells as healthy IVD cells are naturally exposed to higher osmolarity and experience greater osmotic gradients [4, 5] than most of the other cells/tissues for which an increased osmolarity may be a sign of a disease [15, 16]. To conclude, it remains to be elucidated if TRP channels are involve in sensing hyper-osmotic stress and what changes on the mRNA level are induced by hyper-osmotic stimuli. A clear limitation of these data is that they are preliminary and only tackle a narrow scope of the existing knowledge gap. Moreover, a use of healthy human IVD cells in the future studies could be beneficial for translating *in vitro* findings into clinics.

References

1. Schroeder, A., et al., *The RIN: an RNA integrity number for assigning integrity values to RNA measurements.* BMC Mol Biol, 2006. **7**: p. 3.
2. Hampton-Marcell, J.T., et al., *Preparation and metatranscriptomic analyses of host-microbe systems.* Methods Enzymol, 2013. **531**: p. 169-85.
3. Conesa, A., et al., *A survey of best practices for RNA-seq data analysis.* Genome Biol, 2016. **17**: p. 13.
4. Boos, N., et al., *Quantitative MR imaging of lumbar intervertebral disks and vertebral bodies: influence of diurnal water content variations.* Radiology, 1993. **188**(2): p. 351-4.
5. Wuertz, K., et al., *Influence of extracellular osmolarity and mechanical stimulation on gene expression of intervertebral disc cells.* J Orthop Res, 2007. **25**(11): p. 1513-22.
6. Mizuno, S., K. Kashiwa, and J.D. Kang, *Molecular and histological characteristics of bovine caudal nucleus pulposus by combined changes in hydrostatic and osmotic pressures in vitro.* J Orthop Res, 2019. **37**(2): p. 466-476.
7. Richardson, S.M., et al., *Mesenchymal stem cells in regenerative medicine: Focus on articular cartilage and intervertebral disc regeneration.* Methods, 2016. **99**: p. 69-80.
8. Tao, Y.Q., et al., *Potential of co-culture of nucleus pulposus mesenchymal stem cells and nucleus pulposus cells in hyperosmotic microenvironment for intervertebral disc regeneration.* Cell Biol Int, 2013. **37**(8): p. 826-34.
9. Bessac, B.F. and A. Fleig, *TRPM7 channel is sensitive to osmotic gradients in human kidney cells.* J Physiol, 2007. **582**(Pt 3): p. 1073-86.
10. Sadowska, A., et al., *Osmosensing, osmosignalling and inflammation: how intervertebral disc cells respond to altered osmolarity.* Eur Cell Mater, 2018. **36**: p. 231-250.
11. Lee, J.K., et al., *Promoting increased mechanical properties of tissue engineered neocartilage via the application of hyperosmolarity and 4alpha-phorbol 12,13-didecanoate (4alphaPDD).* J Biomech, 2014. **47**(15): p. 3712-8.
12. Chen, J., et al., *Matrix protein gene expression in intervertebral disc cells subjected to altered osmolarity.* Biochem Biophys Res Commun, 2002. **293**(3): p. 932-8.
13. Boyd, L.M., et al., *Osmolarity regulates gene expression in intervertebral disc cells determined by gene array and real-time quantitative RT-PCR.* Ann Biomed Eng, 2005. **33**(8): p. 1071-7.
14. Johnson, Z.I., I.M. Shapiro, and M.V. Risbud, *Extracellular osmolarity regulates matrix homeostasis in the intervertebral disc and articular cartilage: evolving role*

of TonEBP. Matrix biology : journal of the International Society for Matrix Biology, 2014. **40**(2): p. 10-6.
15. Schwartz, L., et al., *Is inflammation a consequence of extracellular hyperosmolarity?* J Inflamm (Lond), 2009. **6**: p. 21.
16. Brocker, C., D.C. Thompson, and V. Vasiliou, *The role of hyperosmotic stress in inflammation and disease.* Biomol Concepts, 2012. **3**(4): p. 345-364.

Chapter 8

8 Synthesis

Low back pain (LBP) is a prevailing musculoskeletal disorder affecting up to 80% of individuals at some point in their lifetime [1]. The prevalence of LBP is growing with the aging-population, thus leading to rising future costs related to the medical intervention, but also lost days of work [1, 2]. Current treatment strategies are limited and mostly focus on the combination of physical therapy, pharmacological therapy and surgery [3, 4]. However, the outcomes are not always long lasting [5, 6]. Painful intervertebral disc (IVD) degeneration, also referred to as degenerative disc disease (DDD), as well as inflammation are believed to be the key contributors to the development of LBP [7], however our understating of it is incomplete. IVD degeneration is a biologically complex process, where the structural, biomechanical and biochemical properties of the IVD predispose it to the degeneration [7]. Even in the healthy IVD, the local microenvironment is rather harsh, as compared to the other tissues: being avascular, the nutrient supply relies on the diffusion *via* cartilaginous endplates (CEP) and as a load-bearing tissue, the IVD experiences high and diurnal osmotic gradients [7]. Consequently, with a limited regenerative capacity, the IVD's health depends on the fine balance of each of its regions (annulus pulposus (AF), nucleus pulposus (NP) and CEP) to maintain their normal function [7]. On the structural level, changes to CEPs lead to a deficiency in the nutrient supply, making the local microenvironment more acidic, whereas the annular fissures may lead to nerve and vessels penetration as well as to the displacement of the inner disc material (known as disc herniation (DH)) [7]. On the molecular level, IVD degeneration is characterized by a decreased extracellular matrix (ECM) production as well as an enhanced production of ECM degrading enzymes and pro-inflammatory cytokine, which is often a target of a pharmacological interventions [7, 8]. Advancing our knowledge on the inflammation in the IVD as well as the identification of novel biomarkers for (painful) IVD degeneration may not only close the existing knowledge gap related to the biology of IVD degeneration, but these biomarkers may also serve as promising therapeutic targets for its treatment [9]. Transient receptor potential (TRP) channels, which are membrane receptors involved in the calcium signaling that can be activated by a multitude of stimuli, have recently emerged as contributors to joint diseases [10-12]. TRP channels have previously been identified as

promising biomarkers and therapeutic targets in many other diseases, e.g. pancreatic or breast cancer [13, 14], but have not been yet widely researched in the field of IVD pathologies. Therefore, the central aim of this thesis was to identify and characterize TRP channels in the IVD. Further, three separate aims were described:

Aim 1: Measure and compare the expression levels of selected pro-inflammatory cytokines, as well as the TRPC6 and TRPV4 channels in human samples from degenerated lumbar and cervical IVD tissue. We hypothesized that significant differences exist between expression levels of tested targets based on specific patient and IVD characteristics.

Aim 2: Measure the gene and protein expression levels of all currently known TRP channels in the human non-degenerated and degenerated IVD tissue. We hypothesized that apart from previously investigated TRPV4 and TRPC6 channels, other members of the TRP channel family are as well expressed in the degenerated IVD tissue. Moreover, we hypothesized that the differences in their expression levels exists between non-degenerated and degenerated IVD tissue and may be influenced by specific patient and IVD characteristics such as the IVD degeneration grade as well as pain intensity and chronicity.

Aim 3: Test and identify members of the TRP channel family, which function as sensors for the hypo-osmotic stress and with RNA sequencing identify genome-wide expression changes caused by the hypo-osmotic stress in the IVD. Reduced tissue osmolarity is a hallmark of the IVD degeneration and we hypothesized that TRP channels may sense osmotic-stress and mediate the gene expression of ECM molecules and pro-inflammatory cytokine under hypo-osmotic stress.

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

The study presented in the chapter 4 was the first one to our knowledge to compare the inflammatory processes and the expression of TRPV4 and TRPC6 channels in the human degenerated cervical and lumbar IVD tissue. In line with the other studies, we could confirm a leading role of the cytokine IL-8, which expression was induced in patients suffering from degenerative disc disease and together with the IL-6 was also more enhanced in patients

undergoing multilevel disc surgery. Moreover, we described the expression levels of so far insufficiently investigated inflammatory candidates from the interferon type I family and IL-15. We found that IL-15 is expressed in a degeneration- and age-dependent manner and its expression is higher in the cervical spine compared to the lumbar spine. Additionally, we were the first to show that TRPV4 and TRPC6 are expressed in the human IVD and both being expressed at similarly high levels to the well-known cytokines. There are relatively few studies focusing on novel pro-inflammatory markers in the IVD. Therefore, the results presented here are an achievement in our collective understating of IVD inflammation by indicating that that inflammation is not limited to commonly investigated targets (IL-6, IL-8 and TNF-α) and it is rather a complex network of pro-inflammatory crosstalk, where selected targets may correlate with each other (e.g. TRPC6 and IL-15/IL-8/IL-6). Hence, more effort should be placed on unraveling these molecular dependencies in the future studies. Furthermore, our data clearly present that the inflammatory profile may be affected by relevant patient characteristics or specific tissue features, however we concluded that due to the overall small differences, the pharmacological therapy for disc pathologies may be applicable independent of the spinal region.

8.2 Differential regulation of TRP channel gene and protein expression by intervertebral disc degeneration and back pain

The study described in the chapter 5 was the first to present a throughout screening of TRP channels in non-degenerated and degenerated human IVD tissue. For the first time in our field, we could demonstrate that 26 out of 28 known TRP channels are expressed in the IVD. Overall, TRPC1, TRPM7, TRPP1 and TRPV4 were the highest expressed TRP channels in the non-degenerated and degenerated IVD tissue. While some TRP channels were undetectable in the non-degenerated tissue, the expression of TRPC4, TRPC6, TRPM7 and TRPML1 was found to be degeneration or Modic grade depended. Interestingly, the expression of TRPM2 was significantly reduced in the patients who underwent prior steroid treatment. Furthermore, we were able to show that the pain intensity and chronicity influenced the gene and protein expression of TRPC6, TRPM2 and TRML1. This is the first study to describe that the expression levels of selected TRP channels are dependent on the intensity and chronicity of discogenic back pain and therefore indicating that TRPC6,

TRPM2 and TRPML1 are promising therapeutic targets. These findings have the potential to initiate pharmacological innovations for the treatment of low back pain by pointing out pain-related and degeneration-affected TRP channels.

8.3 Hypo-osmotic loading induces expression of IL-6 in nucleus pulposus cells of the intervertebral disc independent of TRPV4 and TRPM7

The study presented in the chapter 7 focused on the investigation of the effects of hypo-osmotic stress on the degenerative profile of the IVD as well as the expression and activity of the TRP channels. As the first one in the field, we presented a complete screening of the TRP channels in the bovine non-degenerated IVD cells and a genome-wide screening *via* RNA sequencing of the NP cells exposed to hypo-osmotic stress. Similarly to our earlier study on human IVD tissue (chapter 5), TRPC1, TRPM7 and TRPV4 were the most highly expressed TRP channels in the bovine IVD cells. These results confirmed that the bovine model is suitable for the study of TRP channels in the lack of human non-degenerated IVD cells. Moreover, we have researched the osmosensing role of TRPV4 and thus far not investigated TRPM7 in the IVD. We presented the effect of hypo-osmotic stimuli and pharmacological modulation on TRPV4 and TRPM7 channel activity and expression. In contrast to an earlier publication [15], we demonstrated that it is unlikely that TRPV4 mediates hypo-osmotically induced IL-6 expression. Our data indicate that although TRPM7 and TRPV4 may be sensitive to hypo-osmotic stress, a pharmacological modulation of these channels' activity does not affect the expression levels of aggrecan, ADAMTS9 and IL-6 under hypo-osmotic stress. These findings, together with the genome sequencing, may provide future scientists with a relevant tool to continue research on hypo-osmotic stress in the IVD and TRP channels.

8.4 Limitations and future research

One of the leading limitations of the studies presented as part of this thesis is the lack of or limited availability of human non-degenerated IVD tissue, which could serve as a control. Such tissue is usually obtained *via* organ donation, but their supply to science and medicine is limited not only on the national level, but also globally. To illustrate it, by the end of the year 2018, around 600 patients underwent an organ transplantation and around 1400

patients remained on the waiting list in Switzerland [16]. More importantly, a voluntary donation of body for research purposes is yet in many European countries underdeveloped, which is a result of obsolete regulations, cultural attitudes and ethical as well as legal ambiguity [17, 18]. However, an extensive analysis of non-degenerated human IVD tissue would give an opportunity for defining a "molecular baseline" and thus to differentiate between healthy and diseased conditions on the cellular and molecular level, which in turn could be also used as a benchmark for regenerative therapies.

Degenerated IVD tissue is obtained from patients undergoing spinal surgery and is of high value in spine research. Nevertheless, it also carries certain limitations, which may bias the results. Firstly, nucleus pulposus and annulus fibrosus are often hard to separate precisely, especially in severely degenerated tissue. This clearly hampers the conclusions on zonal differences in degenerated IVDs. Secondly, obtained cell population may be dedifferentiated and a may contain different cell types that penetrated into the IVD due to innervation and vascularization [7, 19]. Lastly, working with human or animal samples often brings donor-to-donor variation, which is rarely experienced when working with cell lines. Some of these limitations may be mitigated by using a larger (in the context of biological research > n=3) and balanced sample size and whenever possible we aimed at fulfilling these criterion (e.g. in the study presented in the chapter 4 n = 51). Nonetheless, collecting a bigger and more balanced sample size requires also more time and financial resources. Therefore, creating a permanent supply chain of test samples to a research laboratory by collaborations with hospitals and doctors as well as designing new cohorts may be advantageous for any future study.

In the presented studies, we have used gold standard techniques (e.g. qPCR), validated reagents (e.g. TaqMan primers) and established protocols (provided by either an assay manufacturer or our internal lab protocols). However, every assay has sensitivity and specificity limitations, which need to be accounted for when drawing conclusions from the results. Moreover, disparities between studies resulting from using different interpretation of spine-research related terms (e.g. degenerative disc disease) or values (e.g. for osmolarity) could be overcome in the future by defining field specific standards and guidelines, which could be formulated by key-opinion leaders and task force groups. Furthermore, RNA sequencing is an outstanding tool, which enables to identify thousands of differently expressed targets in a single test sample. However, at the same time, the amount of

generated data may be difficult to be efficiently processed. Future studies using omics-techniques will require improved tools for data analysis such as data mining, which at the moment are not commonly used in basic biological research. This and other programming-based techniques could help to speed up the data analysis and interpretation time, support decision making as well as perhaps facilitate contrasting these new data with the scientific findings expanding beyond the scope of a given project.

With respect to the TRP channels, their identification and characterization in regard to specific patient and tissue characteristics was an important finding that helped in the selection of the most promising therapeutic targets for future funding applications. So far the past research has focused on testing TRP channels that were proven to be of relevance in other tissue types (e.g. TRPV4 in cartilage [20]). Although this is a safe strategy, presented here broad screening approach will help to move beyond the current state-of-art. Future research endeavors, which extend beyond the scope of this thesis, shall aim at combining herby presented findings with research on e.g. the mechanosensing role of TRP channels and their interaction with other membrane receptors and pro-inflammatory molecules as well as pre-clinical investigations. These studies will have the potential to provide a more holistic understanding of the function of TRP channels in the IVD and DDD in general.

It is the prime motivation of any spine-focused research project to advance treatment strategies for patients suffering from low back pain. In order to make translation happen, the needs of every stakeholder (e.g. patients, medical practitioners and payers) will need to be taken into account. Therefore, even basic *in vitro* research must envision approaches on how to test these newly obtained findings in the next stage of pre-clinical and clinical trials. We have shown that TRP channels and especially TRPC6, TRPM2 and TRPML1 are promising therapeutic targets for low back pain. Further research should investigate the mechanism of action of these (and other) TRP channels as well as continue the development of specific agonists and antagonists, which could be used in the clinical practice. Furthermore, our research has highlighted previously closer not examined patient characteristics, such as BMI, prior steroid treatment or the duration of symptoms, as important factors for consideration in research studies related to DDD. Future research should consider including these and other factors (e.g. epigenetics) in their investigations as well as expanding to cohorts studies. On the one hand, this direction could support the

development of personalized medicine treatment strategies. On the other hand, it could help create a genetic profile of individuals with predisposition to painful IVD degeneration as well as a more detailed profile of the condition itself.

8.5 Conclusion

The high prevalence of the low back pain, in combination with a growing aging population, indicate the need for improvement treatment strategies. The fundamental goal of advancing the low back pain treatment can be achieved by the discovery of new therapeutic targets. Furthermore, a rigorous characterization of the low back pain patient population is needed to determine who will ultimately profit from novel treatments. This thesis has made a significant progress in identifying novel pain (e.g. TRPC6, TRPM2 and TRPML1) and pro-inflammatory targets (e.g. IL-15 or IFNα1) as well as by pointing out specific patient or IVD characteristics (e.g. pain intensity and chronicity, IVD degeneration grade, pathology or spinal level), which may differentiate the outcome. Although further research is required to understand the mechanistic role of these targets and their interaction with other pro-inflammatory molecules, the findings presented in this work not only advance the knowledge on IVD biology and inflammation, but are also of translational relevance. A successful translation of these findings would provide medical practitioners with a novel tool for the targeted treatment for patients suffering from low back pain.

References

1. Freburger, J.K., et al., *The rising prevalence of chronic low back pain.* Arch Intern Med, 2009. **169**(3): p. 251-8.
2. Wieser, S., et al., *Cost of low back pain in Switzerland in 2005.* Eur J Health Econ, 2011. **12**(5): p. 455-67.
3. Ulger, O., et al., *Effectiveness of physiotherapy and minimal invasive technics on functional status and quality of life in geriatric patients with low back pain.* J Exerc Rehabil, 2018. **14**(6): p. 1048-1052.
4. Rozenberg, S., V. Foltz, and B. Fautrel, *Treatment strategy for chronic low back pain.* Joint Bone Spine, 2012. **79**(6): p. 555-9.
5. Atlas, S.J., et al., *Long-term outcomes of surgical and nonsurgical management of sciatica secondary to a lumbar disc herniation: 10 year results from the maine lumbar spine study.* Spine (Phila Pa 1976), 2005. **30**(8): p. 927-35.
6. Leven, D., et al., *Risk Factors for Reoperation in Patients Treated Surgically for Intervertebral Disc Herniation: A Subanalysis of Eight-Year SPORT Data.* J Bone Joint Surg Am, 2015. **97**(16): p. 1316-25.
7. Kepler, C.K., et al., *The molecular basis of intervertebral disc degeneration.* Spine J, 2013. **13**(3): p. 318-30.
8. Pimentel, D.C., et al., *Anti-tumor necrosis factor antagonists in the treatment of low back pain and radiculopathy: a systematic review and meta-analysis.* Pain Physician, 2014. **17**(1): p. E27-44.
9. Khan, A.N., et al., *Inflammatory biomarkers of low back pain and disc degeneration: a review.* Ann N Y Acad Sci, 2017. **1410**(1): p. 68-84.
10. Brederson, J.D., P.R. Kym, and A. Szallasi, *Targeting TRP channels for pain relief.* Eur J Pharmacol, 2013. **716**(1-3): p. 61-76.
11. Ramsey, I.S., M. Delling, and D.E. Clapham, *An introduction to TRP channels.* Annu Rev Physiol, 2006. **68**: p. 619-47.
12. Krupkova, O., J. Zvick, and K. Wuertz-Kozak, *The role of transient receptor potential channels in joint diseases.* Eur Cell Mater, 2017. **34**: p. 180-201.
13. Ouadid-Ahidouch, H., et al., *TRP channels: diagnostic markers and therapeutic targets for breast cancer?* Trends Mol Med, 2013. **19**(2): p. 117-24.
14. Yee, N.S., *Role of TRPM7 in Cancer: Potential as Molecular Biomarker and Therapeutic Target.* Pharmaceuticals (Basel), 2017. **10**(2).
15. Walter, B.A., et al., *Reduced tissue osmolarity increases TRPV4 expression and pro-inflammatory cytokines in intervertebral disc cells.* European cells & materials, 2016. **32**(7): p. 123-36.

16. *Patienten auf der Warteliste und transplantierte Patienten 2018.* 26.03.2020]; Available from: https://www.swisstransplant.org/fileadmin/user_upload/Swisstransplant/Jahresbericht/Jahresbericht_und_Grafiken_2018/2018_Patienten_auf_der_Warteliste_und_transplantierte_Patienten.pdf from https://www.swisstransplant.org.
17. Bin, P., et al., *Donation of the body for scientific purposes in Italy: ethical and medico-legal considerations.* Open Med (Wars), 2016. **11**(1): p. 316-320.
18. Riederer BM, B.S., Brenner E, Bueno-López JL, Circulescu ARM, Davies DC, Caro Gerrits DR, McHanwell PO, Païs S, Paulsen D, Plaisant F, Sendemir O, Stabile E, Moxham I. BJ, *The legal and ethical framework governing Body Donation in Europe—1st update on current practice.* European Journal of Anatomy, 2012. **16**
(1): p. 1–21.
19. Vo, N.V., et al., *Molecular mechanisms of biological aging in intervertebral discs.* J Orthop Res, 2016. **34**(8): p. 1289-306.
20. Phan, M.N., et al., *Functional characterization of TRPV4 as an osmotically sensitive ion channel in porcine articular chondrocytes.* Arthritis Rheum, 2009. **60**(10): p. 3028-37.