

In Vitro and *In Silico* Modeling of Vertebral
Endplate Degeneration Mechanisms

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Abstract

Developed within the Disc4All consortium, this thesis investigates the structural, mechanical, and inflammatory mechanisms driving intervertebral disc endplate degeneration through complementary *in vitro* and *in silico* approaches. Recognizing the endplate as a heterogeneous structure, this work distinguishes its three main regions: the non-calcified cartilaginous endplate, calcified cartilaginous endplate, and bony endplate, highlighting their distinct contributions to degeneration processes. Imaging-based computational approaches are employed to characterize bony endplate microarchitecture and estimate its mechanical properties, while multi-tissue explant models are used to investigate region-specific responses to inflammatory cytokines. Additionally, cell-seeded hydrogel models are developed to specifically assess cartilaginous endplate cell responses to mechanical loading and inflammatory cytokine stimulation. Furthermore, a computational-experimental workflow and a custom perfusion bioreactor concept are proposed to establish a framework for future studies of fluid transport conditions within endplate tissues and their influence on cellular stress responses. By integrating structural characterization, computational modeling, and experimental approaches, this thesis establishes a comprehensive framework linking endplate regional heterogeneity, biomechanics, and cellular responses. These findings contribute to the development of more physiologically relevant models for studying intervertebral disc degeneration and support future investigations into endplate-driven degeneration mechanisms.

Résumé

Réalisée au sein du consortium Disc4All, cette thèse étudie les mécanismes structuraux, mécaniques et inflammatoires impliqués dans la dégénérescence des plateaux vertébraux du disque intervertébral, en combinant des approches complémentaires *in vitro* et *in silico*. Considérant le plateau vertébral comme une structure hétérogène, ce travail distingue trois régions principales — le plateau cartilagineux non calcifié, le plateau cartilagineux calcifié et le plateau osseux — et met en évidence leurs contributions respectives aux processus dégénératifs. Des approches computationnelles basées sur l'imagerie sont utilisées pour caractériser la microarchitecture du plateau osseux et estimer ses propriétés mécaniques, tandis que des modèles d'explants multi-tissulaires permettent d'étudier les réponses spécifiques à chaque région aux cytokines inflammatoires. Par ailleurs, des modèles d'hydrogels ensemencés de cellules sont développés afin d'évaluer spécifiquement les réponses des cellules du plateau cartilagineux aux sollicitations mécaniques et à la stimulation par des cytokines inflammatoires. Enfin, une approche combinant modélisation computationnelle et expérimentations, ainsi qu'un concept de bioréacteur à perfusion sur mesure, sont proposés afin d'établir un cadre pour de futures études des conditions de transport des fluides au sein des tissus du plateau vertébral et de leur influence sur les réponses cellulaires au stress. En intégrant la caractérisation structurale, la modélisation computationnelle et les approches expérimentales, cette thèse établit un cadre global reliant l'hétérogénéité régionale du plateau vertébral, la biomécanique et les réponses cellulaires. Ces résultats contribuent au développement de modèles plus physiologiquement pertinents pour l'étude de la dégénérescence du disque intervertébral et soutiennent les recherches futures sur les mécanismes de dégénérescence impliquant les plateaux vertébraux.

Samenvatting

Dit proefschrift, ontwikkeld binnen het Disc4All-consortium, onderzoekt de structurele, mechanische en inflammatoire mechanismen die betrokken zijn bij de degeneratie van de eindplaat van de tussenwervelschijf, door middel van complementaire *in vitro*- en *in silico*-benaderingen. Hierbij wordt de eindplaat beschouwd als een heterogene structuur, bestaande uit drie hoofdregio's: de niet-verkalkte kraakbeenachtige eindplaat, de verkalkte kraakbeenachtige eindplaat en de benige eindplaat. De verschillende bijdragen van deze regio's aan degeneratieprocessen worden hierbij benadrukt. Op beeldvorming gebaseerde computationele benaderingen worden gebruikt om de microarchitectuur van de benige eindplaat te karakteriseren en de mechanische eigenschappen ervan te schatten, terwijl multi-weefsel-explantaatmodellen worden ingezet om regiospecifieke reacties op inflammatoire cytokinen te onderzoeken. Daarnaast worden hydrogelmodellen met cellen ontwikkeld om specifiek de reacties van kraakbeenachtige eindplaatcellen op mechanische belasting en stimulatie met inflammatoire cytokinen te evalueren. Verder worden een computationeel-experimentele werkwijze en een concept voor een op maat gemaakte perfusiebioreactor voorgesteld om een kader te bieden voor toekomstige studies naar vloeistoftransport binnen eindplaatweefsels en de invloed hiervan op cellulaire stressreacties. Door structurele karakterisering, computationele modellering en experimentele benaderingen te integreren, biedt dit proefschrift een geïntegreerd kader dat de regionale heterogeniteit van de eindplaat, biomechanica en cellulaire responsen met elkaar verbindt. Deze bevindingen dragen bij aan de ontwikkeling van fysiologisch relevantere modellen voor het bestuderen van degeneratie van de tussenwervelschijf en ondersteunen toekomstig onderzoek naar degeneratiemechanismen waarbij de eindplaat een centrale rol speelt.

ملخص

تُعنى هذه الأطروحة، التي طُوّرت ضمن مشروع "ديسك فور اوول"، بدراسة الآليات البنيوية والميكانيكية والالتهابية التي تُحفّز تنكس الصفيحة النهائية للقرص الفقري، وذلك من خلال مناهج تكملية مخبرية وحاسوبية. وانطلاقاً من كون الصفيحة النهائية بنيةً غير متجانسة، يُميّز هذا العمل مناطقها الرئيسية الثلاث: الصفيحة الغضروفية غير المتكلسة، والصفيحة الغضروفية المتكلسة، والصفيحة العظمية، مُسلّطاً الضوء على مساهمات كل منها في عمليات التنكس. وتُستخدم مناهج حسابية قائمة على التصوير الطبي لتوصيف البنية المجهرية للصفيحة العظمية وتقدير خصائصها الميكانيكية، بينما تُستخدم نماذج زراعة الأنسجة المتعددة لدراسة الاستجابات الخاصة بكل منطقة للسيتوكينات الالتهابية. إضافةً إلى ذلك، طُوّرت نماذج هيدرولوجيل محمّلة بالخلايا لتقييم استجابات خلايا الصفيحة الغضروفية تحديداً للتحميل الميكانيكي وتحفيز السيتوكينات الالتهابية. علاوة على ذلك، يُقترح منهجٌ حسابي-تجريبي ومفهومٌ لمفاعل حيوي مُصمّم خصيصاً للتروية، وذلك لوضع إطار عملٍ للدراسات المستقبلية حول ظروف نقل السوائل داخل أنسجة الصفيحة النهائية وتأثيرها على استجابات الخلايا للإجهاد. ومن خلال دمج التوصيف البنيوي والنمذجة الحاسوبية والأساليب التجريبية، تُرسيّ هذه الأطروحة إطار عملٍ شاملٍ يربط بين عدم تجانس مناطق الصفيحة النهائية، والميكانيكا الحيوية، والاستجابات الخلوية. تُسهم هذه النتائج في تطوير نماذج أكثر ملاءمةً من الناحية الفيزيولوجية لدراسة تنكس الأقراص بين الفقرات، وتدعم الأبحاث المستقبلية في آليات التنكس الناجمة عن الصفيحة النهائية.

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وَلَيْسَ أَخُو عِلْمٍ كَمَنْ هُوَ جَاهِلٌ
صَغِيرٌ إِذَا التَّقَتْ عَلَيْهِ الْجَافِلُ
كَبِيرٌ إِذَا رُدَّتْ إِلَيْهِ الْمَحَافِلُ

تَعَلَّمَ فَلَيْسَ الْمَرْءُ يُوَلَدُ عَالِمًا
وَإِنَّ كَبِيرَ الْقَوْمِ لَا عِلْمَ عِنْدَهُ
وَإِنَّ صَغِيرَ الْقَوْمِ إِنْ كَانَ عَالِمًا

— الإمام الشافعي

The Intersection of AI, Art, and Science

As this thesis explores the intersection of engineering, biology, and computational methods, it feels fitting to acknowledge another technology that has become part of my writing journey. Artificial intelligence was used to support selected aspects of the presentation of this thesis, but not its scientific content.

Several of the creative, non-scientific visuals throughout this thesis, including the cover page, chapter divider pages, quote pages, and back cover, were created with the assistance of Google's Gemini 1.5 Flash. These illustrations were intended solely to enhance the visual presentation of the thesis and reflect my appreciation for the creative possibilities that modern computational tools can offer.

In addition, ChatGPT (OpenAI) was used to assist with English-language refinement and to improve the clarity and readability of the text.

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List of Abbreviations

AA antibiotic–antimycotic

ABC avidin–biotin complex

ABM agent-based model

AB Alcian blue

AC articular cartilage

ACAN aggrecan

ADAMTS a disintegrin and metalloproteinase with thrombospondin motifs

ADAMTS4 a disintegrin and metalloproteinase with thrombospondin motifs 4

ADAMTS-5 a disintegrin and metalloproteinase with thrombospondin motifs 5

ADC analog-to-digital converter

AF annulus fibrosus

AI artificial intelligence

ALP alkaline phosphatase

ANOVA analysis of variance

API application programming interface

ASCII American Standard Code for Information Interchange

BEP bony endplate

BHC beam hardening correction

BM-MSC bone marrow mesenchymal stem cell

BMP bone morphogenetic protein

BNB blood–nucleus pulposus barrier

BSA bovine serum albumin

BV bone volume

CaP calcium phosphate

CaSR calcium-sensing receptor

cCEP calcified cartilage endplate

cDNA complementary DNA

CEP cartilaginous endplate

CFD computational fluid dynamics

CI confidence interval

cLSM confocal laser scanning microscopy

CNN convolutional neural network

COL2 collagen type II

CPU central processing unit

CSV comma-separated value

CT computed tomography

DAB 3,3'-diaminobenzidine tetrahydrochloride

DALY disability-adjusted life year

DAPI 4',6-diamidino-2-phenylindole

DMEM Dulbecco's Modified Eagle Medium

DMMB dimethylmethylene blue

DNA deoxyribonucleic acid

DOF degree of freedom

DTT dithiothreitol

ECM extracellular matrix

eDAPS endplate-modified disc-like angle-ply structures

EDTA ethylenediaminetetraacetic acid

ELISA enzyme-linked immunosorbent assay

EP endplate

ERK extracellular signal-regulated kinase

ESR Early Stage Researcher

EZH2 enhancer of zeste homologue 2

FAK focal adhesion kinase

FBS fetal bovine serum

FCD fixed charge density

FCS fetal calf serum

FE finite element

FEM finite element modeling

FGF fibroblast growth factor

GAG glycosaminoglycan

H&E hematoxylin and eosin

HG-DMEM high-glucose Dulbecco's Modified Eagle Medium

I²C inter-integrated circuit

ICD-11 International Classification of Diseases

IgG immunoglobulin G

IHC immunohistochemistry

IL-1 interleukin-1

IL-17 interleukin-17

IL-1 α interleukin-1 alpha

IL-1 β interleukin-1 beta

IL-6 interleukin-6

IL-8 interleukin-8

IQR interquartile range

IVD intervertebral disc

IVDD intervertebral disc degeneration

LBP low back pain

LG-DMEM low-glucose Dulbecco's Modified Eagle Medium

LMM linear mixed-effects model

MAPK mitogen-activated protein kinase

MC Modic change

MC1 Modic type 1 change

MC2 Modic type 2 change

MC3 Modic type 3 change

μ CT micro-computed tomography

MIF macrophage migration inhibitory factor

miRNA microRNA

MMP matrix metalloproteinase

MMP13 matrix metalloproteinase 13

MMP3 matrix metalloproteinase 3

MMP8 matrix metalloproteinase 8

MMP9 matrix metalloproteinase 9

MRI magnetic resonance imaging

mRNA messenger RNA

MTB metaphyseal trabecular bone

MTC Masson's trichrome

NCBI National Center for Biotechnology Information

ncCEP non-calcified cartilage endplate

NO nitric oxide

NP nucleus pulposus

OR odds ratio

OSM osmolarity

PBS phosphate-buffered saline

PCL poly(ϵ -caprolactone)

PCM pericellular matrix

PEG polyethylene glycol

PG proteoglycan

PGE₂ prostaglandin E2

PMMA polymethyl methacrylate

PSU polysulfone

PTHrP parathyroid hormone-related protein

P0 passage zero

qPCR quantitative polymerase chain reaction

RBM red bone marrow

REML restricted maximum likelihood

RNA ribonucleic acid

RNM regulatory network model

ROI region of interest

ROS reactive oxygen species

RS-485 Recommended Standard 485

RVF reactive fluid volume flux

RVT reaction total fluid volume

SafO safranin O

SE standard error

SEM scanning electron microscopy

Shh Sonic Hedgehog

SI International System of Units

SIMPLE Semi-Implicit Method for Pressure-Linked Equations

SLS selective laser sintering

SOX9 SRY-box transcription factor 9

ST soft tissue

STB subchondral trabecular bone

STL stereolithography

T2WI T2-weighted imaging

TBS Tris-buffered saline

TEPS total endplate score

TGF- β transforming growth factor beta

3D three-dimensional

TIFF tagged image file format

TIMP1 tissue inhibitor of metalloproteinases 1

TIMP2 tissue inhibitor of metalloproteinases 2

TIMP3 tissue inhibitor of metalloproteinases 3

TLR toll-like receptor

TLR2 toll-like receptor 2

TLR4 toll-like receptor 4

TNF tumor necrosis factor

TPMS triply periodic minimal surface

TRPV4 transient receptor potential cation channel subfamily V member 4

TV total volume

2D two-dimensional

USB Universal Serial Bus

UTE ultrashort echo time

UV ultraviolet

VB vertebral body

WSS wall shear stress

YBM yellow bone marrow

CHAPTER — 1

Low Back Pain and the Intervertebral Disc: Pathophysiology and Research Approaches

This chapter is partially based on published content:

Bermudez-Lekerika, Paola, Alminnawi, Ahmad, et al. *Immunomodulatory effects of intervertebral disc cells*. *Frontiers in Cell and Developmental Biology* 10 (2022): 924692.

The chapter starts with a brief discussion on the etiology and pathophysiology of low back pain (LBP) and the intervertebral disc (IVD), which will be elaborated in the following chapter. It then examines the immune-privileged nature of the IVD, emphasizing the role of the cartilaginous endplate (CEP) as a physical and biochemical barrier that regulates nutrient transport while limiting immune access, as well as the therapeutic importance of preserving or restoring endplate integrity to maintain immune homeostasis and slow disc degeneration. Finally, it reviews the state-of-the-art *in vitro* and *in silico* tools that can be used to investigate the pathophysiology of the IVD in general and the bony endplates (BEPs) and CEPs in particular.

1.1 Low back pain

LBP is a major and rising source of socioeconomic burden worldwide, with an annual cost of €272 million in Belgium [1] and \$100 billion in the United States alone [2–6]. It is one of the most common reasons for visiting primary care physicians and receiving opioid prescriptions [2, 7–12], and it ranks as the fifth most frequent reason for doctor visits, affecting up to 37% of patients [4, 13]. Approximately 80% of the global population experiences LBP at some point in life [14], and over 10% of these individuals develop chronic disability [15, 16], which results in substantial treatment costs and a reduced ability to work or engage socially, thereby diminishing quality of life [13]. The global burden continues to rise, with years lived with disability increasing from 58.2 million in 1990 to 83 million in 2010 [17, 18]. Beyond physical impairment, LBP also negatively affects psychological well-being, as chronic cases are often linked with anxiety and depression, complicating treatment and prolonging recovery [16, 19], which further limits daily function and reduces productivity.

Despite its high lifetime prevalence, the precise underlying mechanisms of LBP remain poorly understood [2, 4]. One highly recognized etiologic factor in nonspecific LBP is degeneration of the lumbar IVD [2–5, 18, 20], which has been directly related to 40% of LBP cases [3]. To emphasize the critical nature of the issue, intervertebral disc degeneration (IVDD) is reported to affect almost every individual past the age of 70 [4], but it is also prevalent in younger age groups, including adolescence [4, 13].

Current management strategies for LBP encompass a range of interventions, including physical therapy, pharmacological treatments, and surgical options

aimed at addressing degenerated IVDs through procedures such as resection and nerve root blocking [15]. While multidimensional rehabilitation strategies addressing both physical and psychosocial aspects of recovery have been implemented, they primarily relieve symptoms rather than facilitate regenerative healing of the IVDs. Emerging regenerative therapies show promise but remain largely experimental, signaling the need for continued research and innovation in IVD degeneration and LBP management [13].

1.2 The intervertebral disc

IVDD is a multifactorial condition influenced by various pathophysiological, biochemical, and biomechanical factors, as well as biological sex and ethnicity [21–25]. These factors collectively drive a cascade of events that modify disc morphology, ultimately contributing to conditions such as osteoarthritis and LBP [4, 5, 13, 18, 20, 26, 27]. However, the precise mechanisms by which these factors interrelate remain poorly understood [5, 13, 20]. The consequences of these degenerative processes are often manifested by a loss of cellularity, degradation of the extracellular matrix (ECM), and, in the long term, notable morphological changes and impaired biomechanical properties [20]. This section describes elements related to IVD development, physiology, and pathology that are relevant for this doctoral project. A more detailed review of the specific elements related to the endplate (EP) are provided in the next chapter.

1.2.1 The components of the IVD

The human vertebral column comprises 23 functional units known as IVDs, which lie anterior to the neural arch that encases the spinal cord, collectively forming the vertebral foramen [26]. These IVDs serve as heterogeneous and composite tissues that form joints to separate adjacent vertebrae within the spinal structure. Each adult IVD is composed of three primary components, a centrally located gel-like core called the nucleus pulposus (NP), a thick and tough peripheral ring made of orthogonal concentric layers known as the annulus fibrosus (AF), and an osteochondral boundary known as the EPs that connect the IVD to the adjacent vertebral bodies (VBs) cranially and caudally [2, 4, 15, 18, 20, 26] (Figure 1.2.1.).

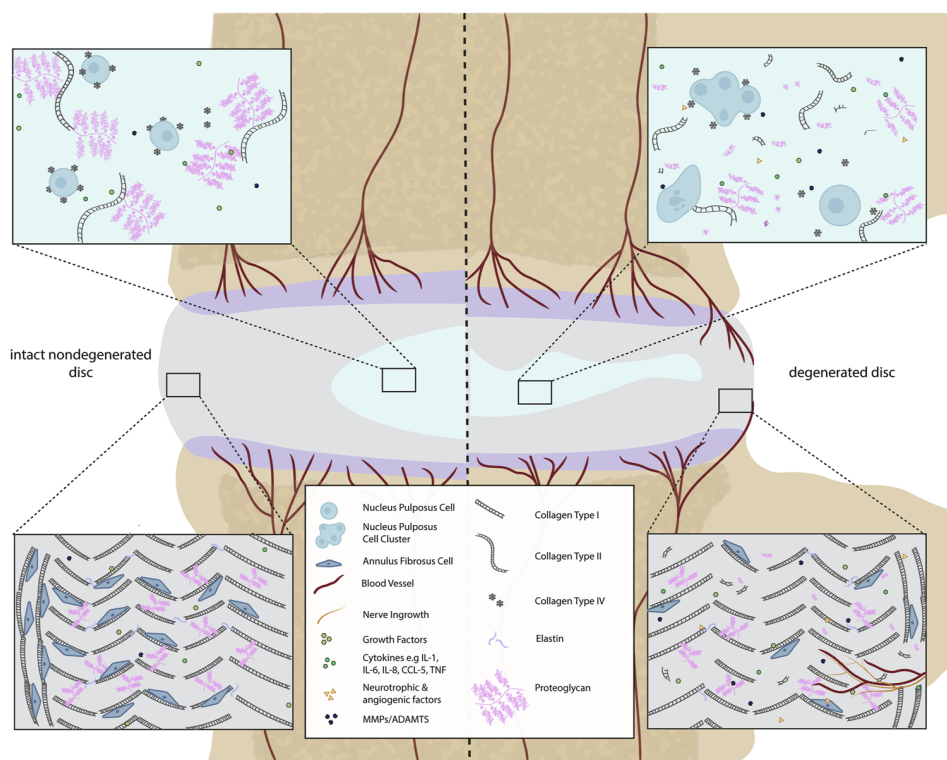


Figure 1.2.1: Comparison of a healthy and a degenerated IVD. In the intact IVD, the NP matrix mostly contains PGs and non-oriented type II collagen fibers. The PGs contain negatively charged sulfated groups, leading to an intradiscal osmotic pressure that is crucial for the basal hydration of the NP and the biomechanical function of the IVD. Within the degenerated disc, the total content of PG decreases. Small non-aggregating PGs are present. This drop in PG content negatively affects the swelling capacity of the disc. Additionally, during disc degeneration, the production of catabolic cytokines, matrix-degrading enzymes, and neurotrophic as well as angiogenic factors occurs due to cellular changes. This can lead to blood and nerve vessel ingrowth in the AF. The AF is composed of highly oriented concentric lamellae of type I collagen, whereas the cell density is higher in intact discs than in degenerated discs.

1.2.2 The notochordal origin of the IVD

During embryonic development, all chordate embryos feature a notochord, an elastic rod-like structure derived from mesodermal tissue, which serves as a precursor to the vertebral column [28, 29]. In many species, including humans, the notochord undergoes significant transformations, regressing within the IVD to give rise to the NP [4, 30, 31]. Meanwhile, the surrounding mesenchyme, which is a loosely organized connective tissue, gives rise to the other structural components of the vertebral column. Condensed mesenchyme develops into the AF, while uncondensed mesenchyme forms the VB [4, 30, 32]. The development of the EP is attributed to the annular epiphysis of the VB prior to its ossification [4, 33]. It is hypothesized that the notochord possesses progenitor properties, as species that lose their notochord exhibit an inability to regenerate IVD tissues [4, 34–38]. Thus, understanding the development and structural composition of the post-notochordal IVD is critical for understanding its implications of IVD degeneration.

1.2.3 The avascular and aneural nature of the intervertebral disc and its dependency on diffusion

The IVD has been described as the largest avascular structure in the human body [39] since it usually has blood vessels during embryonic development regress rapidly after birth, leaving the disc dependent on passive nutrient transport mechanisms [40]. The healthy adult IVD is characterized by its avascular and aneural properties, features that contribute to its ability to facilitate pain-free movement of the spinal column [2, 4, 13]. This lack of innervation is partly due to the presence of high levels of aggrecan (ACAN), a type of proteoglycan that has been shown to inhibit nerve growth and may reduce the sensitivity of surrounding tissues to mechanical stress and injury [5, 41, 42]. As a result, the IVD can withstand significant loading without the activation of pain pathways typically associated with neural structures. Blood vessels terminate at the vertebral endplates and outer AF, failing to penetrate deeper disc regions [43, 44]. Due to these structural characteristics, the IVD relies entirely on the diffusion of nutrients, oxygen, and metabolic waste products through the ECM between the disc and the capillary networks located in the subchondral bone. This diffusion allows for effective nutrient and waste transport which are essential for maintaining physiological conditions within the disc, including appropriate levels

of lactate, pH balance, and osmotic pressure [5, 13]. This diffusion dominates transport for small solutes (e.g., glucose, oxygen, and lactate), whose movement occurs along concentration gradients from capillary beds in the EP and outer AF toward the disc center [44–46]. Additionally, convection, which is the bulk fluid flow, is usually driven by mechanical loading and aids mainly larger solute transport (e.g., growth factors, proteins) [45–47]. The EP is widely regarded as the primary diffusion gateway, where calcification or degeneration reduces nutrient influx, leading to accelerated NP dehydration which, in turn, can lead to mechanical failure [40, 44, 45, 48, 49]. In conclusion, this high reliance on diffusion for metabolic needs can be compromised by changes in the ECM, nutrient supply, or physical loading patterns, potentially leading to IVDD.

1.3 The immune-privileged nature of the IVD

1.3.1 Maintenance of immune privilege

IVDD is a complex, multifactorial process characterized by a metabolic transition of the disc from an anabolic state, dominated by matrix synthesis, to a catabolic state, marked by matrix degradation. This catabolic shift is accompanied by elevated production of cytokines and chemokines by native disc cells, a response often misinterpreted as inflammation in the literature despite occurring in the absence of immune cell infiltration. The IVD is considered an immune-privileged organ [50, 51], mainly due to its isolated anatomy and specialized cellular microenvironment. The EP, specifically the CEP, plays a crucial role in maintaining this immune-privileged property, acting as both a physical [48, 52, 53] and biochemical [2, 48, 50, 52, 54–56] barrier for various molecules. Together with the AF, the EP forms part of the blood–nucleus pulposus barrier (BNB) [50], a structural and molecular interface that restricts vascular and immune access to the NP. This barrier is reinforced by the dense ECM and by anti-angiogenic and anti-neurogenic factors secreted by native disc cells. Under physiological conditions, the CEP facilitates the movement of metabolites, small molecules, and waste between the IVD and the neighboring blood vessels located in the BEP. In other words, the CEP allows small, neutrally charged solutes like oxygen and glucose in addition to cations like sodium and calcium to penetrate the disc [53, 57]. The BNB on the other hand, prevents exposure of NP antigens to the systemic immune system. This mechanism helps in

avoiding immune recognition and inflammatory activation which in turn regulate communication between the avascular NP and the vascularized vertebral bone marrow. The IVD environment is harsh, characterized by low oxygen, low glucose, and low pH levels. Limited nutrition and high lactic acid levels contribute to the catabolic shift, while hypo-osmolarity can activate pro-inflammatory and catabolic factors [55, 58–63]. Native cells within the IVD, including CEP cells, display immune-modulatory activity despite this isolation. They constitutively express cytokines and chemokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor (TNF), and interleukin-17 (IL-17), which contribute to matrix turnover and homeostatic catabolic–anabolic balance [64–71].

1.3.2 Breach of the barrier and immune activation

IVDD is often accompanied by loss of CEP integrity representing a pivotal event in the breakdown of the IVD immune privilege. As IVDD progresses, the CEP becomes thinner, more fissured, with reduced collagen and glycosaminoglycan (GAG) content [50, 54]. These morphological changes alter the physiology and performance of the CEP, specifically changing its permeability and disrupting its selective barrier function [55, 72, 73]. Any structural failure, such as thinning, fissuring, or ruptures, would allow blood vessels and nerve fibers to infiltrate the IVD creating entry routes for blood vessels and immune cells from the subchondral bone into the disc (Figure 1.3.1). The infiltration of macrophages, T cells, and mast cells transforms the local catabolic state into a truly inflammatory state [74], characterized by the release of additional cytokines and proteases that accelerate ECM degradation [67]. This immune infiltration establishes a vicious feedback loop where CEP damage facilitates vascular ingrowth and immune activation, which in turn aggravates CEP degradation through inflammatory mediators. The ensuing crosstalk between the IVD and the adjacent vertebral bone marrow can be demonstrated radiologically as Modic changes, particularly Type 1 lesions that correspond to an inflammatory phenotype and are strongly associated with pain [75–77]. Consequently, CEP pathology is now recognized as a more reliable indicator of discogenic pain than the degree of NP degeneration or IVDD itself [2, 55].

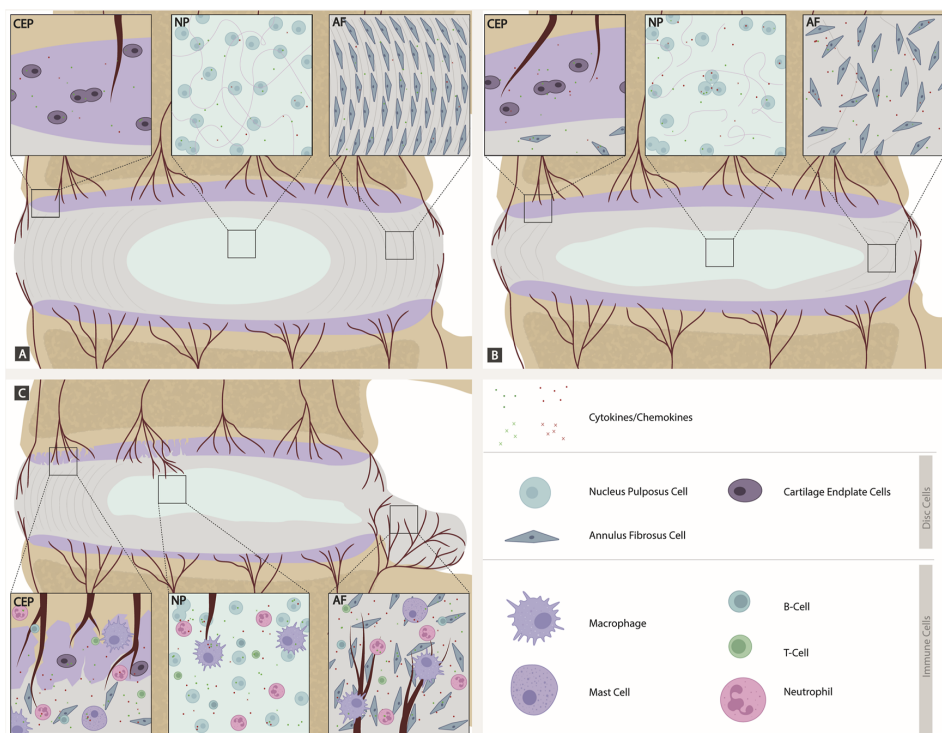


Figure 1.3.1: Comparison of healthy, degenerated, and herniated IVDs (focused on cellular involvement). (A) Intact IVD: Native disc cells produce a plethora of cytokines and chemokines expressing the corresponding receptors and maintaining homeostasis in a para and autocrine manner. The CEP is intact with blood vessels. The NP has a high number of proteoglycans. AF cells align. (B) Degenerated IVD: Shift to catabolic environment. Cytokines are expressed by disc cells themselves. The CEP has a higher amount of blood vessels than in the intact IVD. Proteoglycan number decreases in the NP. In the AF, there is a loss of alignment and support for AF cells. (C) Herniated IVD with crack in CEP: As soon as the AF or CEP is ruptured during injury or disc degeneration, a route for migration of immune cells into the IVD is provided. Immune cells, including T cells, B cells, macrophages, neutrophils and mast cells, contribute to an inflamed environment within the disc, further increasing the cytokine and chemokine expression and leading to a vicious circle of inflammatory driven catabolism. A crack in CEP allows blood vessels to grow into the AF and NP. The AF herniates/bulges, which is where blood vessel in-growth primarily occurs.

1.3.3 Immunomodulatory implications for therapy

Given its dual role as both barrier and mediator, the CEP stands at the interface between immune homeostasis and inflammation in the disc. Preservation or restoration of CEP integrity is therefore critical for maintaining immune privilege and preventing inflammatory-driven degeneration. Therapeutic strategies targeting CEP regeneration or reinforcement may restore the diffusion barrier, limit immune infiltration, and reestablish the metabolic and immunological equilibrium of the IVD. Yet there is a lack of studies focused on the EP regeneration therapies. Computational and experimental models that simulate CEP permeability and its relationship with immune crosstalk will be essential for developing such targeted interventions.

1.4 Preclinical animal model selection for intervertebral disc research

Given the ethical and practical limitations of obtaining healthy human osteochondral IVD samples, such as the lack of associated clinical information in cadaveric samples and the inadequacy of surgically discarded tissues for control studies [3], it is crucial to explore animal models for disc research. Various preclinical animal models, including bovine [78], canine [78, 79], feline, porcine [80], alpaca/llama, equine, kangaroo [81], ovine [80], caprine [80], leporine [82, 83], and rodent [27, 83], have been recognized as a valuable source of IVD tissue when studying the mechanobiology, cell biology, and pathogenesis of human IVDD. They form interesting research subjects due to their anatomical and biochemical similarities to human IVDs, such as comparable cell phenotypes, tissue composition, and mechanical properties [18], as well as genetic parallels that enhance their utility in exploring the structural and mechanical environments of IVDs and their associated pathomechanisms [27, 84]. Given the ethical and practical limitations of obtaining healthy human osteochondral IVD samples, such as the lack of associated clinical information in cadaveric samples and the inadequacy of surgically discarded tissues for control studies [3], it is crucial to explore these animal models in disc research. Selecting the appropriate animal model for studying IVDD and related phenomena is critical, as each species exhibits specific deviations in IVD characteristics that can influence research outcomes. The choice of model should align with the specific biological, biomechanical, or pathological aspects under

investigation taking into account the research objective, experimental design, and species availability [80, 85–89]. For example, the human notochord disappears by approximately 30 days gestational age, leading to the regression of notochordal cells within the NP as mentioned earlier, while in rodent models, such as mice, notochordal cells persist within the NP throughout their lifespan [4]. This distinction creates a different biological environment for investigating mechanobiological and pathological processes. Nevertheless, rodents remain widely used in genetic and molecular studies because of their genetic similarity to humans [90]. Canine models, especially chondrodystrophic breeds, exhibit spontaneous disc degeneration similar to humans, making them valuable for translational research [80]. Sheep have discs of similar sizes to those of humans and, despite their quadruped stature, experience comparable mechanical loading (stresses), allowing for surgical and implant studies [80]. Bovine models have proven effective for understanding IVDD and evaluating potential interventions due to their size, tissue composition, and biomechanical properties that closely mimic those of human discs making them suitable for testing surgical techniques and the effects of biochemical agents on disc health [84, 91]. Due to this close similarity with human discs, and their availability in large quantities from abattoirs, bovine is one of the most utilized sources for *ex vivo* or organ culture experiments in controlled laboratory studies, particularly the coccygeal discs from the tail [91–97].

1.5 Experimental approaches for studying the intervertebral disc

1.5.1 Advanced three-dimensional (3D) culture systems and bioreactors

3D culture systems and bioreactor technologies have become essential tools, offering more physiologically relevant environments than traditional two-dimensional (2D) cultures. They play a pivotal role in the study of IVD degeneration and regeneration, providing a controlled environment that mimics physiological conditions. Different designs have been developed to address specific research needs, enabling the investigation of mechanical, biochemical, and cellular dynamics within the IVD. Cells, explants, and whole-organ culture of IVDs are increasingly utilized in bioreactor systems to mimic physiological loading conditions and the native microenvironment

due to their pre-clinically relevance, thus bridging the gap between *in vitro* and *in vivo* studies [57, 98–102].

Organ culture and hydrogel cell culture

IVD research has increasingly turned to organ culture and hydrogel cell culture systems because of their ability to facilitate the assessment of disc biology and the investigation of potential therapeutic interventions in a controlled environment compared with cell monolayer or pellet cultures. Whole-organ culture preserves the native architecture and cellular environment by maintaining the structure and cell-matrix interactions of the whole IVD *ex vivo*. This enabled the investigation of tissue-level responses, interactions between the NP, AF, and EP, and the impact of mechanical loading or biochemical insults [102–104]. Systems range from unloaded (free-swelling) to complex mechanically loaded setups, including static, cyclic, and multiaxial loading, to simulate physiological or pathological conditions [102, 105, 106]. Hydrogel-based cell culture involves encapsulating IVD cells (NP, AF, EP, or stem cells) within 3D, ECM-mimicking hydrogels. Hydrogels like alginate, collagen, and hyaluronic acid provide a 3D microenvironment that supports cell viability, phenotype maintenance, and matrix production, better reflecting the *in vivo* setting than traditional monolayer cultures [102, 107]. These gels allow for cell type isolation and can be tuned for stiffness, degradation, and bioactivity to study cell-matrix interactions or screen biomaterials for tissue engineering and have been used for developing injectable therapies for disc regeneration [107, 108].

Dynamic flow bioreactors

Dynamic flow bioreactors, such as spinner flasks or direct perfusion systems, are employed to facilitate the 3D culture of IVD-derived cells, providing more uniform nutrient distribution and promoting rapid, large-scale propagation of disc cells while maintaining their physiological characteristics [109, 110]. This type of bioreactor can be used to investigate cellular behavior under dynamic conditions, mimicking the physiological environment of the IVD. They are especially useful in studies involving cell integration and ECM production. These bioreactors have also been shown to enable effective cell propagation while promoting matrix synthesis, demonstrating distinct advantages over traditional monolayer cultures [109, 110]. These systems

can be integrated with more advanced bioreactors that apply mechanical loading to better simulate the *in vivo* disc environment [102, 111]. Such findings underscore the utility of these bioreactors in understanding IVD pathophysiology and in assessing the effects of various mechanical loading scenarios on disc degeneration.

Axial loading bioreactors

Axial loading bioreactors are commonly used to apply dynamic mechanical loading to IVDs, simulating the physiological conditions under which IVDs operate *in vivo*. Traditional bioreactors primarily applied uniaxial compressive loads to mimic the basic physiological loading of IVDs [112, 113], but next-generation systems now incorporate multiaxial loading, including six degrees of freedom (DOFs), to more accurately simulate the complex, multidirectional forces experienced by the spine during daily activities [111, 114, 115]. These bioreactors allow researchers to evaluate cellular responses to mechanical stimuli and nutrient flow across the endplates, which is essential for maintaining disc health. Axial loading bioreactors demonstrated that discs maintained significant cell viability under loading, promoting nutrient flow and tissue viability [116].

Organ-on-a-chip microfluidics

Organ-on-a-chip systems are microfluidic *in vitro* models that recreate key features of IVD tissue, such as cell organization, nutrient transport, mechanical loading, and inflammatory signaling. They are used to study IVDD, immune-cell infiltration, and pain-related pathways while reducing reliance on animal models. Organ-on-a-chip systems can mimic IVD physiological conditions more realistically by combining controlled flow, gradients, and mechanical cues making them more relevant than simple 2D cell culture and more flexible than animal models for mechanism studies [117–120].

3D bioprinting

Although not considered a culture bioreactor, 3D bioprinting technology has emerged as a groundbreaking method for creating bioengineered IVD models that replicate the anatomical and functional characteristics of natural tissues.

It enables biomimetic scaffold fabrication that imitates the macroscopic and microscopic architecture of the native IVD [121, 122]. By precisely depositing cells, biomaterials, and growth factors, 3D bioprinting allows for the creation of complex disc structures that can be used to study cellular interactions and mechanobiology in a controlled environment [123–125]. Bioprinting utilizes a variety of bioinks, often combining natural hydrogels, such as gelatin, alginate, and hyaluronic acid, with synthetic polymers to achieve both mechanical strength and biocompatibility [126]. Recent advancements in this area have the potential to create patient-specific models for regenerative therapies.

Limitations of advanced 3D culture systems and bioreactors

Despite their advantages over conventional monolayer culture, these technologies cannot fully reproduce the structural and biological complexity of a living vertebral motion segment. Hydrogel and cell-based systems isolate selected cell populations from the native organization of the NP, AF, CEP, BEP, bone marrow, ligaments, and other surrounding tissues. Consequently, they cannot completely reproduce tissue–tissue interactions, spatial gradients, native ECM architecture, or the coupled mechanical behavior of the vertebral complex. Dynamic flow systems improve mass transport but can expose cells to non-physiological flow and shear conditions, while the observed response remains strongly dependent on scaffold composition, cell source, and culture parameters [107–110]. Similarly, organ-on-a-chip and bioprinted models reproduce selected features of the IVD rather than the entire organ system. Their simplified geometry, restricted number of cell and tissue types, material-dependent properties, and limited long-term maturation constrain direct translation to the native human disc [121, 123–126].

Whole-organ culture under multiaxial loading currently provides one of the closest approximations of native IVD architecture and mechanical stimulation because it preserves several tissue interfaces and permits the controlled application of complex loads [102, 104, 111, 114, 115]. Nevertheless, even this approach remains an isolated *ex vivo* system. Culture media, oxygen tension, nutrient gradients, waste removal, and loading histories can approximate but cannot remain identical to the continuously changing *in vivo* environment. Harvesting also disrupts vascular, neural, marrow, and surrounding soft-tissue connections. The explant consequently receives no physiological supply or recruitment of circulating immune cells, progenitor cells, or other systemic cell populations, and it lacks endocrine and

whole-body regulatory inputs. Cell and tissue viability therefore declines with culture duration, particularly when transport through the endplates is impaired, limiting the study of long-term degeneration, remodeling, and repair [102–104, 116].

These platforms should therefore be regarded as complementary model systems rather than complete replacements for the living vertebral complex. The appropriate system ultimately depends on the research question as reductionist cell and chip models provide experimental control and mechanistic resolution, whereas organ culture and multiaxial bioreactors offer greater structural and mechanical fidelity at the cost of lower control, finite viability, and limited access to systemic interactions. Findings should consequently be interpreted within the boundaries of each model and, where possible, validated across complementary *in vitro*, *ex vivo*, computational, animal, and clinical approaches.

1.6 The spectrum of *in silico* technologies in IVD research

Computational (or *in silico*) tools have become increasingly integral in IVD research, providing innovative approaches to simulate biological processes, assess mechanical properties, and investigate the effects of various interventions on IVD health and degeneration. The development and refinement of these models can enhance understanding of IVD pathophysiology and facilitate the exploration of potential therapeutic strategies. The spectrum of *in silico* technologies ranges from white box, (knowledge driven and first principles based models) to black box (data driven approaches based on statistical methods, machine learning, and artificial intelligence) with numerous hybrid approaches combining elements of both paradigms. One of the key advantages of these tools is that they allow the integration and interrogation of the observations from pre-clinical and clinical studies. The following subsections provide a more detailed discussion of specific types of computational models and their applications in IVD and LBP research.

1.6.1 Regulatory network models

Regulatory network models (RNMs) are computational frameworks that represent biological entities as nodes and their regulatory interactions as directed edges to simulate complex biological systems and predict their dynamic behaviour. Their primary focus is on the interactions and regulatory relationships within a network of interconnected nodes rather than on the behaviour of individual entities. By identifying stable system states, or attractors, these models can simulate how perturbations, such as therapeutic interventions or changes in the cellular microenvironment, shift cells toward healthy or degenerative phenotypes [127–130].

1.6.2 Agent-based models

Agent-based models (ABMs) are computational frameworks that simulate the behaviors and interactions of individual agents, e.g., cells and molecules, within the complex microenvironment of the IVD. In contrast to RNMs, the primary focus of ABMs is on the behaviors of autonomous agents. These models are particularly valuable for studying the multifactorial and dynamic processes underlying disc degeneration, which are difficult to capture *in vivo* or *in vitro*. They have been used to mimic the behavior of IVD cells in 3D environments, integrating multiple biochemical and mechanical stimuli. This allows researchers to study how IVD cells respond to factors such as inflammation, nutrient deprivation, and changes in tissue properties [131, 132]. These models can be used to predict cell death under specific biochemical conditions and to quantify catabolic shifts in gene expression under adverse conditions [68, 133].

1.6.3 Finite element modeling

Finite element modeling (FEM) (or Finite Element Analysis) is widely utilized to solve complex physical or biochemical processes by spatially discretizing systems. They are used to model the mechanics of the IVD, allowing researchers to study stress distribution, deformation patterns, and the biomechanical response of discs under various loading conditions. FEM enables the creation of detailed models that can represent the heterogeneous structure of the IVD, including the AF and NP, and can incorporate different material properties, fiber orientations, and even stages of degenera-

tion [134, 135]. FEM can simulate the impact of different spinal postures and movements on the IVD, helping to identify risk factors for degeneration and develop optimized rehabilitation strategies. The value of FEM was demonstrated in assessing stress distribution in discogenic back pain, showing how specific movements affect mechanical loading [136]. The effects of osteoporotic vertebral degeneration on lumbar spine stability were also evaluated through 3D FEM, indicating that modified loading conditions can influence the stability of IVDs. Such models provide valuable insights into the relationship between degeneration in vertebral bodies and the overall health of the IVD [137]. Additionally, these methods are utilized to test the impact of surgical interventions, spinal implants, or regenerative therapies in a virtual environment before clinical application [138].

1.6.4 Imaging and artificial intelligence techniques

Various imaging and image analysis tools are used to quantify features from medical or laboratory images. Magnetic resonance imaging (MRI) remains the gold standard for assessing IVDD due to its superior soft tissue contrast and ability to visualize disc structure and hydration status [139–141]. Standard T2-weighted imaging (T2WI) is commonly used for qualitative evaluation, but it has limitations in reproducibility and correlation with clinical symptoms [142]. Computed tomography (CT) is also valuable for detecting disc calcification, extrusion, and bony changes, particularly in cases where MRI is contraindicated [141, 143]. Advanced imaging techniques, combined with artificial intelligence (AI) such as deep learning models, are revolutionizing the field of IVD research as they excel in pattern recognition and predictive modeling. AI models have been trained to classify disc degeneration according to established grading systems, e.g., Pfirrmann grading, showing high sensitivity and specificity and supporting clinical decision-making [144]. They are capable of automatic detection of narrowed IVD spaces in X-ray images, enhancing the initial screening of IVD disease and herniation by providing quick and accurate assessments [145, 146]. Other models, particularly convolutional neural networks (CNNs) and architectures such as U-Net, are used to automatically segment spinal structures and measure parameters such as disc height and spinal canal diameter from MRI scans. These methods improve efficiency, accuracy, and reproducibility compared to manual analysis [147, 148].

1.6.5 Multiscale modeling

Multiscale modeling in IVD research integrates several types of computational approaches to understand the complex structure–function relationships of the disc across multiple biological scales, from the molecular and cellular level up to tissue and organ levels [68, 149]. It bridges the gap between cellular processes, ECM composition, tissue mechanics, and organ-level function. This allows researchers to study how changes at the micro-level, e.g. fiber orientation and cell signaling, influence macroscopic disc behavior and vice versa [68]. These models can also incorporate multiphasic mechanics, accounting for solid, fluid, and ionic phases within the disc. This is vital for simulating osmotic behavior, water-content changes, and their effects on disc mechanics and degeneration [149, 150].

Computational models are advancing towards a more personalized approach and future models are expected to simulate patient-specific CEP shape and environments to more accurately simulate pathophysiological processes such as water and nutrient transport [48, 151–157].

1.7 Conclusion and outlook

This chapter established the clinical relevance of LBP and outlined the developmental, biological, and biomechanical characteristics of the IVD that contribute to its degeneration. It described how the isolated environment of the IVD is maintained and how disruption of its barriers can permit immune activation. In addition, the chapter reviewed the principal preclinical, *in vivo*, and *in silico* approaches used to investigate these processes. Together, these perspectives support the view that IVDD cannot be understood through a single tissue, mechanism, or model and highlight the EP as an important interface linking nutrient transport, mechanical function, and communication between the disc and vertebral body.

Building on this context, Chapter 2 focuses specifically on the CEP, examining its structure, composition, permeability, mechanical roles, and changes during degeneration. This transition from a broad overview of IVD pathophysiology to a detailed assessment of the CEP establishes the rationale for the experimental and computational investigations presented later in this thesis.

CHAPTER — 2

Cartilaginous Endplates: A Comprehensive Review of an Understudied Structure in Intervertebral Disc Research

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*Shared first authorship.

The cartilaginous endplates (CEPs) are key components of the intervertebral disc (IVD) necessary for sustaining the nutrition of the disc while distributing mechanical loads and preventing the disc from bulging into the adjacent vertebral body. The size, shape, and composition of the CEP are essential in maintaining its function, and degeneration of the CEP is considered a contributor to early IVD degeneration. In addition, the CEP is implicated in Modic changes, which are often associated with low back pain. This review aims to tackle the current knowledge of the CEP regarding its structure, composition, permeability, and mechanical role in a healthy disc, how they change with degeneration, and how they connect to IVD degeneration and low back pain. Additionally, the authors suggest a standardized naming convention regarding the CEP and bony endplate and suggest avoiding the term vertebral endplate. Currently, there is limited data on the CEP itself as reported data is often a combination of CEP and bony endplate, or the CEP is considered as articular cartilage (AC). However, it is clear the CEP is a unique tissue type that differs from articular cartilage, bony endplate, and other IVD tissues. Thus, future research should investigate the CEP separately to fully understand its role in healthy and degenerated IVDs. Further, most IVD regeneration therapies in development fail to address, or even consider the CEP, despite its key role in nutrition and mechanical stability within the IVD. Thus, the CEP should be considered and potentially targeted for future sustainable treatments.

2.1 Introduction

2.1.1 What is the CEP?

The IVD provides the spine with flexibility and operational mechanical support. Depending on the speed or dynamism of the mechanical loads, it can store or dissipate energy and allows movement in the vertebral column. The IVD comprises three anatomic regions: a gelatinous core, the nucleus pulposus (NP); the annulus fibrosus (AF), a fibrocartilage that confines the NP laterally; two CEPs that are thin hyaline-like cartilage layers, covering the cranial and caudal ends of NP and the inner part of the AF. The human CEP is 0.1–1.6 mm (~ 0.06 in) thick. It separates the IVD from the adjacent endplates of the vertebral bone, i.e., the bony endplates (BEPs) [47, 52, 158, 159]. The CEP thickness varies greatly even within healthy IVDs, according to age, location in the spine (disc level), position

in the IVD (cranial, caudal) and region in the tissue (central, peripheral). Its extracellular matrix consists of mainly type II collagen, proteoglycans, and water [160].

The CEP plays a key mechanical role in preventing the disc from bulging into the adjacent vertebral body (VB) [161] and providing cranial and caudal anchorage for the fibers of the inner AF and NP of the innermost part of the AF (Table 2.1.1) [162–164]. In addition, the CEP provides a key path for the diffusion of nutrients from the peripheral vasculature to the IVD and waste out of the IVD, which is crucial as it is the largest avascular tissue in the human body [48, 165]. While AC relies on diffusion from the subchondral bone and via synovial fluid for nutrition [166, 167], the CEP relies on diffusion from neighboring blood vessels. Solutes, including oxygen and glucose, have been hypothesized to be predominantly transported into the disc through the CEP and their availability is regulated by the bone marrow contact channels that cross the BEP [168]. Often, the combined CEP and BEP are referred to as the vertebral endplate; however, the term vertebral endplate is also used interchangeably to refer purely to the BEP. Although the CEP and BEP have been recognized as distinct tissues since the 1930s, many studies do not distinguish the CEP from the BEP [169], e.g., when reporting fluid transport in the IVD [170] or radiological signs of IVD degeneration [171] even if the authors acknowledge that the vertebral endplate is a bilayer of cartilage and bone [172, 173]. Arguably, it is difficult to isolate the CEP from the BEP experimentally, and this must be done very carefully [78, 174]. Clinically, the distinction of the two tissues on medical images is also very challenging. Thus, when reporting methods, it should be clearly stated what tissue or construct (CEP, BEP, or a combination thereof) is being used and a clear consistent nomenclature should be used, to avoid confusion [175]. Further, it is the authors' recommendation to define the CEP and the BEP independently where possible, or otherwise explicitly define the vertebral endplate as a construct of two tissues.

2.1.2 Developmental biology of the CEP

The CEP, AF and vertebral bodies develop from the mesoderm, specifically from the sclerotome [209]. AC is also derived from the mesoderm (Table 2.1.1) [176, 177]. In contrast, the NP develops from the notochord [73]. The mesoderm (paraxial) undergoes somitogenesis promoted by precise and cyclic temporal and spatial regulation of Notch and Wnt, and fibroblast growth factor (FGF) signaling pathways, respectively [210]. The Sonic

Table 2.1.1: Differences between the cartilaginous tissues CEP, AC, NP, and AF. Part A: general differences.

A. General			
Characteristic	Cartilaginous endplate (CEP)	Articular cartilage (AC)	Nucleus pulposus (NP)
Tissue origin	Mesoderm (sclerotome) [73]	Mesoderm [176, 177]	Notochord [73]
Vascularity	Vascular at birth, avascular in adulthood [73, 178, 179]	Avascular [166, 180] regardless of the stage of development [181]	Mesoderm (sclerotome) [73] Vascular at outer one-third of the AF and avascular at inner AF [40]
Nutrition source	Diffusion from neighbouring blood vessels [182]	Diffusion from synovial fluid [166, 167] and the subchondral bone [167]	Anaerobic glycolysis [91], diffusion from CEP [91, 97, 182]
Imaging*	Hypointense on T2-weighted magnetic resonance imaging (MRI), indistinguishable from bony endplate. Specific sequences such as ultrashort echo time (UTE) assist in differentiating CEP from bony endplate on MRI [183–185]	Hypointense on T2-weighted MRI and distinguishable from adjacent soft tissue and bone [186]	Hypointense on T2-weighted MRI, indistinguishable from CEP, with a gradient in the boundary between AF and NP in healthy IVD [183]

* Multiple imaging modalities are relevant in distinguishing these structures, especially when degenerative features are present, e.g. computed tomography (CT) in CEP sclerosis.

B. Biochemical composition

Characteristic	Cartilaginous endplate (CEP)	Articular cartilage (AC)	Nucleus pulposus (NP)	Annulus fibrosus (AF)
Cell density	15×10^6 cells/mL [187]	$14\text{--}15 \times 10^6$ cells/mL [188]	4×10^6 cells/mL [187]	9×10^6 cells/mL [187]
Water content	1.585–1.666 mg water/mg dry wt [47] and 22.1–62.4% [164, 189]	70.7% [190]	70%–90% [191]	50%–70% [191]
Proteoglycan	7.2–13.4% sulfated glycosaminoglycan (GAG) $\mu\text{g}/\text{mg}$ dry weight [47] and 4.37–18.48% $\mu\text{g}/\text{mg}$ dry weight [189]	5–15% GAG by dry weight [192]	30%–50% GAG of dry weight [191]	10% GAG of dry weight [191]
Collagen	681 ± 171 $\mu\text{g}/\text{mg}$ dry weight [47] and $329.0\text{--}886.9$ $\mu\text{g}/\text{mg}$ dry wt [189]	60–70% collagen by dry weight [192]	20% of dry weight [191]	70% of dry weight [191], aligned with alternate orientations of an average of $\pm 30^\circ$ [193]
Cell morphology	Rounded and slightly elongated in the direction of the collagen fibers [2]	Superficial and mid zone: rounded and slightly elongated in the direction of the collagen fibres; deep zone: round [176]	Fibrochondro-cyte-like cells [194]	Rounded chondrocyte-like cells (inner AF) and elongated, fusiform, fibroblast-like cells (outer AF) [195]
Gene markers	extracellular signal-regulated kinase (ERK), bone morphogenetic protein (BMP), aggrecan (ACAN), COL1A1, COL2A1 [91]	GDF10, CYTL1, IBSP, FBLN1 [196]; ACAN, PTN [197]	PAX1, FOXF1, HBB, CA12, OVOS2 [196]; KRT19 [198]; ACAN, VCAN, TNMD, BASP1, TNFAIP6, FOXF2, and AQP1 [197]	COL1, VCAN, PTN, TNMD, BASP1, TNFAIP6, FOXF1, FOXF2, and AQP1 [197]
Pericellular matrix	Randomly arranged [164]	Columnar organization [164]	Single cells in lacunae [3]	Single cell, paired, or multiple cells in contiguity [199]

C. Mechanics and permeability			
Characteristic	Cartilaginous endplate (CEP)	Articular cartilage (AC)	Nucleus pulposus (NP) Annulus fibrosus (AF)
Primary mechanical function	Resist fluid flow in and out of the disc and maintain a uniform stress distribution across the IVD [200–202], and prevent the disc from herniating or bulging [164, 201, 202]	Distribute load during joint movement and provide lubricated (low-friction) movement [180]	Withstand compressive loads to the IVD and maintain the BEP–CEP interface through fluid pressure [158] Confine the NP laterally [162] and anchor the IVD to the VB [203]
Primary permeability function	Main gateway of nutrients and waste into and out of the disc [48, 97, 161, 169, 204, 205], and prevent loss of large proteoglycan molecules from the disc [201]	N/A	N/A Secondary gateway of nutrients and waste into and out of the disc [48, 97, 169]
Permeability	1.27×10^{-16} and $1.66 \times 10^{-14} \text{ m}^4/\text{Ns}$ [47, 48, 182]	$(0.76 \pm 0.42) \times 10^{-14} \text{ m}^4/\text{Ns}$ [192]	$0.67 \pm 0.09 \times 10^{-15} \text{ m}^4/\text{Ns}$ [206] $0.23 \pm 0.19 \times 10^{-15} \text{ m}^4/\text{Ns}$ [206]
Tensile modulus	0.5–21.8 MPa [189]	1–30 MPa [207]	1–1.66 MPa [208]
Bone interaction	Parallel collagen fibers that make it weak and susceptible to detachment [203]	Anchored by perpendicular collagen fibers making them strongly attached [203]	N/A Outer AF extends to the VB, anchoring the disc to the vertebral rim [203]

Hedgehog (Shh) spatiotemporal regulation led by the notochord further differentiates somite to sclerotome and simultaneously promotes spine segmentation [211]. After sclerotome segmentation, cells proliferate, condense, and undergo chondrogenesis to form the vertebral bone (through the endochondral bone process), the AF and CEP. This complex regulation is governed by the coordinated action of Shh, SOX5/6/9, Pax1/9 and BMP pathways [209].

At birth, the human CEP is thicker and takes up approximately half of the intervertebral space, which is reduced to about 5% by adulthood [73]. Additionally, blood vessels are present at infancy, but are replaced over time by cartilaginous extracellular matrix (ECM) and almost disappear by skeletal maturity [73, 178, 179]. In humans, the CEP acts as a growth plate for the vertebrae, but this is lost after teenage years, so that only a thin layer of hyaline cartilage remains [73, 178]. This is different in many animals such as sheep or bovine, in which the growth plate has been shown to persist into adulthood, and is separated from the CEP by the BEP [212]. The shape of the lumbar (L4–L5) CEP also changes with age, starting with a biconvex shape at infancy, but evolves to a concave shape beginning around the age of two or three years when children start to walk [213–216]. Weight bearing and movement have been shown to influence the shape of the vertebrae and IVDs [213]. The CEP is vascularized during fetal development; however, by the age of 10 there is a substantial decrease in blood vessels, which are lost by adulthood [40]. Similarly, blood vessels have been shown to be present in the outer third of the AF up until the age of two, but decrease by age 30 unless there is damage that allows for revascularization [40]. In contrast, AC [166, 180] and the NP [50] are avascular regardless of the stage of development [181]. Throughout the 20s and after adulthood, calcification is observed, often in focal points in the CEP. These calcified sections can drive revascularization and bone formation which occurs following activation of matrix metalloproteinases (MMPs) which degrade the ECM [178, 179]. Furthermore, oxidative stress has been shown to induce CEP calcification through the p38/ERK/p6 pathway [217], which acts in conjunction with mitogen-activated protein kinase (MAPK) stimulation. This pathway is also involved in cartilage calcification in osteoarthritis [218], and is implicated in embryonic endochondral ossification in coordination with the transforming growth factor beta (TGF- β) and BMP families [217, 219]. Similarly, AF and CEP have both been demonstrated to be the source of pathological fibrocartilage in the NP [220]. Thus, the ability of CEP to move towards bone phenotype (calcification) and to equivalent AF cell fate (fibrocartilage) suggests that CEP cells maintain developmental-like plasticity, and con-

sistent tissue homeostasis to maintain their healthy phenotype. Similarly, this de-differentiation capacity has been shown in AC, where significant differential expression in the ERK and BMP pathway genes is observed [221].

2.2 Healthy CEP

2.2.1 Structure and composition of the CEP

Throughout life, the composition and anatomy of the CEP and BEP continuously change. During early life, ossification of the VB occurs. While the vertebra-sided part of the endplates becomes ossified forming the BEP in young adults, the disc-sided part remains cartilaginous forming the CEP [179]. The BEP is a layer of porous, coalesced trabecular bone containing pockets of vascularized bone marrow enabling the two-way transport of nutrients and cellular metabolic products [222]. In adults, the structure is avascular but has a base that contains a dense network of capillaries formed by terminal branches of metaphyseal and nutrient arteries [223]. The thicker peripheral section of the BEP forms a junction between the CEP and AF with the vertebral body [224].

The structural integration of the CEP into the BEP, AF, and NP varies [158]. At the bone interface there is minor integration, and the bone–cartilage junction is seen as a straight line with no gaps with collagen fibers of the CEP aligned parallel to the bone [158, 203, 225]. This is different than AC–bone interfaces, where the collagen fibers of the cartilage are perpendicular to the bone, anchoring the tissue types together [203]. However, in a healthy disc, the fluid pressure of the NP maintains the CEP and the BEP pressed together and thus, under normal loading (i.e. compression), the limited integration of the CEP and BEP is enough to maintain the BEP–CEP interface [158, 203]. Also at the BEP, there are capillaries that penetrate the pores of the subchondral bone and terminate by looping before the CEP junction [91, 97]. The capillaries are denser at the center of the vertebral endplates above the NP, which is where the IVD is thickest [204]. As the mature IVD is considered as avascular, the nutrient and waste exchange occur by diffusion from these capillaries [182]. Although diffusion can occur through the outer AF, the CEP is considered as the main gateway for nutrients into the disc and waste out of it [48, 97, 161, 169, 204, 205]. The diffusion distance between the CEP and the cells in the center of the NP

can reach 8 mm [168], which provides a shorter route for diffusion than through the AF.

In addition, collagen fibers from the outer AF extend into adjacent vertebrae and serve as anchor for the IVD to the rim of the vertebral bone [203]. The outer AF connects directly into the bone while the inner AF and NP connect to the CEP [158, 203, 226]. The integration at the inner AF, however, is more complex as the collagen fibers of the AF lamellae are continuous with those of the CEP (Figure 2.2.1) [203, 227]. Additionally, scanning electron microscopy (SEM) analysis of ovine discs has shown that the collagen fibers of the AF branch, which strengthens the annulus–endplate (EP) anchorage by increasing the interface area over which shear forces are distributed [228]. These fibers also intertwine and sometimes merge with the fibrils of the CEP. The strength of this connection is essential for the CEP ability to resist tensile loading [158, 225]. Collagen fibers in the CEP differ from both the NP and AF [229]. The collagen network of the CEP is denser than that of the NP; and tends to be arranged mainly parallel to the vertebrae, although not as highly oriented as in the AF [164, 229]. Additionally, the collagen fibers at the inner CEP are stronger and more interconnected than those at the outer CEP, which could play a role in the anisotropic flow resistance of the CEP [230]. Additionally, the highly convoluted collagen fibers within the NP penetrate at least partially into the CEP, providing the resistance to tensile force at the NP–CEP interface [162–164].

Interestingly, the caudal CEP is usually thinner than cranial one and both are rich in type II collagen, proteoglycans, and water [164]. Measurements of cadaveric human lumbar CEPs properties found that collagen content was $681 \pm 171 \mu\text{g}/\text{mg}$ dry weight [47], or within a range of 32.9–88.69% dry weight [189]. In comparison, the collagen content of AC has a smaller range of 60–70% [192] while that of the NP was around 20% [191]. Collagen content of the AF was around 70% [191] and has a specific alternating aligned orientation of an average $\pm 30^\circ$ [193]. The average sulfated GAG content was $103 \pm 31 \mu\text{g}/\text{mg}$ dry weight [47], or within a range of 4.37% and 18.48% dry weight [189]. The GAG content is similar to that of AC, which ranges between 5% and 15% [192], and that of the AF which is 10% [191]. The NP GAG content, on the other hand, is much higher, ranging between 30% and 50% [191]. CEP hydration was 1.585–1.666 mg water/mg dry weight, and CEP porosity was 0.648 ± 0.069 [47]. The water content of the CEP, which ranges between 22.1–62.4% [164, 189], is much lower than that of AC, at 70.7% [190], the NP, at 70%–90%, and the AF,

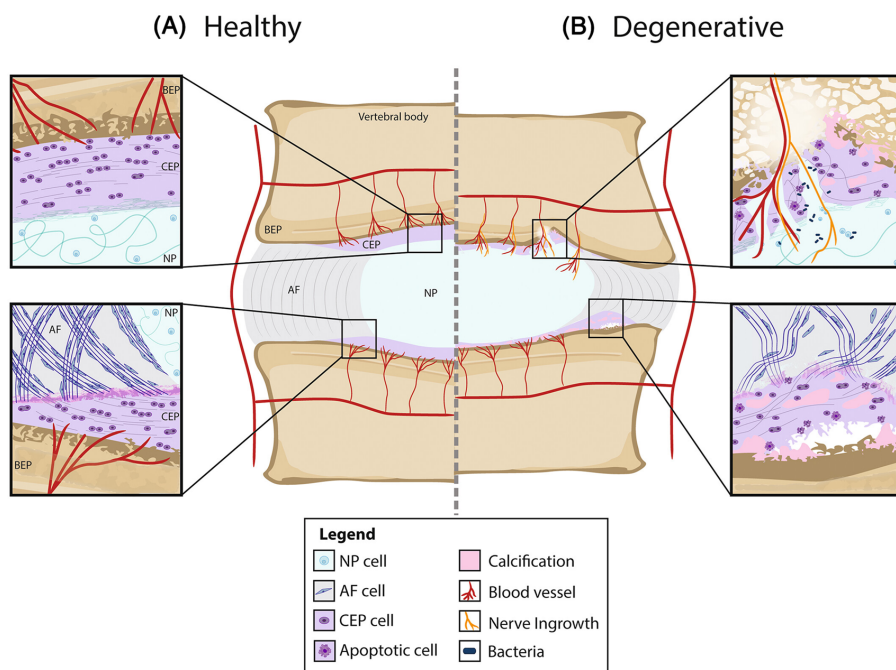


Figure 2.2.1: Healthy (A) versus degenerative (B) CEP.

at 50%–70% [191]. Chondrocytes are distributed throughout the CEP and are responsible for maintaining the ECM and, thus, providing stability to the tissue [52]. Macroscopically, CEP chondrocytes are typically rounded, although slightly elongated in the direction of the collagen fibers, more similar to the chondrocytes of the mid or superficial zone of AC than to the chondrocytes of the deep zone of AC [2, 176]. The cell density of the CEP is $\sim 15 \times 10^6$ cells/mL with the highest density closest to the vertebral bone. Although similar to the cell density of AC ($1.4\text{--}1.5 \times 10^7$ cells/mL [188]) this is nearly four times the cell density of the NP (4×10^6 cells/mL) and two times that of the AF (9×10^6 cells/mL) [187]. Recent single-cell analysis of human IVD cells have demonstrated that there are different IVD cell clusters non-randomly distributed in the AF, NP, and CEP [231]. The interactions between these IVD tissues at a mechanistic level are essential to understand the pivotal role of CEP chondrocytes in a healthy CEP structure. The main components of the pericellular matrix around the CEP chondrocytes are hyaluronan, proteoglycans and type VI collagen [232], while interstitial collagen is mainly composed of type II collagen [233] and proteoglycans, where the type chain, length, and quantity of GAGs determine the water content [234]. Type X collagen, a calcium-binding collagen which is a key marker of hypertrophy, increases with age

and is associated with increased calcification of the CEP [235]. Additionally, depletion of one of the collagen type II gene (*collagen type II (COL2)*) alleles has been shown to promote calcification of the CEP in mice [236]. Unlike AC, the CEP has a randomly arranged pericellular matrix that does not follow a columnar organization [164]. In comparison, the NP has single cells in lacunae [3], and the AF has single cell, paired, or multiple cells in contiguity [199]. In addition, the CEP is less hydrated and has lower GAG content than AC [164], which is why it should be considered a different and unique tissue. Negatively charged proteoglycans represent approximately 15% of the dry weight of CEP tissue [47]. CEP cells exhibit an elongated morphology aligned to the collagen-rich ECM and are arranged parallel to the VB, similar to superficial zone chondrocytes of AC, while deep cartilage AC cells present a round morphology and are arranged perpendicular to the adjacent bone [2, 176]. In contrast, the NP has fibrochondrocyte-like cells [194]. The cells of the AF differ between the inner AF, where cells are rounded and chondrocyte-like, and the outer AF, where cells are elongated, fusiform, and fibroblast-like [195]. Furthermore, AC cells present a decreased expression of ECM genes (*ACAN*, *COL1A1*, *COL2A1* genes) relative to CEP cell expression [2]. Other possible gene markers suggested for the CEP include ERK, BMP [91]. Genetic markers of AC have been proposed as *GDF10*, *CYTL1*, *IBSP*, *FBLN1* [196], *ACAN*, and *PTN* [197] while NP markers are considered as *PAX1*, *FOXF1*, *HBB*, *CA12*, *OVOS2* [196], *KRT19* [198], *ACAN*, *VCAN*, *TNMD*, *BASP1*, *TNFAIP6*, *FOXF1*, *FOXF2* and *AQP1* [197]. Genetic markers of the AF have been indicated as *COL1*, *VCAN*, *PTN*, *TNMD*, *BASP1*, *TNFAIP6*, *FOXF1*, *FOXF2* and *AQP1* [197].

2.2.2 Mechanics and permeability within the CEP

Effects of pressurization on CEP mechanics and permeability

Mechanical forces are crucial in maintaining cartilage homeostasis [237]. Chondrocytes respond to the mechanical environment, which contributes to the regulation of cell metabolism. As with other cartilage tissues, mechanical loading, such as compressive, tensile, and shear forces as well as pressure from fluid flow, is essential for the function of the CEP [238]. While overloading of the disc will result in vertebral endplate fractures or another injury, lack of mechanical stimuli will also impair disc homeostasis [239]. As it sits above and below the NP, the CEP acts as a mechanical barrier that adds resistance to the flow of fluid from the IVD to the VB, allowing for the pressurization

of interstitial fluid in response to compression while preventing the disc from bulging into the adjacent VB [229, 240, 241]. This fluid pressurization helps to maintain a uniform stress distribution across the IVD [200]. Thus, the permeability of the CEP is essential in maintaining the intradiscal pressure. In contrast, AC mechanically distributes load during joint movement and provides lubricated (low friction) movement [180]. However, it does not play an important role in permeability.

The reported permeability of cartilaginous tissue varies significantly. AC has been reported to have a permeability of $0.76 \pm 0.42 \times 10^{-14} \text{ m}^4/\text{Ns}$ [192] while the NP has a permeability of $0.67 \pm 0.09 \times 10^{-15} \text{ m}^4/\text{Ns}$, [233] and the AF has a permeability of $0.23 \pm 0.19 \times 10^{-15} \text{ m}^4/\text{Ns}$ [206]. The permeability of the CEP ranges between 1.27×10^{-16} and $1.66 \times 10^{-14} \text{ m}^4/\text{Ns}$ [47, 48, 182], depending on the CEP location in the IVD, the animal model, and the region of the sample within a CEP. Rodriguez et al. also reported a permeability of $1.19 \times 10^{-10} \text{ m}^4/\text{Ns}$ in human CEPs, and explained the significantly lower measured permeability as due to inhomogeneities and focal cartilage lesions common in degenerated human samples [204]. Despite the considerable variation in the reported CEP permeability, it is still at least an order of magnitude less than that of the BEP ($\sim 2.21 \times 10^{-9} \text{ m}^4/\text{N} - \text{s}$) [47, 91, 204, 242]. However, a more accurate representation would consider the CEP permeability as a gradient which exponentially increases from the NP towards the BEP according to proteoglycan content [48]. Early studies using disulfine blue dye also demonstrated higher permeability across the CEP at the center than across the lateral regions [169]. However, despite this, the central region of the NP experiences low nutrition and high lactate concentrations due to the large diffusion distances across discs, particularly in the lumbar region [205]. Further, there are differences in permeabilities in the CEPs of the same IVD, in which the cranial CEP is significantly more permeable than the caudal one [164] possibly due to the differences in loading experience.

Effects of biochemical composition on CEP mechanics and permeability

The biochemical composition is fundamental in determining the material properties of the CEP, and therefore the permeability and response to mechanical loading [189, 241]. It has been proposed that there is an optimal range of biochemical composition that balances both the biomechanical and nutritional demands of the CEP [189]. To this extent, the CEP must be

stiff enough to hold the disc together but porous enough to allow for solute transport. Therefore, the CEP tensile properties have been found to be inversely related to the transport properties [206]. Additionally, within the bovine CEP, the biochemical composition, and therefore the biomechanical properties, were found to vary in the central region located next to the NP, compared to the lateral CEP, which is stiffer and thus could withstand a more significant portion of loading [241].

The tensile stresses within the CEP occur through Poisson's effects when the CEP is pressed against the BEP by the NP pressure, and/or through direct peripheral pulling by the inner AF fibers that blend with the CEP. At the inner AF and NP, the collagen is not as highly oriented as the outer AF, and therefore does not exert high tensile forces on the CEP [158], although the central CEP will experience transverse shear and some tensile stress when the NP bulges laterally during compression [225]. The tensile modulus of the CEP was found to be 5.9 ± 5.7 MPa, ranging from 0.5–21.8 MPa [189]. In comparison, the tensile modulus of AC ranges from 1–30 MPa [207], NP from 1–1.66 MPa and AF from 2.56–12.29 [208]. The tensile modulus of the CEP positively correlates to the collagen content; however, water and GAG content have been shown to have minimal effects [189]. In contrast, water content is expected to play a more prominent role than GAG content in the extrinsic viscoelastic, or poro-elastic, properties, i.e., aggregate modulus and hydraulic permeability, and diffusion of the CEP, alongside other cartilage tissues including the NP, AF, and AC [164, 229, 241]. Yet, GAG content is considered more important in osmotic properties, rather than elastic properties in the CEP [164]. In particular, multi-physics models showed that this characteristic might provide the GAG with an important role in the effective control of the fluxes of fluid between the BEP and the NP, by the CEP [48]. Interestingly, GAG quantity has been shown to not correlate with water content [164], suggesting that the type and quality of the GAGs are more important than the quantity.

CEP transport properties depend on the porosity and collagen, GAG, and water content of the CEP matrix [91, 161], while solute transport into and out of the IVD depends on solute size [97], shape, weight [161], and charge (Figure 2.2.2). For small molecules, net charge is the determining factor of diffusivity through the CEP [160]. The charge of the molecules are important due to the Donnan osmosis effect, in which small positive ions from the interstitial fluid migrate into proteoglycan-rich tissues [243]. Water enters the tissue to equilibrate the chemical potential, and the tissue swells as much as allowed by the collagen network and surrounding tissue

constraints. Thus, electrical charge of small particles impacts the diffusivity [244, 245]. Further, multiphysics models suggest that proteoglycan content has a greater effect than collagen content on the macroscopic hydraulic permeability of the CEP [48]. Nevertheless, when biopsies or cores of CEP are used it is challenging to control potential GAG loss and tissue swelling in altered osmotic environments *in vitro* compared to those seen *in vivo*. Thus, future studies where GAG release into media is prevented and osmotic pressure is controlled are essential to further the understanding of CEP permeability [246].

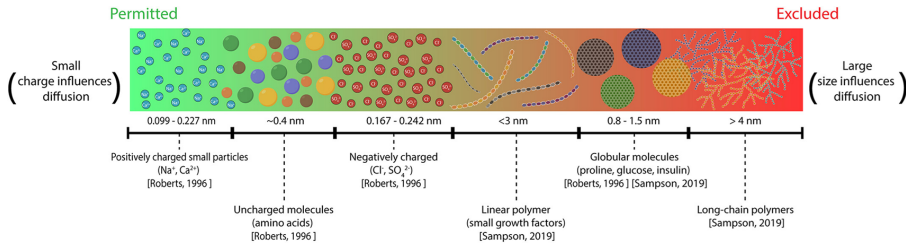


Figure 2.2.2: Healthy CEP diffusivity to different types of solutes, governed primarily by charge for small molecules and by size and shape for larger molecules.

Conversely, for large electrically uncharged molecules, size is the determining factor of diffusivity as the space between GAG chains of aggrecan is only ~ 3 – 4 nm [161]. Due to their ability to bend, linear polymers have a relatively high diffusivity compared to spherical molecules of the same molecular weight [47]. However, long-chain polymers have a relatively low diffusivity compared to globular molecules of the same, or even higher, molecular weight. For example, starch, a globular 10 kD molecule, diffuses more than polyethylene glycol (PEG), a long-chain 4 kD polymer [161].

Additionally, dynamic loading influences convective solute transport of large solutes, particularly in less porous CEPs [47, 229, 247, 248]. However, dynamic compression has been shown to have a minimal effect on small molecule, such as glucose or lactate, transport as this acts primarily through diffusion [47, 246]. In contrast, static compression can decrease diffusivity and inhibit nutrient transport because the tissue gets compacted, and thus porosity decreases [229, 238].

In silico investigation of CEP multiphysics

In silico simulations, such as finite element modeling (FEM) and computational fluid dynamics (CFD) analyses, have been widely used to determine the multiphysics mechanisms involved in biological tissues such as the ones of the IVD, in response to external mechanical loads. Models are useful in understanding the particularity of CEP mechanics. However, despite its importance, the CEP is often disregarded in *in silico* simulations, simplified to a boundary condition of the IVD [249], or given homogenized properties [250]. Since experimental data on the CEP is limited, equations and material properties determined for the IVD or AC are often used to represent the CEP [48]. Osmo-poroelastic models have been demonstrated to be generic enough to use for cartilaginous tissues made up of proteoglycans and collagen fibers, however, CEP-specific data should be used when possible.

Nevertheless, models have given insight into how the IVD gains water during 8 hours of rest at night much faster than it loses water during 16 hours of activity during the day. The compression of the CEP against the BEP is considered to close the porosity of the CEP and limit water out-flow when the IVD is compressed, while opening the porosity and favoring fluid in-flow into the IVD when unloaded [251]. Coined as the “intervertebral disc valve theory”, this mechanism might explain functional anisotropic, or direction-dependent, flow resistance mechanism in the CEP [252] and has been backed up by experimental measurements [252] and independent permeability measurements [182]. Further, *in silico* simulations used composition-dependent CEP modeling and incorporated cranio-caudal gradients of compositions measured in a healthy human disc to support the theory [52]. These simulations found that CEP porosity changes additionally induced by the composition gradients reinforced the resistance to the mass flow of water that reaches the BEP when the NP is pressurized [48]. However, there are contradictory findings on whether the favored direction of flow at the CEP is in-flow or out-flow [246, 253].

2.3 Importance of CEP in IVD degeneration

The integrity of the CEP and BEPs are key to the homeostasis of the motion segment as they form an interface to exchange nutrients and metabolites from the IVD to the external circulation and play an essential role in the mechanical stability of the motion segment. Thus, pathological processes

that occur to the CEP and BEP can alter the mechanical and nutritional environment of the IVD, triggering degeneration. Although most studies have focused on changes to the BEP with degeneration, which are visible on MRI images, these are likely associated with CEP changes as well. Indeed, cells derived from CEP adjacent to degenerated discs have very similar properties (morphology, immune phenotyping, proliferation and gene expression) to bone marrow mesenchymal cells from the same patients [174]. BEP defects have been associated strongly and independently with IVD degeneration [254], where they have been hypothesized to be an initiating factor to degeneration of the disc [215, 255–257].

2.3.1 Imaging endplate defects

BEP defects include several key recognizable features, which are normally identified on MRIs (Figure 2.3.1). CT has been used to identify the presence of endplate sclerosis in Modic change (MC) but is not usually suitable for clinical diagnosis or epidemiological studies [258]. Generally, the term “endplate changes” in literature describing clinical T1- and T2-weighted MRI features refers to changes seen in the bone marrow adjacent to the CEP, typically referred to as MC. Atypical changes that affect the endplate which are detectable from MRIs can be classified into three categories: focal, corner and erosive [259]. Focal changes, in which Schmorl’s nodes are included, are defined as local hollow regions on the endplate with NP protrusion into the subchondral bone; whilst corner defects are changes in anterior or posterior end of the BEP with the compromise of the vertebral trabeculae. Finally erosive defects are characterized by an irregular extensive alteration of the endplate on T2-weighted images [259]. However, some features, such as endplate changes in the upper lumbar spine may have a developmental rather than degenerative origin [260]. Moreover, in a large population-based study BEP damage was strictly associated with MC, rather than other endplate defects [261].

MC, although by definition connected to the CEP, are at best an indirect reflection of CEP status and do not correlate well with histology [262, 263]. The CEP itself is not clearly visible with conventional MRI sequences. Cartilage has short T2 values, so the CEP signal is not detected or is very hypointense. Additionally, its size ranges from 0.1–1.6 mm [158], which is close to the typical pixel size of a sagittal T2-weighted MRI of 0.5 mm² [52]. This poses a significant challenge for identifying the role, if any, of the CEP in low back pain (LBP) through imaging [264]. In contrast, AC is

distinguishable from adjacent soft tissue and bone [186]. In healthy discs, the position of the CEP can be inferred as a line of hypo-intensity between the hyper-intense NP and the VB on T2-weighted MRI but it is impossible to distinguish this from the BEP (see figure in Law et al. (2013) [183]). However, this feature is obscured when the AF is hypointense, which is characteristic of the early stages of degeneration.

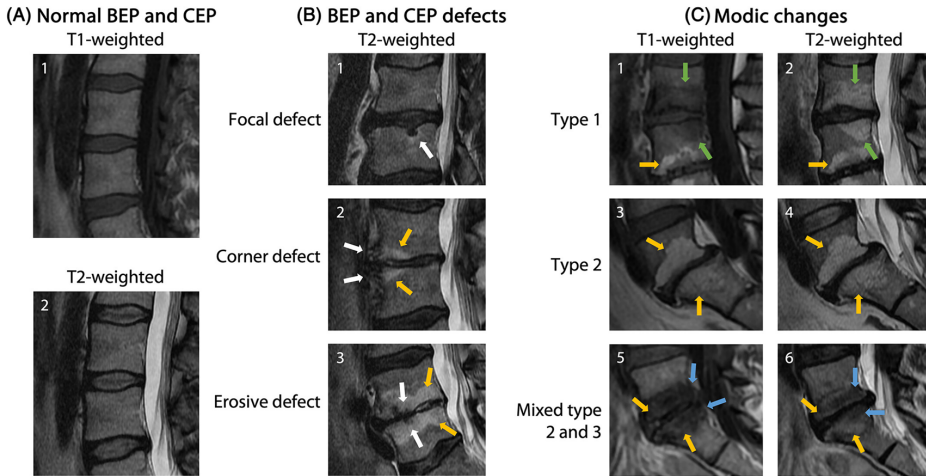


Figure 2.3.1: Examples of CEP and BEP appearance on standard-of-care MRI from 47-year-old participants in the Northern Finland Birth Cohort 1966. (A.1–A.2) Characteristic appearance of healthy CEP and BEP on T1- and T2-weighted MRI. (B.1) Schmorl’s node as an example of a focal defect at the L3–L4 IVD caudal BEP (white arrow). (B.2) Corner defects at the anterior edges of the L4–L5 IVD cranial and caudal BEP (white arrows) with accompanying type 2 MC (orange arrows). (B.3) Erosive defects at the L4–L5 IVD cranial and caudal BEP (white arrows) with accompanying type 2 MC (orange arrows). (C.1–C.2) Type 1 MC extending from the L3–L4 IVD cranial and caudal BEP (green arrows) with type 2 MC also visible in the L4 vertebral body (orange arrows). (C.3–C.4) Type 2 MC extending from the L5–S1 IVD cranial and caudal BEP (orange arrows). (C.5–C.6) Mixed type 2 and type 3 MC (orange and blue arrows, respectively) extending from the L5–S1 IVD cranial and caudal BEP.

The terms “Schmorl’s nodes” and “endplate damage/disruption” are often used to describe specific MRI features at the boundary between the IVD and the VB seen on MRI, making them more specific to the CEP. However, the multiple proposed classification schemes for these imaging features reflect a poorly defined phenotype [214]. Considering the limitations of T1- and T2-weighted sequences and CT for analyzing CEP features, alternative quantitative MRI approaches have been implemented [265]. UTE MRI is the most widely reported as an effective means of visualizing the CEP and can show *in vivo* the delineation of the BEP and CEP [183, 184].

Outside of the clinical setting, both CT and MRI are widely used in *ex vivo* animal or human studies. For example, MRI has been used in *ex vivo* human cadaveric spine segments to characterize the structure of the CEP in detail [266]. micro-computed tomography (μ CT) has also been used in such settings to confirm compositional characteristics of the CEP seen in MRI such as sclerosis [267], or for detailed morphological and biochemical characterization using contrast enhanced μ CT [27]. These *ex vivo* imaging approaches are needed to better understand the role of early CEP changes in the pathogenesis of IVD degeneration, while clinically applicable sequences and accompanying analyses capable of detecting early CEP changes are needed for identifying at-risk patients and early intervention targets.

2.3.2 Significance in LBP

The evidence for innervation of the vertebral bone marrow extending to the endosteal surface gives a biological basis for the endplate as a source of nociception in vertebrogenic pain [268]. Areas of vertebral endplate damage may trigger neoinnervation [268–270], with concomitant MC also shown to be innervated [268, 271–273]. While these sensory fibers generally terminate in or near the endosteal surface of the BEP, the CEP has also been shown to have small vascular spaces containing nerve fibers in some cases [269] and the CEP specifically can be accessed by nerves and blood vessels in the case of damage [274].

Endplate sensory fibers may be activated mechanically and chemically in the case of endplate damage. Disc/vertebra crosstalk as a consequence of the breakdown of the barrier provided by the CEP can contribute to nerve irritation with exposure to pro-inflammatory and neurogenic factors and the by-products of NP anaerobic metabolism such as lactic acid [172]. Additionally, damage to the endplate alters the distribution of IVD stress and the response to spinal loading [172, 222], contributing to local mechanical nerve activation. Associated changes to the paraspinal muscle quality may further interfere with segmental biomechanics and play an aggravating role in endplate nociception [275].

Although established as a plausible source of nociception, as with other spine image phenotypes, the observed relationship between visible defects of the vertebral endplate and the experience of pain is unclear. Multiple grading schemes for qualitative grading have been put forward [276, 277], but no consensus exists for endplate MRI image phenotype nomenclature [214],

which makes it more challenging to interpret and aggregate pain association study results. There is also a limitation in the ability to specifically detect CEP changes using clinical diagnostic tools (e.g., standard T1- and T2-weighted MRI sequences), and these cannot distinguish changes specifically associated with neoinnervation. Alternative sequences such as UTE [265] are not widely used in the clinical setting, and few methods for quantitative image analysis of the endplate have been tested [264, 278].

2.3.3 MC: definition, prevalence, natural course, and pain association

MC are MRI signal intensity changes of the vertebral bone marrow around a degenerated IVD [256, 279] and independently associate with chronic LBP [280–284]. A meta-analysis showed that MC prevalence in LBP patients is about seven times higher than in the non-clinical population (43% versus 6%). MC occur predominantly in the lower lumbar spine [285]. There are three interconvertible types of MC depending on their appearance on T1- and T2-weighted MRI. Modic type 1 changes (MC1) (Figure 2.3.1) are hypointense on T1-weighted images and hyperintense on T2-weighted images and represent oedema, fibrovascular granulation tissue, infiltration of immune cells and expansion of pro-fibrotic stromal cells [279, 286–288]. Modic type 2 changes (MC2) are hyperintense on T1- and T2-weighted images and represent fatty marrow conversion with presence of fibrotic tissue [279, 288]. Modic type 3 changes (MC3) are hypointense on T1-weighted and T2-weighted images and are sclerotic changes [279, 288]. The reported prevalence for MC1, MC2, and mixed type (MC1/2, MC2/3) are highly variable. Median prevalence is around 15–20% for MC1, 25–65% for MC2, and < 5% for MC3 [281, 289]. Mixed type MC1/2 are also frequent (15–20%), whereas mixed type MC2/3 are rare. The prevalence generally increases with age and peaks in the 60s [290, 291]. MC can inter-convert over time [279, 292–295]. MC1 are the least stable, where within four years, most MC1 either convert to MC2, increase in size, or resolve. MC2 are more stable, while MC3 are a terminal stage. Smaller MC lesions are more likely to resolve than larger lesions [292].

The association of chronic LBP with MC has been extensively studied and reviewed [296]. About half of the studies report a significant association with an odds ratio (OR) ranging from 1.53 (95% confidence interval (CI) 1.02–2.29) to 83.10 (95% CI 4.85–1424) Only one study reported a significant negative association with MC2 with an OR of –3.2 (CI –5.39–0.1). The

association of MC with discography concordant pain has a specificity of > 94% in 5 of 6 studies with an OR of 4.01 (1.52–10.61) in a meta-analysis [172, 296]. Larger lesions had a stronger association with discography-concordant pain [297]. Overall, these data show an association of MC with LBP, in particular of MC1.

Increased innervation of the CEP in MC1 and MC2 is believed to cause increased pain sensitization at MC levels and is often referred to as verte-brogenic LBP [268, 272]. Low back vertebral endplate pain (DM54.51) has recently been added to the International Classification of Diseases (ICD-11) as a subclassification of patients with LBP and MC.

2.3.4 Association of BEP changes with CEP changes

Despite the importance of CEP and BEP in the onset of spinal pathologies, the relationship between CEP and BEP is poorly understood due to the difficulty of evaluating the CEP with imaging techniques. A study on cadaveric lumbar spines using UTE MRI was used to enable investigation of CEP morphology within IVDs with BEP lesions, demonstrating abnormalities of the CEP were statistically associated with BEP lesions [298]. After needle induction of IVD degeneration in a rabbit animal model, the CEP progressively thickened and showed increased collagen accumulation [299]. Along with changes in cartilaginous tissue, the bone interface was modified, specifically an increase in bone volume fraction. These findings suggest that CEP could have a role in the development of BEP lesions or vice versa. It has been shown that poor CEP composition can affect disc health with and without defects in the VB [300]. However, a precise understanding of the sequelae of changes in the CEP and BEP is still missing. There is comparably little data available about the integrity and damage of the CEP in MC. Fields et al. (2014) [268] showed that in cadaveric human spines, CEP damage is associated with histological changes consistent with MC. Still, not all specimens with histopathological changes had MC on MRI. In another human cadaveric study, Heggli et al. (2022) [273] showed that CEP and BEP damage are strongly associated with MC2. Supporting evidence for CEP damage in MC stems from studies assessing CEP fragments in surgically removed herniated disc tissue at the MC level. CEP fragments can co-herniate with disc tissue in cases of avulsion-type herniations, where the CEP is torn from the BEP. These CEP avulsions were found to associate with MC [301, 302].

2.3.5 Mechanisms of CEP damage in MC

While damage to the BEP is believed to be caused mainly through mechanical cues, mechanisms of CEP damage are poorly understood [303]. In MC, local biological reactions seem to contribute to CEP damage. In an animal model of MC1, immune reactions in the MC1 bone marrow caused damage to the adjacent endplate. This is noteworthy because it demonstrates that MC1 are not just reactive changes to disc degeneration, but that MC1 themselves can cause CEP damage and maintain the cross-talk of the bone marrow with the adjacent disc [304]. Recent studies confirm the possibility that activated immune cells in MC1 bone marrow can lead to CEP damage [305]. Increased lactate dehydrogenase activity and increased concentration of C-reactive protein and of complement factors in MC bone marrow indicate also a humoral immune response related to local tissue damage [273, 306]. On a cellular level, CEP cells at MC levels express more tumor necrosis factor (TNF), a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS-5), macrophage migration inhibitory factor (MIF), and its receptor CD74 [272, 307, 308]. TNF upregulates MIF in CEP cells, and MIF upregulates the secretion of pro-inflammatory cytokines by CEP cells, through an autocrine mechanism involving CD74. The existence of this positive inflammatory feedback loop suggests that the CEP has the capability to escalate inflammation in MC independently from the disc and the bone marrow. Together, these data evidence that CEP damage in MC is not a pure mechanical mechanism but that the local inflammatory processes in the bone marrow and of the CEP itself can lead to progressive CEP damage.

Occult disc infection, mainly with the *Cutibacterium acnes* (Gilchrist 1900) and other coagulase-negative staphylococci are discussed as a potential etiology of MC1, at least in a subset of patients [291, 309]. This is based on reports in which disc tissue from microdiscectomy of herniated discs was analyzed for the presence of *C. acnes* [291, 310–312]. *C. acnes* has been isolated from discs adjacent to MC1 and the presence of this bacteria was predictive for the development of new MC1 after one year. *C. acnes* is assumed to migrate to structurally damaged discs through hematogenous spread from a distant infection or from the skin and other epithelial surfaces through the blood after innocuous lesions, e.g., tooth brushing [313, 314]. Disc herniation and endplate damage, both structural damages present in MC, can represent disc damage which allow bacteria to enter discs. Once in the disc, the low oxygen tension and low pH in the disc favor the proliferation of *C. acnes*. Furthermore, *C. acnes* is unlikely to colonize the MC1 bone

marrow because of too high oxygen tension in the bone marrow. Rat and rabbit models have demonstrated the biological plausibility that *C. acnes* injected into disc can trigger MC1-like changes [315–317]. For example, injecting a *C. acnes* strain, which had been isolated from a human MC1 disc triggered hallmarks of MC1 within two weeks after injection, (i.e. MC1-like MRI changes in the bone marrow, disc degeneration, fibrotic-inflammatory bone marrow changes, and almost complete resorption of the CEP) [317].

The exact mechanism of how intradiscal *C. acnes* causes CEP resorption is still unclear. It has been shown that disc cells respond to *C. acnes* with the release of pro-inflammatory and neurotrophic factors through a toll-like receptor 2 (TLR2)-dependent pathway [317–320]. Stimulation of TLR2 on disc cells also upregulates matrix proteases that can degrade the disc and CEP matrix. Additionally, *C. acnes* secrete different virulence factors such as proteinases, hyaluronidases, lipases, and neuraminidases [321, 322]. Proteinases and hyaluronidases can directly degrade ECM components of the CEP. Lipases hydrolyze triacylglycerides into glycerol and free fatty acids. Free fatty acids, in particular saturated free fatty acids, are highly pro-inflammatory by signaling through toll-like receptor 4 (TLR4) [323, 324]. Neuraminidases disrupt the ternary complex of toll-like receptors (TLRs) with the membrane components CD24 and SiglecG/10 and abolish the inhibition of TLR signaling by SiglecG/10 [325]. Together, *C. acnes* has the capacity to cause CEP damage, yet the precise mechanism remains unclear.

2.3.6 Role of CEP damage in MC

Damage to the CEP compromises its function as a sieve for cells and macromolecules. In MC, where endplate damages are present, the degenerating disc can cross-talk with the adjacent bone marrow [304]. Pro-inflammatory and pro-osteoclastic cytokines that are produced at higher rates from MC discs can more easily escape into the adjacent bone marrow [303, 304, 326]. Marrow-sided leukocytes can in turn aggravate degenerative changes in the disc, even without infiltrating the disc itself [303]. Consequently, CEP damage in MC facilitates an inflammatory cross-talk between the disc and the bone marrow that contributes to the rapid degeneration of MC segments [256]. Furthermore, increased concentrations of cytokines and chemokines produced during disc degeneration [70, 327] could more easily diffuse out of the IVD into the adjacent bone marrow leading to chemotaxis gradients and activation of immune cells.

2.3.7 Microscopic changes to the CEP

During IVD degeneration, key structural and cellular changes occur within all areas of the IVD, with key features identified within the NP, AF, CEP and BEP (Figure 2.2.1). While macroscopic structural changes can be identified by imaging techniques such as MRI, these often fail to identify changes to the CEP. Furthermore, cellular and fine ECM changes require microscopic examination to be identified. (Figure 2.3.2) During the development of the recently published standardized histopathology scoring system for human IVD degeneration [3], each of the regions (NP, AF, CEP and BEP) were scored independently with equal weighting, demonstrating the recognition of the critical role of the CEP and BEP in IVD degeneration [3]. Within the CEP the degenerative features identified histologically included scoring for cellularity, lesions and ECM structure [3]. Cellularity changes seen during degeneration included: abnormal cellular clusters, empty lacunae, extensive neovascularization and presence of apoptotic, necrotic and senescent cells [3]. Changes within ECM can be identified histologically, and include loss of endplate thickness; avulsions from BEP; cracks and fissures; loss of normal matrix staining; fibrosis and calcification [3]. These visible microscopic changes within the CEP are due to alterations in the biomechanical and cellular regulation of the CEP.

Additionally, through histological analysis Huang et al. (2021) [5] identified physical microdamage in 40% of degenerated CEPs. Further, pain and disability scores were significantly higher in these micro-damaged CEPs than in degenerated IVDs without damaged CEPs. Six main patterns of micro-damage were used to classify the CEPs, including fissures, vascular mimicry, NP herniation into the CEP, NP herniation and incorporation of bone tissue into the CEP, incorporation of bone tissue into the CEP, and traumatic nodes [5].

Another methodology used to categorize the endplate is the total endplate score (TEPS) [328]. MRI scans are used to quantify the damage, which was given a score based on diffusion patterns and the presence of endplate breaks or defects, focal thinning, MC, and irregularities or sclerosis. Five visually distinct diffusion patterns were identified through the study as well, that reflected disc marrow contact, focal leakage into the subchondral bone or NP, and pooling of liquid. These diffusion patterns correlated with degeneration level as well as the TEPS. As the TEPS increased, the diffusion pattern increased towards pooling. The TEPS was shown to correlate with Pfirrmann's grading regardless of the age and levels of disc. Unfortunately,

there was no distinction between CEP and BEP in that study.

2.3.8 Biomechanical regulation of CEP degeneration

There are two main theories about the contribution of mechanical loading to IVD degeneration; the overload theory, which states that excessive mechanical loading damages the IVD over time, and the immobilization theory, which states that low mobility causes the IVD to adapt and leads to tissue weakness and degeneration [239]. There is evidence that both overloading and immobilization contribute to IVD degeneration, and it is considered that there is a range of mechanical loading in which the disc remains healthy. Outside of this range, the metabolism shifts to catabolism.

The CEP is essential in maintaining the mechanics of the disc. Damage to the CEP barrier alters the hydration and could allow water to escape from the NP under loading, leading to NP decompression and degeneration [158]. FEM simulations of the disc, performed in the 90s, suggested that initial failure always occurred in the endplates, demonstrating their vulnerability within the disc under loading [329]. Nevertheless, experimental testing of disc failure show that although most failures happen at the CEP–bone interface or disc–CEP interface, some primary failures also occur within the subchondral bone [203], which was not predicted by such early FEM models [329]. Additionally, endplate herniation through the CEP is a key feature seen in patients and is the most common type of herniation [330, 331]. However, after endplate damage, additional failures could propagate to the annulus or lead to delamination [329]. This highlights the importance of CEP integrity; preventing CEP damage early on could also avert damage to the rest of the disc and prevent disc degeneration.

Cyclic compression has been shown to lead to the development of microstructural voids at the CEP–BEP border [332]. Microdamage accumulation occurred more often in flexed-joint postures in comparison to neutral postures. Following the damage, there was also a decrease in type I and II collagen content. Thus, overuse injuries could occur in the CEP and lead to altered biochemical content.

The IVD is most vulnerable to failure under bending, in which stretching puts the disc under tension [158, 203]. Studies have found that in cadaveric thoracic IVD samples under tension, 71% of primary failures occur at the CEP–BEP interface [203]. The next most common failure was at the IVD–

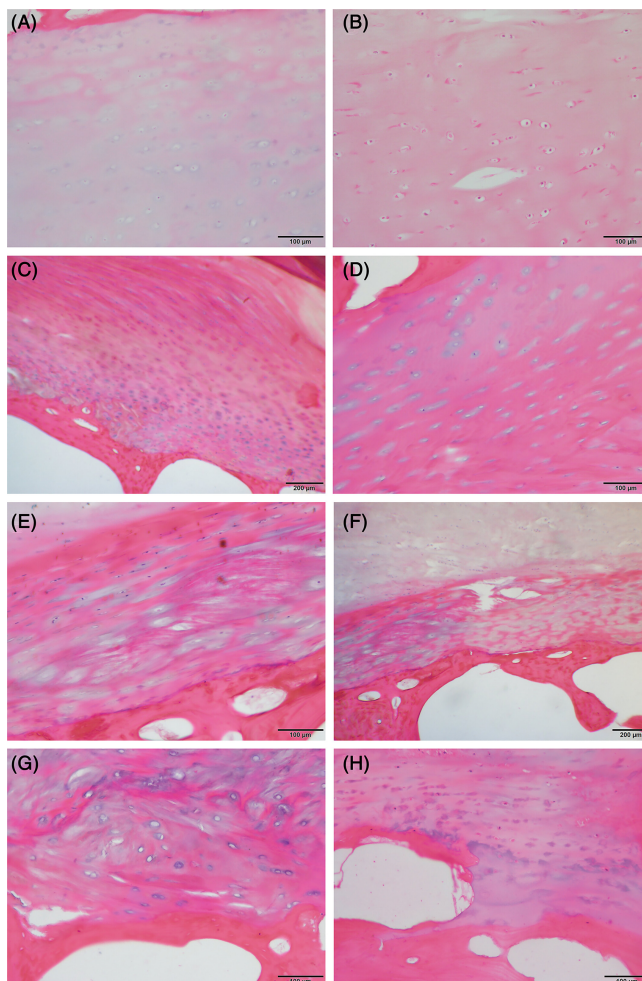


Figure 2.3.2: Hematoxylin and eosin staining of human CEPs demonstrating key histological features of non-degenerate and degenerate CEP. (A) Non-degenerate CEP with the BEP in the upper left of the image and the CEP within the NP region. (B) CEP within the NP region demonstrating excellent maintenance of eosin staining. (C) CEP–AF enthesis within a non-degenerate region, showing the BEP at the bottom left and the CEP in the middle connecting into the AF. (D) CEP within the AF region demonstrating a change in matrix organization at the bottom of the image. (E) Abnormal CEP demonstrating clear disorganization and fibrosis. (F) Abnormal CEP showing fissures and disorganization of the CEP. (G) Abnormal CEP showing disorganization of the extracellular matrix. (H) Abnormal BEP–CEP enthesis with bony avulsion. Scale bars: 100 μm in (A), (B), (D), (E), (G), and (H); 200 μm in (C) and (F).

CEP interface (21%), while the rest failed within the subchondral bone. When pulled in tension from the vertebral bone on each side, the tensile failure strength of the CEP–BEP interface is 0.4 MPa [203, 225], while failure strain, calculated from force–displacement data normalized to the initial specimen height, was found to be $38.5 \pm 20.3\%$ with the highest principal strains occurring in the mid-AF [203]. The rate of loading also affects the failure; slower rates cause disc pressurization with localized stretching and failure in the AF, while under rapid loading the AF does not have time to stretch and thus pulls the CEP from the bone [333]. Further, the position of the disc affects the failure location; neutral discs, which would be seen during normal standing, tear at the CEP–inner AF interface, while flexed discs, which would be seen during bending movements, fail at the outer AF–endplate interface or in the BEP [333, 334].

The thickness, porosity, and curvature of the CEP also influence the biomechanics [203, 225]. Further, Thompson Grade and bone density have been correlated with failure strength [203]. Additionally, the bone volume fraction, expressed as bone volume (BV)/total volume (TV), has been shown to positively correlate to the failure strength, with stress increased at the higher BV/TV end of the VB [203, 222, 335]. In humans, the cranial endplates have a lower BV/TV than caudal endplates, and therefore they tend to fail before the caudal endplate fail [222]. The microarchitectural features of endplate concavity are also significant predictors of failure strength [335]. Specifically, when the concavity of the CEP is wider, more voluminous, and less steep, it is capable of tolerating higher loads before failure [335]. Overall, CEPs are stronger when they are thicker and denser with a higher concave curvature that allows more space for the NP [203, 225, 335, 336]. Thickness has even been suggested to be used as a clinical risk measure for avulsion-type herniation [203]. It should be noted that quadrupeds have a different curvature than humans, as well as additional material properties such as BV/TV, and therefore it is essential to consider the animal model used during mechanical tests [335, 336].

2.3.9 Animal models of the CEP

As human CEP samples are not always available, it is important to consider animal models which can be used to elucidate the properties and functions of the CEP. Interestingly, rats and mice have only a CEP with no BEP [212]. Rabbits and goats have a very thin CEP (1–3 cell layers) with a larger BEP. Larger animals such as dogs, cows, and sheep have both; however, the CEPs

are thinner and the BEPs thicker than those of humans [78]. Bovine and canine CEPs have been shown to have similar biochemical content to human CEPs, although canine CEPs have been shown to have significantly more sulfated GAG than those of humans [78]. Further, bovine CEPs have been shown to have more proteoglycans in the outer AF–EP region in comparison to the NP–EP region similar to the human CEP, while canine CEPs show the opposite pattern. Bovine CEP cells are rounded and organized in stacked columns, in contrast to canine cells which have no organization and human CEP cells which are along the collagen fibers parallel to the disc. Thus, the molecular similarities of the bovine and human CEPs make the bovine a more suitable model for investigating mechanics and transport in the CEP [78]. Additionally, baboon CEPs have been demonstrated to have similar biochemical content, including GAG, water and collagen, as those of humans, and thus could also be a good model to study mechanics with the CEP [200]. Rabbits have also been validated as a model to investigate initial endplate failure, although rabbit endplates have a higher BV/TV and a steeper, narrower concavity which should be considered when translating results to humans [335]. Particular care should be taken with diffusivity studies, taking into account the difference in thickness and CEP:BEP ratio compared to the human endplate [78]. Overall, no single animal model provides a complete representation of the human CEP and caution should be taken when extrapolating data [212].

2.3.10 Cellular regulation of CEP degeneration

With increasing degeneration of the CEP, its composition undergoes several changes that could reduce its permeability and limit nutrient transport. In tissue adjacent to degenerated discs, the calcium concentration was shown to be higher. There is a delicate balance between the nutrient demand and the nutrient supply, which is imposed by the cell population and controlled by the permeability of the CEP [91, 205]. The classical paradigm is that any reduction in CEP permeability leads to nutrient retention and accumulation of lactate that in turn decreases the pH of the disc. A decrease in pH is directly related to hydrogen ion concentration. CEP permeability reduction or loss would impair disc oxygenation [169], and subsequently, reduce cell survivability and activity, being possibly a major regulator of IVD cell populations [91].

Increased levels of calcium showed to enhance the cleavage of aggrecan by ADAMTS-5 [337]. Not only was a decrease in aggrecan observed, but also

a change in its composition changing from a 1:1 ratio of keratan sulfate to chondroitin sulfate to a 3:1 ratio [234]. Those compositional changes result in a decrease in the net hydrophilic property of the tissue. Furthermore, a positive correlation between the degenerative state of the tissue and increased denaturation of type II collagen has been shown [338]. Moreover, a mouse spondylosis model demonstrated that with increased age, apoptosis of chondrocytes in the CEP lead to a markable decrease in cell density. Subsequently, the disappearance of the CEP structure [339]. This was corroborated in human CEP samples. In addition to decreased cell density and a higher rate of MC in degenerated CEP, expression of matrix metalloproteinase 3 (MMP3), matrix metalloproteinase 9 (MMP9), interleukin-1 alpha (IL-1 α) and interleukin-1 beta (IL-1 β) was increased [340]. In addition, tissue inhibitor of metalloproteinases 3 (TIMP3) also showed increased expression in degenerated CEP, suggesting a compensatory mechanism to regulate the increased a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) expression. Interestingly, tissue inhibitor of metalloproteinases 1 (TIMP1) and tissue inhibitor of metalloproteinases 2 (TIMP2), but not TIMP3, were overexpressed in degenerated AF and NP compared to non-degenerate tissues [341]. CEP chondrocytes have also been demonstrated to have a different response than AC chondrocytes to the same stimuli [342]. In response to hypertrophic stimuli such as Wnt agonist, CEP chondrocytes did not undergo the morphological changes seen in AC chondrocytes. However, they did show hypertrophic gene and protein expression and a decrease in proteoglycans. Oxidative damage-induced stress was also shown to induce apoptosis and promote calcification in the human CEP [343]. Neidlinger-Wilke et al. (2014) [344] showed in an *in vitro* model stimulating NP cells with conditioned media of CEPs a significant increase in interleukin-6 (IL-6), interleukin-8 (IL-8) and MMP3, as well as matrix metalloproteinase 13 (MMP13). Aggrecan and type II collagen were significantly decreased in NP cells exposed to the CEP-conditioned media [344]. Those findings indicate the interactions between the CEP and the NP tissue via molecular factors influencing the pathophysiology of disc degeneration. Limited studies have been performed on the expression of cytokines within the isolated CEP. In addition, there is strong evidence of genetic regulation of CEP cell fate through non-coding ribonucleic acid (RNA), which includes microRNA, short interference RNA and circular RNA. Proliferation, apoptosis, migration, and autophagy of CEP cells are the processes shown to be targeted by those RNAs and promoters of its degeneration [345–348]. There is also evidence for epigenetic roles in CEP degeneration regulation and in extension, IVD degeneration. Overexpression of the histone methyltransferase enhancer of zeste homologue 2 (EZH2)

in CEP cells produces reduced expression of *COL2*, *ACAN* and *SRY-box transcription factor 9 (SOX9)* genes and increased *ADAMTS-5* and *MMP13* genes in rat CEP cells [349].

Furthermore, reduced CEP permeability, and consequently reduced nutrition and lowered pH, can downregulate both catabolic and anabolic gene expression of the NP cells negatively affecting ECM homeostasis [91, 205]. Specifically, the messenger RNA (mRNA) expression for *ACAN*, *COL2A1*, and *MMP2* in the NP reduces [91]. Additionally, increased Ca^{2+} deposition leads to the activation of calcium-sensing receptor (CaSR), causing increased catabolism through the suppression of collagen and GAG synthesis and can induce calcification of CEP tissue through upregulation of alkaline phosphatase (ALP) [337]. Further, deposition of Ca^{2+} in the IVD has been associated with increased parathyroid hormone-related protein (PTHrP) signaling, which drives calcification [350].

Calcification and influence on permeability

Increased Ca^{2+} deposition in the CEP is seen with increased IVD degeneration in humans [337, 350]. Calcification of the CEP is associated with decreased nutrient transport into the disc and waste transport out of the disc, which leads to nutrient starvation disc cells and a decreased pH within the disc, respectively [52]. Further, calcification can lead to lower porosity, hydration, and permeability [47]. With lower porosity and hydration, diffusion is impaired, and thus, dynamic loading has a greater effect on nutrient transport [47, 246]. Static compression, however, reduces the CEP porosity and leads to reduced oxygen and greater lactate accumulation in the disc, limiting nutrient transport and gas exchange [238, 246]. Altered nutrient transportation through the CEP has thus been suggested to be a significant factor in the pathogenesis of IVD degeneration [351]. Preserving sufficient metabolite transport through the CEP is essential for the IVD to maintain its ECM and biochemical environment [204]. Degenerative changes in the CEP could lead to up to 70% decrease in CEP permeability and, ultimately cell death [164]. Overall, within calcified discs, dynamic compression improves disc nutrition while static compression impairs nutrition and leads to further degeneration. However, calcification is not always present in degenerated discs. When the CEP degenerates, GAG and collagen concentration decrease, which causes higher porosity and thus increased solute transport [189, 352]. Dynamic compression has been shown to have less effect on higher porosity CEPs [47, 205]. Nevertheless, the decreased matrix

content also decreases the tensile modulus of the CEP, losing its ability to withstand mechanical forces [189].

Degeneration has not been found to be correlated to GAG or water content separately, but rather to fixed charge density (FCD), which is a property related to both. Higher FCD hinders transport through creating steric and ionic barriers. It has been found to be directly proportional to IVD degeneration and inversely proportional to CEP permeability [164]. This agrees with findings that CEPs with low permeability have high levels of collagen, aggrecan, and minerals which can physically block the solutes [91]. Shirazi-Adl et al. (2010) [97] demonstrated that IVD cells start dying when CEP permeability decreases below 30% and the death rate increases exponentially as CEP permeability decreases further. The NP is the tissue most severely affected by CEP permeability changes [48]. While the NP periphery is adjacent to the CEP, its center can be as far as 8–10 mm in the IVD [91]. While there is also some diffusion through the outer AF, it is not enough to compensate for an impermeable CEP due to calcification [47].

While calcification and dehydration have been shown to reduce the permeability of the CEP [91, 161], the effect of calcification in the CEP has also become a topic of debate recently. On one side, there is the hypothesis that calcification prevents fluid from flowing into the IVD to transport nutrients. Opposingly, there is the hypothesis that fluid movement, and thus the effect of calcification, has a negligible effect on nutrient transport. Supporters of the first hypothesis claim that calcification leads to a reduction of the pore volumes creating a physical impermeable barrier that obscures the fluid path [161]. That, in turn, leads to reduced nutrient supply to the cells and, ultimately IVD degeneration [97, 161, 205]. Supporters of the latter hypothesis claim that advection, or the movement of fluid, through the CEP has minimal effect on nutrient supply since the nutrient concentration in the IVD is controlled by diffusion. Diffusion occurs regardless of fluid velocity [242], especially when mechanical loading is present [157]. Further, some studies claim that severe nutritional deprivation does not appear until calcification causes a 50% blockage [48]. This level of blockage only occurs at late stages of degeneration, which contrasts other hypotheses that the depletion of CEP ECM might promote early degenerative mechanisms in the IVD through local cell starvation in the NP [48]. The lack of blockage at early stages of IVD degeneration suggests that calcification as a physical barrier has minimal effect on IVD degeneration, however, cell catabolism induced by Ca^{2+} could induce IVD degeneration [204].

Nonetheless, there is evidence that there is a strong positive correlation between CEP porosity and hydration [47, 161]. Within extreme IVD degeneration, the CEP permeability barrier would be negligible due to the infiltration of blood vessels into the disc after damage. Therefore, both hypotheses could be correct depending on the age of the subject, degeneration level of the CEP, degree of calcification, and vascularization. This topic requires more intensive research to fully understand the effects of calcification on the IVD. Additionally, it is important to consider the poromechanical interactions among the BEP, the CEP and the NP to understand the mechanotransport of nutrients [48].

2.4 Emerging therapies

Nutritional supply controls the population and activity of the IVD cells to synthesize and maintain the ECM. Thus, for any disc cell therapy to succeed, maintenance of the nutrition supply is crucial. Furthermore, even a successful attempt to biologically repair the IVD can fail in the long run if the nutrition–population balance is not maintained.

Most therapies aim to surgically remove the source of LBP or re-establish IVD mechanical functions overlooking the sustainability of such operations, which is part of why they have a low success rate [91]. Intradiscal biological therapy is a non-invasive alternative that consists of injecting genes, growth factors, and other molecules to the IVD that aim to boost the cell population and produce ECM to restore a physiological environment [91]. Yet, increasing the cell number without increasing the nutrient supply is unsustainable because it will lead to the supply–demand disturbance, as previously mentioned [91]. A FEM analysis study demonstrated that cell injection could lead to increased and accelerated degeneration in the IVD due to a higher supply–demand disturbance that is directly related to the state of the CEP, i.e. whether it is healthy, calcified, or thinned [205]. Thus, the harsh nutrient environment of the IVD, particularly concerning the state of the CEP, must be accounted for to make any cell therapy beneficial. They suggested reducing the CEP thickness to enhance nutrition in the IVD, but this could compromise the mechanical stability of the IVD.

Treatments targeting the CEP are often focused on enhancing permeability. One possible method would be decalcification of the CEP through injecting compounds that can bind calcium [353]. Other methods suggested include

enzymatic treatment, such as trypsin or hyaluronidase, to remove large proteoglycans from the CEP [353]. However, chemical injection with enzymes is also used to induce degeneration in animal models [327]. Therefore, the use of enzymes for CEP therapies should be strictly restricted to the CEP. Excessive/untargeted protease activity as in antiquated chemonucleolysis can even trigger MC within six weeks [354]. Dolor et al. (2019) [352] treated the CEP with matrix metalloproteinase 8 (MMP8) to reduce the matrix and enhance solute uptake and nutrient diffusion. MMP8 is selective for type II collagen and aggrecan, which are the two main matrix components of the CEP. However, these are also the main components of the NP, and therefore it is crucial that using MMP8 or another enzyme does not induce degeneration within the NP or AF due to off-target digestion or matrix fragments triggering a catabolic response. One method Dolor et al. (2019) [352] considered to avoid this was using targeted delivery through injection and linking the enzyme to bulky nanoparticles that cannot migrate to other tissues. Nevertheless, this treatment was only performed in human cadaveric CEPs, so it is unknown whether a catabolic response will be produced testing *in vivo*.

Although therapies addressing the CEP are still preliminary, studies have shown that incorporating the CEP into a tissue engineered disc improves the performance [355]. While development of functional NP and AF replacements is important, these tissues must be integrated into the CEP to allow for successful and functional IVD replacement [353, 356]. Studies have shown that using direct contact co-culture of AF [356] or NP cell-seeded scaffolds [357] with chondrocyte-seeded scaffolds produced native interface characteristics. However, although type I collagen, type II collagen, and aggrecan distribution were like native tissue, the apparent mechanical strength was 57-times weaker than in native tissue segments, which means it would not function well under daily mechanical loads [355]. Obtaining comparable mechanical properties of a native disc has been a large problem in tissue engineered scaffolds [125, 358]. Gullbrand et al. (2018) [355] have tested endplate-modified disc-like angle-ply structures (eDAPS) as replacement discs in rat and goat animal models. In the eDAPS, the endplate was made up of acellular, porous poly(ϵ -caprolactone) (PCL) foams which was combined with the NP and AF components [355]. They showed that after 20 weeks with external fixation, native cells from neighboring tissues could migrate into the CEP structure and start producing matrix components and sparse vascularization [355], which is a focus area of CEP treatments [353]. However, it should be noted that while vascularization is important for the BEP and CEP, it can lead to increased degeneration and pain if angiogenesis

occurs in the NP [63]. Although Gullbrand et al. (2018) [355] observed improvements in tensile properties, the failure strain of the eDAPS was only 50% that of native values. However, it was shown that the constructs which included a CEP structure outperformed those without [359].

Bioprinting is also a popular technique for developing tissue engineered constructs but has the same issue of sub-optimal mechanical properties [125]. Printing using bioinks with reinforcement structures such as carbon fibers or alumina platelets has been considered for recreating load-bearing tissues such as the CEP, although low printing resolutions can limit the functionality [125]. Using decellularized ECM is another option for tissue engineered constructs, which addresses the problem of low printing resolutions, and could help with the design of three-dimensional (3D) printed scaffolds [358]. Nevertheless, mechanics of chemically modified decellularized ECM also have weak mechanical properties that do not approach the Young's modulus of native tissues [358]. Further, scalability and reproducibility alongside high manufacturing costs are limiting in the bio fabrication of IVD constructs [125].

Recently, Liu et al. (2011) [174] have identified the presence of progenitor cells in the CEP. After culturing in agarose, cells isolated from degenerated human CEPs were found to be positive for stem cell markers OCT-4, NANOG, and SOX-2 as well as common bone marrow mesenchymal stem cells (BM-MSCs) markers CD105, CD73, CD90, CD44, CD166 and Stro-1 [174]. Further, the group found that NP cells that were stimulated with CEP progenitor cells isolated from healthy subjects showed a decrease in apoptotic rate by releasing exosomes that activate the PI3K/AKT signaling pathway [360]. Thus, CEP progenitor cell-derived exosomes could be a possible therapeutic tool in the treatment of IVD degeneration.

Several studies have also found potential targets for the treatment of the CEP with regard to IVD degeneration, although additional research needs to be done before testing. For example, the HIF1A/MIF pathways has been shown to play a role in promoting chondrogenesis, while also inhibiting osteogenesis [55]. EZH2 inhibition has also shown promise as a therapeutic target to combat CEP degeneration through upregulation of SOX9 [349]. Managing oxidative stress and damage could be another novel therapy target. For this, research has found inhibiting reactive oxygen species (ROS) reduced apoptosis in CEP cells under oxidative stress [343] and, similarly, enhancing the Nrf/Keap1 pathway in CEP cells increases antioxidants that can combat damage from ROS [361]. Certain microRNAs (miRNAs), specifically miR-

495-3p [347] and miR-34a [345], have been found to play a role in ECM degradation and chondrocyte apoptosis, respectively. Therefore, silencing these miRNAs could be a novel treatment for CEP and IVD degeneration.

2.5 Conclusion

The CEP is a unique tissue distinct from other cartilaginous tissues in morphology, gene expression, and mechanical and transport properties. It is an essential component of the IVD and is considered to play a key role in the early stages of IVD degeneration due to its fundamental role in nutrient transport [48, 362]. However, most data regarding intervertebral disc degeneration (IVDD) have focused on the NP and AF tissues. Further, research that does include the CEP often does not distinguish it from the BEP. New MRI techniques [183, 184] as well as the standardization of histopathology scoring in the CEP [3] allow for the characterization of the CEP separate from the IVD or BEP, and thus future research should aim to investigate the CEP as an independent tissue type. In particular, the role of the CEP in MC should be investigated further as most research is limited to the BEP or the combined BEP and CEP.

Additionally, the CEP is often considered to be the same as AC particularly in modeling and simulations [48]. However, as detailed in this review, the CEP differs from AC in function, cellular response, biochemical content, and material properties [2, 164, 176, 187–189, 207, 342]. Thus, future research should aim to characterize the CEP itself, and avoid assumptions that the CEP will behave and/or respond the same as AC.

Much is still unknown about the mechanics and transport properties of the CEP, and reported values show a large variation. The wide range of reported values is due to various testing methods, environmental conditions, species, and degree of degeneration [47, 48, 182, 204, 230], and thus highlights the need for standardized, reproducible methods and guidelines for investigating the CEP. Likewise, the authors recommend to standardize specifying the terms cartilaginous endplate (CEP) and bony endplate (BEP) and advise avoiding the term vertebral endplate. Further, it is essential that researchers clearly state which tissue is being investigated, whether it is CEP, BEP, or a combination of both.

Calcification should also be a focus of future research, as there is contro-

versy regarding the role it plays in CEP permeability. It is accepted that calcification occurs in degeneration. However, it is unclear whether disc damage occurs at an early stage due to impaired nutrient transport induced by calcification, cellular level changes caused by excess Ca^{2+} in the environment [48, 91, 157, 161, 204, 242], and/or due to early depletion of the CEP ECM (possibly related with MC) disrupting the functional fluid exchange between the vertebrae and NP under mechanical loads [48].

Overall, much is still unknown about the CEP and the mechanisms of CEP degeneration. Additional research is needed to elucidate the mechanical and transport properties, gene expression, cellular response, and how these traits change with degeneration and age. Understanding the CEP is essential to develop therapies that target or include the CEP. Notably, the CEP should be considered in any treatment of the IVD, as the nutrient and waste transport must be functional for any therapy targeting the NP or AF to be successful. Thus, any long-lasting and sustainable therapy aiming to reverse IVD degeneration should target the CEP first or simultaneously with the NP and AF to rescue the IVD from a pathological environment.

CHAPTER — 3

Research Goals and Methodological Strategy

This chapter outlines the overall research objectives aimed at investigating endplate (EP) degeneration in the intervertebral disc (IVD) through experimental and computational approaches, as well as how they are addressed by this thesis as part of the Disc4All consortium. The chapter also details the main methodological protocols, including bovine EP harvesting, cell isolation, culture media preparation, hydrogel carrier fabrication, bioreactor development, computed tomography (CT)-based modeling, and computational fluid dynamics (CFD) analysis.

3.1 Challenges in understanding the role of the endplate in low back pain (LBP)

Chapter 2 identified several interconnected challenges that continue to limit understanding and treatment of IVD and EP degeneration, particularly when considering the endplate as a heterogeneous structure composed of distinct subregions (Figure XX). Most IVD research has focused on the nucleus pulposus (NP) and annulus fibrosus (AF), while the cartilaginous endplate (CEP) and the bony endplate (BEP) remain understudied, as shown by the targeted PubMed bibliometric analysis (Figure 3.1.1). In research, the CEP is frequently combined with the BEP, or is represented using properties derived from articular cartilage (AC). Inconsistent terminology, difficulty isolating the thin CEP without causing damage or compositional changes, limited availability of healthy human tissues, and substantial variations in species, spinal level, degeneration, loading, and testing conditions hinder comparisons between studies, emphasizing the need for standardized and reproducible methods. Conventional clinical imaging cannot reliably distinguish the CEP from the surrounding tissues, while Modic change (MC) provide only an indirect indication of EP ($EP = CEP + BEP$) condition, and their relationship with pain remains uncertain. Computational models similarly tend to omit the CEP, homogenize its properties, or reduce it to a boundary condition, and no animal model fully reproduces human EP anatomy, composition, mechanics, and transport. Fundamental uncertainty also remains regarding the interactions among calcification, matrix degradation, permeability, inflammation, nutrient deprivation, and tissue failure. Collectively, these challenges demonstrate the need for a multiscale framework that examines cellular responses, intact tissue interactions, EP microarchitecture, mechanical loading, and fluid transport using complementary experimental, and computational approaches. This framework forms the rationale for the research objectives and methodological strategy

presented in this chapter.

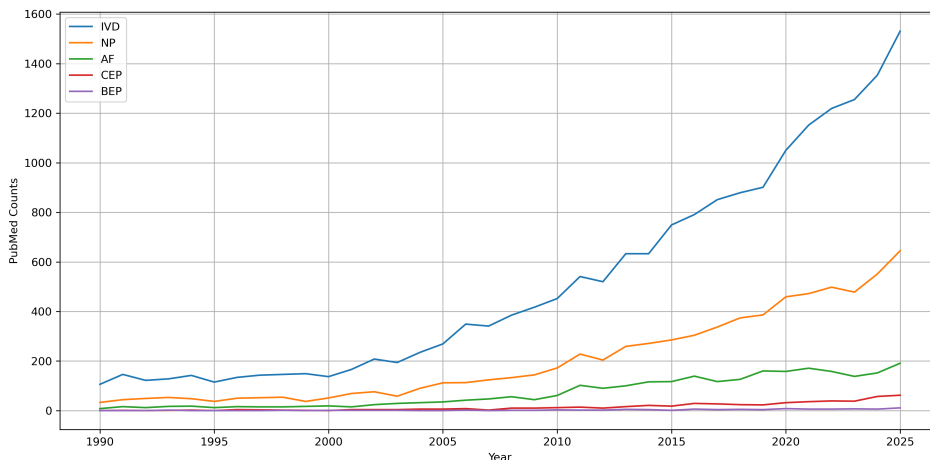


Figure 3.1.1: Annual number of PubMed publications matching title/abstract searches for IVD and related anatomical structures—NP, AF, CEP, and BEP—from 1990–2025. Publication counts were obtained using the NCBI Entrez API. In 2025, the results were LBP, 5,254; IVD, 1,531; NP, 645; AF, 191; CEP, 62; and BEP, 11.

3.1.1 The Disc4All consortium: Mission, objectives, and activities

The Disc4All consortium was a multidisciplinary, international research network funded through the European Union’s Horizon 2020 Marie Skłodowska-Curie Actions Innovative Training Networks programme (Grant Agreement No. 955735). The consortium brought together 15 Early Stage Researchers (ESRs) across 11 primary institutions and eight partner organizations, creating a collaborative network that integrated expertise from academia, industry, and clinical practice. Its overarching aim was to address the translational gap in the understanding and management of LBP by investigating the multifactorial mechanisms underlying intervertebral disc degeneration (IVDD). To achieve this aim, Disc4All combined expertise in molecular biology, biomechanics, computational modeling, data science, and clinical medicine to develop a holistic, patient-specific approach to spinal health.

The consortium focused on integrating large-scale biological and clinical datasets, developing advanced computational and *in silico* models to investigate disc degeneration and predict disease progression, translating these

approaches into clinically relevant decision-support tools, and providing interdisciplinary training to a new generation of researchers. This doctoral thesis was conducted within the Disc4All project, contributing to the investigation of the role of EP sub-tissues, with a particular focus on nutrient and waste transport to and from the IVD through these sub-tissues. As part of the consortium, several experimental and computational protocols were standardized and optimized to ensure compatibility across the research projects of the ESRs, enabling cross-referencing of results and facilitating the comparison and integration of findings.

3.2 Research aim and specific objectives

The overall aim of the research carried out in this PhD is to investigate the degeneration mechanisms of the EP in the IVD through a combined experimental and computational approaches. The study aims to bridge the gap between cellular responses, tissue-scale biomechanics, and fluid transport phenomena within the EP, ultimately contributing to a better understanding of EP degeneration and its role in IVD pathology. To achieve this overall goal, the following specific objectives were tackled.

This overall aim is broken down into several specific objectives. The first specific objective is to developing a computational modeling framework capable of reconstructing EP geometry from CT scans. This will enable the quantitative analysis of morphological features such as porosity, permeability, and structural heterogeneity. The second objective pertains to the evaluation of the biological and structural changes in full EP explants exposed to pro-inflammatory cytokines (tumor necrosis factor (TNF) and interleukin-1 beta (IL-1 β)). This will enable the simulation of degenerative conditions and the quantification of biochemical and histological alterations. The third objective is the characterization of the mechanobiological response of EP cells cultured within three-dimensional (3D) gel carriers under controlled mechanical compression. This will enable the assessment of loading conditions and their influence on cell viability, metabolism, and extracellular matrix (ECM) production. The fourth objective is to design and manufacture an *ex vitro-in silico* workflow to investigate the effect of fluid-induced wall shear stress (WSS) on EP explants.

Through these objectives, this research seeks to establish a multiscale framework linking mechanical and transport phenomena to biological outcomes in

the EPs, thereby providing new insights into its degeneration mechanisms and potential therapeutic targets.

3.3 Thesis outline and chapter overview

This thesis is divided into four parts (Figure 3.3.1). Part I establishes the foundation of the thesis and comprises Chapters 1 and 2, which introduce the clinical and scientific background, review the current state of the art, and define the knowledge gaps addressed in this work. Part II presents the research strategy and consists of Chapter 3, which outlines the research objectives, hypotheses, and overall study design. Part III forms the core of the thesis and comprises Chapters 6–7, which present the multiscale experimental and computational investigations. The organization and objectives of these chapters are described in more detail in the following subsection. Finally, Part IV (Chapter 8) integrates the findings from all preceding chapters discusses the overall contributions, limitations, and future research directions.

Chapter 4 presents a semi-automated computational workflow that was developed to characterize the bovine BEP microarchitecture. Twenty-eight samples from four bovine subjects were imaged using micro-computed tomography (μ CT) and divided into central and peripheral zones. Porosity, pore radius, connectivity, and tortuosity were quantified, and used as inputs to estimate zone-specific transport and mechanical properties based on literature-derived relationships. The workflow also assessed spatial, intra-subject, and inter-subject variability using advanced statistical analysis.

Chapter 5 presents a novel *ex vivo* multi-tissue culture system to investigate cytokine-driven degeneration across the intact IVD and vertebral interface. A previously validated signal transduction network model was used to predict tissue responses under degenerative and cytokine conditions. To validate these predictions, intact bovine explants containing the NP, AF, CEP, and BEP were cultured under standard, degenerative, and cytokine-stimulated conditions. Structural, biochemical, cellular, and molecular changes across the different tissues were then evaluated using imaging, biochemical assays, histological analyses, quantitative cell density assessment, and immunohistochemistry (IHC).

Although this study established a robust platform for investigating tissue-

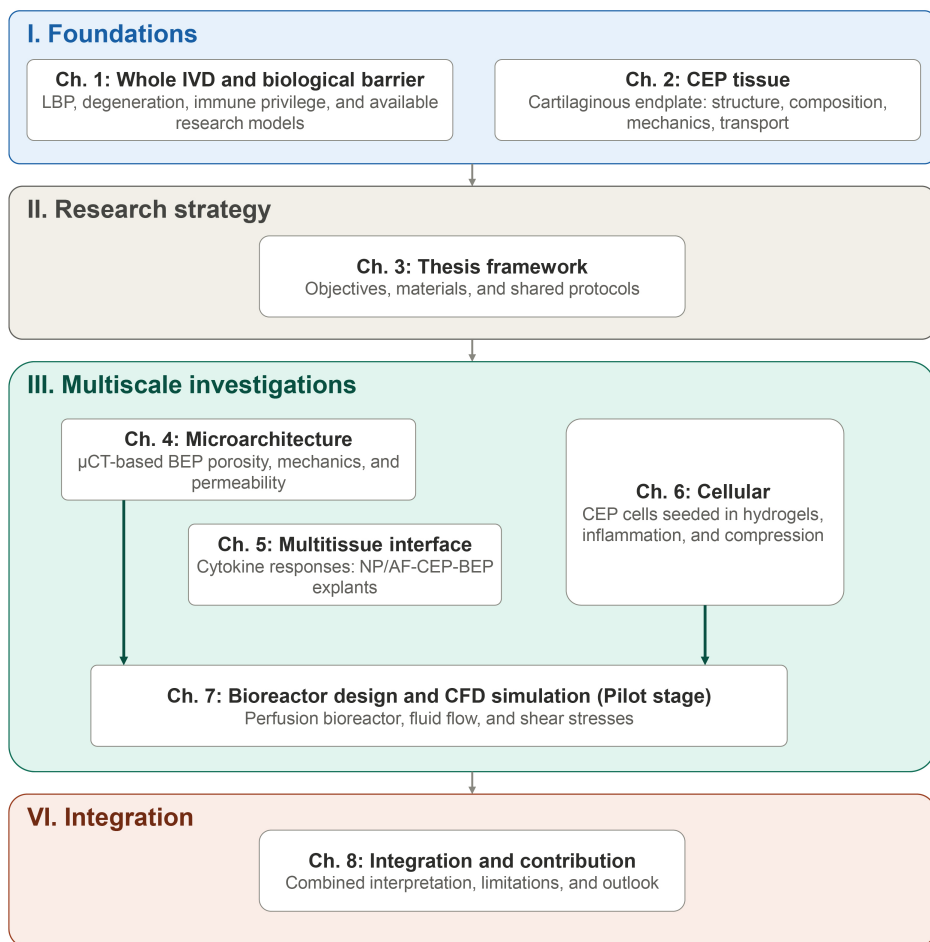


Figure 3.3.1: Structural overview of the thesis, showing the progression from foundational knowledge to integrated conclusions and the specific scale, focus, and contribution of each chapter.

level responses to cytokine stimulation, evaluating biological responses at the individual cell level proved challenging, as molecular analyses such as quantitative polymerase chain reaction (qPCR) could not be reliably performed. To gain a better understanding of how cells respond to mechanical and degenerative cues within their microenvironment, a collaborative study was undertaken with colleagues from the Disc4All consortium using a complementary experimental setup specifically designed to investigate cellular responses under controlled loading conditions, as presented in **Chapter 6**. Human CEP cells were encapsulated in either 2% agarose or blended agarose–collagen hydrogels and cultured under unloaded or dynamic-compression conditions, with and without TNF stimulation. Cell viability, metabolism, matrix production, gene expression, inflammatory mediators, and mechanotransduction markers were assessed. Regulatory-network and finite-element models were also used to simulate cellular signaling, hydrogel deformation, mechanical forces, and fluid transport.

Chapter 7 presents a custom perfusion bioreactor that passes different culture media through bovine osteochondral EP punched out sample (from here on abbreviated as “punch”) under controlled pressure or flow conditions. The system incorporated a recirculating peristaltic pump, flow dampener, medium reservoir, pressure and flow sensors, and real-time data acquisition. In addition, an automated CFD pipeline was developed to characterize the mechanical environment within the punches during perfusion. Together, the bioreactor and CFD pipeline establish an integrated experimental and computational platform for future studies investigating fluid transport and mechanobiological responses in the osteochondral EP. Pilot experiments on the setup were performed.

3.4 Tissue and cell harvesting protocol

In this thesis, both human-derived and bovine-derived biological materials were used to address the various objectives and investigate the EP at cellular and tissue levels. Human EP cells were isolated from small IVD fragments obtained from organ donors or patients undergoing surgery following traumatic incidents. These samples provide clinically relevant human cell populations while avoiding the use of healthy donor tissue solely for research purposes. For whole-tissue experiments, full EP explants were obtained from bovine sources. The bovine EP was selected due to its high structural and compositional similarity to human tissue, its wide availability,

and its suitability for experimental manipulation as mentioned in Chapter 1. The use of bovine explants also offers an ethically preferable alternative to sourcing intact healthy human EPs.

3.4.1 Endplate harvesting from bovines

The harvesting protocol was adapted from Samantha C.W. Chan et al. [363] and Shaghayegh B. et al. [364] where EP explants were harvested from bovine tails obtained from a local slaughterhouse on the day of harvesting. The tails were collected without skin to minimize the risk of contamination (Figure 3.4.1). All tools and surfaces were sterilized before the procedure, and the dissection was carried out on a sterile workstation prepared with sterile sheets placed over a sturdy table suitable for mechanical manipulation. The cutting board was sprayed with 70% ethanol, wrapped with a sterile sheet, and positioned on the workstation. Surgical instruments, including scalpels, tweezers, chisels, hammers, and bone saw blades were sprayed with ethanol and arranged on the sterile surface.

A 1% Betadine solution or 70% ethanol was prepared in a large container (4 L total volume). The entire bovine tail was immersed in this disinfectant solution for approximately five minutes, then briefly dried with sterile gauze before being placed on the sterile surface. The proximal and distal ends of the tail, which contain excessively large and small IVDs, respectively, were removed. Surrounding muscle and ligament tissues were carefully dissected using scalpels and tweezers to expose the vertebral column. The position of each IVD was identified by gently flexing the tail, as the NP could be recognized as the softest region between vertebral bodies.

To isolate the EPs, a chisel and hammer, or surgical oscillating bone saw (NS1011 from Ruijin) when available, were used to cleave one side of the IVD approximately 2 mm into the adjacent vertebra. The NP was transected with a scalpel, and the same procedure was repeated on the opposite side of the disc to separate it completely. The remaining NP and AF were carefully removed with a scalpel and tweezers, leaving the bony and cartilaginous EP structures intact (Figure 3.4.1). Each isolated EP was transferred into a Petri dish containing phosphate-buffered saline (PBS). Residual bone fragments were trimmed away using a scalpel or chisel, and PBS was regularly applied to the tail to prevent desiccation during dissection. This process was repeated until the desired number of IVDs, typically six with diameters between 10 and 20 mm, was obtained.

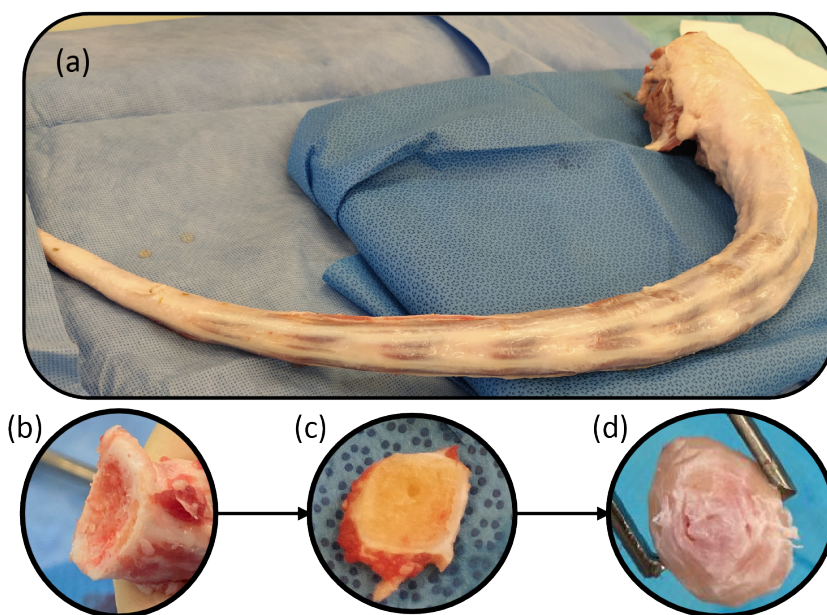


Figure 3.4.1: Fresh bovine tail (a) for EP harvesting where (b) shows the EP still attached to the tail, (c) shows the EP after extraction and (d) shows the EP after cleaning.

Following dissection, the EP samples were rinsed thoroughly using a pressure pump with sterile PBS or shaken well in a falcon tube to remove residual tissue debris and blood. The cleaned EPs were then punched using an 8 mm bone biopsy puncher. To reduce potential microbial contamination, each EP was briefly dipped in the disinfectant solution, rinsed with PBS, and transferred to sterile Petri dishes. The EPs were then sequentially incubated for five minutes in two separate Petri dishes containing PBS supplemented with antibiotic–antimycotic (AA) or penicillin/streptomycin (10×) and taken under a laminar flow hood for further processing.

3.4.2 Cell extraction

Cells were isolated from previously harvested EP tissue using sequential enzymatic digestion with pronase and collagenase II. The tissue was first cut into small fragments in a sterile Petri dish and washed twice with PBS to remove any debris. The minced tissue was then transferred into a T75 culture flask containing 25 mL of low-glucose Dulbecco's Modified Eagle

Medium (LG-DMEM) supplemented with 10% fetal calf serum (FCS) and 47 mg of sterile pronase. The tube used for transfer was rinsed with the remaining pronase solution to ensure complete recovery of the enzyme, and the wash was added to the same flask. The flask was placed on a shaker inside a humidified incubator (37°C, 5% CO₂) and gently agitated at 12 rpm for one hour to initiate tissue digestion.

After the pronase digestion, the content of the flask was transferred to a 50 mL Falcon tube, filled with PBS, and centrifuged to pellet the tissue fragments. The supernatant was aspirated, and the pellet was washed once more with PBS. Residual tissue adhering to the flask was also rinsed with PBS to recover all fragments. The washed pellet was then resuspended in 25 mL of LG-DMEM containing 10% FCS and collagenase type II (285 U/mg; 137 mg per gram of tissue, corresponding to approximately 39,000 U), and the suspension was returned to the shaker in the incubator for overnight digestion (approximately 12 hours).

Following enzymatic digestion, the cell-tissue mixture was filtered through a 100 μ m cell strainer to remove undigested material, then washed once with PBS. The isolated cells were resuspended in 5–10 mL PBS and counted using a hemocytometer or automated counter. Depending on the experimental needs, the cells were either cryopreserved at passage zero (P0) or seeded directly into T-flasks at an appropriate density in LG-DMEM supplemented with 10% FCS and 1% AA solution for expansion.

CHAPTER — 4

Semi-Automated μ CT-Based Modeling of Vertebral Bony Endplate Microporosity and Mechanical Properties: Linking Microstructure to Inferred Mechanical Performance

This chapter is published as:

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The bony endplate (BEP) plays a role in regulating both mechanical load transfer and nutrient transport to the intervertebral disc (IVD), functions strongly influenced by its microstructural porosity. Variations in porosity impact bone strength, elasticity, and solute diffusion, affecting its overall mechanical competence. A semi-automated modeling workflow was established to quantify pore geometry and connectivity of the BEP and relate them to zone-specific mechanical and transport properties using relationships previously established in the literature. Six to eight BEPs from four bovine subjects were imaged using micro-computed tomography (μ CT). The central zone and two peripheral zones of each BEP were segmented and their microstructure, including tortuosity, porosity, and pore radii, were quantified using the pipeline to assess BEP heterogeneity. These metrics were then used to infer the BEP's zone-dependent density, Young's modulus, compressive strength, and shear modulus. The BEPs showed spatial, intra-subject, and inter-subject morphological variability, which affected their predicted mechanical and transport properties. These findings highlight the limitations of purely idealized BEP representations *in silico* simulations and support the incorporation of uncertainty modeling strategies that account for physiologically relevant variations in the BEP. This semi-automated modeling approach represents a tangible step toward more realistic *in silico* simulations of vertebral endplate function.

4.1 Introduction

Degeneration of the IVD and vertebral endplate is associated with compromised mechanical load transmission and impaired nutrient transport, contributing to disc degeneration, low back pain, and adjacent tissue failure [179, 225, 365, 366]. The vertebral endplate is divided into a BEP, which lies on the subchondral trabecular bone (STB) of the vertebral body (VB), followed by a layer of cartilage called the cartilaginous endplate (CEP) that is usually calcified towards the BEP and uncalcified towards the IVD. The entire endplate plays a central role in the aforementioned pathological processes by governing both load transfer and solute exchange to the avascular IVD. These functions are highly sensitive to the endplate's microstructural porosity and connectivity, which has been investigated experimentally [57, 99, 116, 367] as well as through computational modeling and simulations [242, 368–370]. Even modest alterations in endplate porosity have been shown to substantially reduce mechanical strength while simultaneously modifying transport pathways [43, 242, 371, 372], highlighting the

need for accurate microarchitectural characterization.

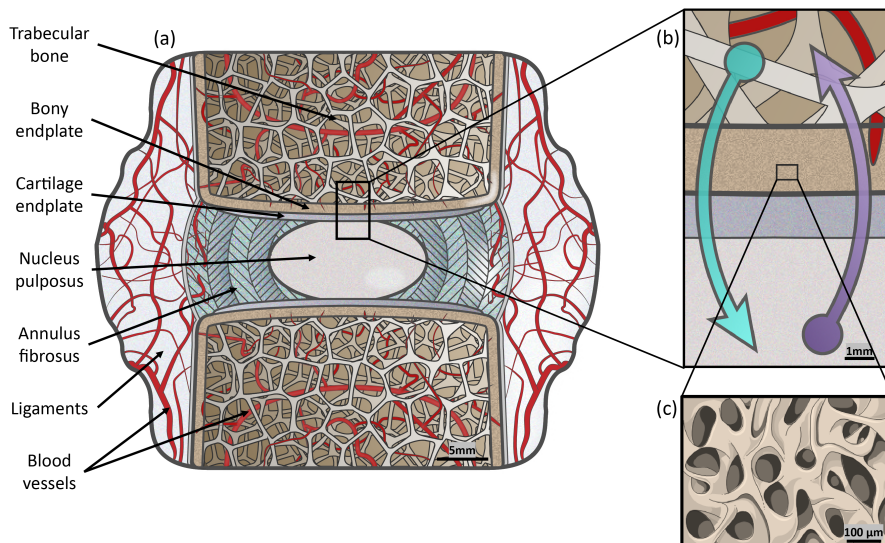


Figure 4.1.1: Intervertebral disc sandwiched between its vertebral bodies (a) showing the path of nutrients (blue) and waste (purple) between the disc and the blood vessels in the vertebral bodies (b), diffusing through the microporosity of the bony endplate (c).

The BEP, despite its thinness (0.2–1 mm [172, 373, 374]) (Figure 4.1.1), contributes 30–60% of bone mass in the VB and bears 45–75% of the compressive loads [375]. In dense bone, even modest increases in porosity can disproportionately reduce tensile and compressive strength, elasticity, and toughness [375–379]. For example, porosity accounts for approximately 70% of the reduction in elastic modulus and more than half of the decrease in yield stress of cortical bone [377]. It is well established that increasing porosity leads to a reduction in bone strength and elasticity, typically following power-law relationships [377, 380–383] as these properties are affected by the structure, geometry, and organization of the pores [383–385].

Beyond its load-bearing role, the BEP has a highly porous microarchitecture, which is specialized for fluid transport and load distribution [43, 372, 386–389] as it is the last vascular structure before the IVD. The BEP–CEP interface is considered the primary pathway for nutrient delivery and waste removal in the IVD, the largest avascular structure in humans [39, 40, 43, 44, 48, 390, 391]. Porosity is increasingly regarded as a quantifiable and predictive marker of bone fragility, with potential to identify at-risk individuals not captured by standard osteoporosis classifications [392, 393], especially since degeneration, microcracks, and Schmorl’s nodes are common

through the BEP and are linked to disc pathology [255, 394–397]. As BEP microstructure directly governs mass transport between the culture medium and the IVD, interpreting experimental measurements after organ culture perturbation without the microstructural information at the BEP level is inherently limited as was shown by Crump et al. [367].

Recent advances in imaging now enable accurate visualization of BEP morphological heterogeneity. μ CT has become the gold standard for high-resolution bone microarchitecture visualization at the sub-micron scale, and computed tomography (CT)-based analysis of BEP pore geometry and connectivity can support model-based predictions of its mechanical and transport parameters. A computational modeling approach offers key advantages over direct experimental measurement. It is non-destructive, preserving sample integrity for downstream analyses such as histology and immunohistochemistry. More importantly, it enables flexible spatial segmentation within each sample, allowing spatially resolved analysis and yielding a richer, more comprehensive characterization of spatial, intra-subject, and inter-subject variations in mechanical and transport properties. This facilitates detailed assessment of tissue heterogeneity and supports the integration of organ culture models with computational simulations. In this study, we applied a semi-automated pipeline to analyze 84 zones obtained from 28 BEPs across four bovine subjects. The workflow enabled reproducible segmentation of bone microporosity and direct quantification of morphological parameters, including tortuosity, porosity, and pore radii. These measures were then used to infer pore-dependent mechanical and transport properties, including permeability, tortuosity, Young’s modulus, compressive strength, shear modulus, and density across the BEP samples. These properties, coupled with the generated three-dimensional (3D) meshes, provide a foundation for future sample-specific computational models and enable mechanistic simulation-based predictions of BEP influences on the disc health, for example, within IVD organ culture models [367].

4.2 Methods

4.2.1 Biological BEP acquisition and microstructural imaging

Bovine bony endplate harvesting and preparation

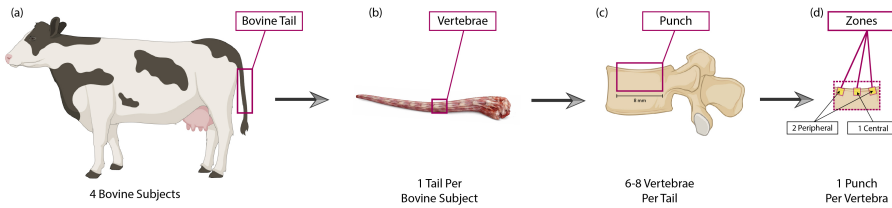


Figure 4.2.1: Workflow illustrating the sample preparation: a bovine tail was harvested (a) and processed by removing skin, muscles, and ligaments (b), followed by isolation of vertebrae (c). Cylindrical punches were extracted to be scanned using μ CT, and three zones were highlighted (one central and two peripheral) for subsequent analysis. The bovine and vertebra illustrations were created in BioRender. Ivanets, M. (2026) <https://BioRender.com/p4sbp46>.

In this study, bovine BEPs were used to test and validate the pipeline, as they have been extensively used as a substitute for human studies in lumbar disc research because of their availability and experimental reproducibility [78, 398–401]. To minimize ethical and animal-welfare concerns, four fresh, full-length bovine tails were obtained from the local slaughterhouse on the day of slaughter, and VBs were harvested from each tail using modified protocols previously published [363, 364]. Briefly, the tails were acquired without skin to reduce the risk of contamination and sterilized with betadine solution before being placed under the laminar flow hood for processing. All muscles, ligaments, and outer connective tissues were removed using a scalpel and tweezers. Intact VBs, including BEP, STB, and cortical bones, were extracted from the middle section of each tail. These VBs were then placed into containers with phosphate-buffered saline (PBS) and shaken 3–6 times until the bone appeared white, indicating the complete removal of the bone marrow and blood clots. On average, two 8 mm punched out sample (from here on abbreviated as “punch”) were then taken from each VB (Figure 4.2.1), at the caudal and cranial BEP sites, using a T-handle bone trephine biopsy punch with ejector. After accounting for samples lost during handling, a total of 28 VB punches were harvested (6–8 per subject). As different tail segments were obtained from the slaughterhouse for each

bovine subject, vertebral level could not be standardized across samples and the punch numbers were therefore assigned as identifiers regardless of vertebral level. Using μ CT imaging and 3D meshing, three microstructural parameters were quantified, with porosity as the primary focus due to its dominant role in governing transport and mechanical properties [242, 402], alongside tortuosity and mean pore radius.

μ CT imaging

The VB punches were fixed in 4% formaldehyde for 48 hours and subsequently immersed in a 40% ioxaglate (Hexabrix[®]) solution for up to two weeks. Hexabrix, an anionic, low-osmolality, and low-viscosity contrast agent [403], is electrostatically repelled by the negatively charged glycosaminoglycans (GAGs) in cartilage [404, 405]. This differential distribution enhances contrast between calcified and non-calcified regions [406], thereby improving tissue delineation in the scans. Each VB punch was subsequently wrapped in parafilm and scanned using a high-resolution μ CT scanner, the Phoenix Nanotom[®] by Baker Hughes (Waygate Technologies). The punches were scanned with in-house optimized settings using a diamond-coated tungsten target and a 0.2 mm Aluminum filter at a voxel size of 4–7 μ m. Each VB punch was scanned at a voltage of 60 kV and a current of 170 μ A at an exposure time of 500 ms. 1800 images were acquired over 360° using the fast scan mode. During reconstruction, the beam hardening correction (BHC) was set to 8 and the inline median filter, the region of interest (ROI) CT filter, and the filter volume were turned on. 16-bit tagged image file format (TIFF) images were exported immediately after reconstruction.

Bone porosity consists of two distinct systems: vascular channels (Haversian and Volkmann canals) and the lacuno-canalicular network. However, connected vascular porosity is the dominant contributor to mechanical and transport properties, as the volume fraction of lacunae and canaliculi is negligible in comparison [407]. Therefore, the pore network of interest in this study primarily comprises the larger vascular channels, which are resolvable at a voxel size of 4–7 μ m.

4.2.2 Semi-automated pipeline

The full set of algorithms covering all tools mentioned below is available for download on our biomech website at <http://www.biomech.ulg.ac.be/software/> and in the public GitHub repository [408], along with a user manual and the full unprocessed output dataset to support reproducibility. The Python-based pipeline (Figure 4.2.2a), implemented in Jupyter notebooks and integrated with 3D Slicer 5.0.3 [409, 410], generates 3D pore structures from μ CT datasets, through a nine-step procedure. The steps are numbered 00 to 08 in the user manual and the scripts are numbered accordingly in the repository. A description of the steps is provided in the following subsections.

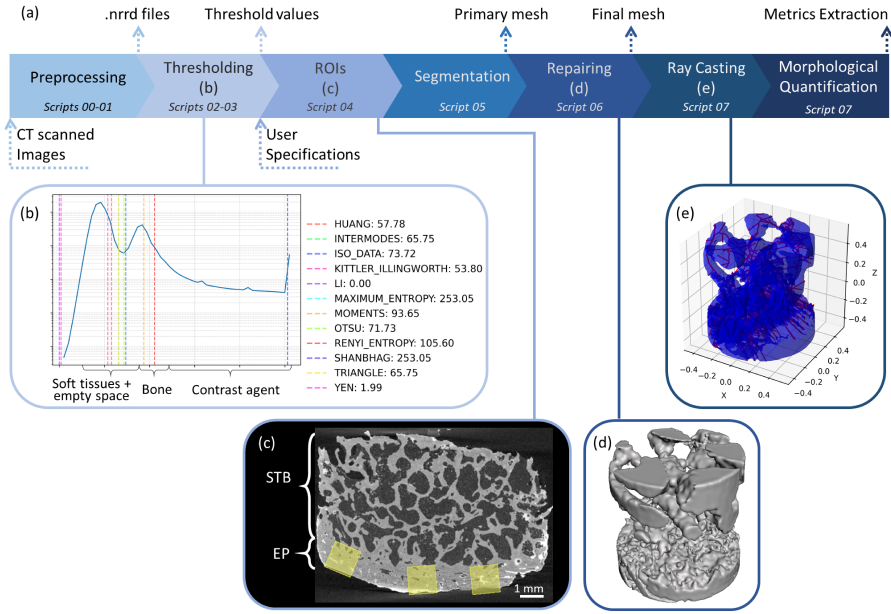


Figure 4.2.2: Porous tissue structure extraction and mesh generation workflow. (a) Overview of inputs and outputs at each step. (b) Automatic thresholding histogram produced by all twelve methods, showing the three ranges: empty space combined with soft tissues, bone, and contrast agent. (c) a VB punch with the 3 ROI zones, the central and two peripheral ones. (d) The cleaned final mesh generated for a zone in the VB punch, representing the pores. (e) The skeletonization and ray casting approach measuring pore radii.

Structure extraction and mesh generation pipeline

In preprocessing, the exported TIFF files were imported to 3D Slicer and stacked using the ImageStacks module of SlicerMorph [411]. They were converted into two volumetric datasets (.nrrd files), one low quality dataset with voxel spacing of 16–28 μm for quick manual handling and confirmation (script 00), and another high-quality dataset with voxel spacing automatically extracted from metadata to be used in the pipeline (script 01). Segmentation was performed using a two-step automatic thresholding strategy, optimized by evaluating 12 global thresholding algorithms, that were Otsu [412], Huang [413], Intermodes [414], IsoData [415], KittlerIllingworth [416], Li [417, 418], MaxEntropy [419], Moments [420], RenyiEntropy [419], Shanbhag [421], Triangle [422], and Yen [423] (Figure 4.2.2b).

Threshold ranges were determined on a sample-specific basis. For each sample, all the automatic thresholding methods were evaluated (script 02), each producing a candidate intensity threshold. For the lower bound, the candidate threshold closest to the first local minimum of the intensity histogram was selected. Voxels with intensities below this threshold were subsequently excluded. The thresholding algorithms were reapplied to the truncated intensity distribution (script 03). For the upper bound, the candidate threshold closest to the second peak of the histogram was selected, provided that the associated peak frequency was lower than that of the lower-bound region. Two intensity intervals were defined: first, from minimum intensity to the lower bone threshold as “void pores”, and second, from the upper bone threshold to maximum intensity as “Hexabrix-filled pores”. The voxels that fall within these two ranges were segmented and merged to generate a unified pore segmentation. Since the void and Hexabrix segmentation intensity ranges are separated by bone intensities, and to mitigate partial volume effects at pore interfaces, a one-voxel morphological dilation was applied to the unified segmentation.

To reduce the computational load associated with analyzing the vertebral BEP samples, and to evaluate the heterogeneity of each sample, cylindrical ROIs were defined interactively within each scan to isolate three zones of the BEP: one central and two peripheral zones (Figure 4.2.2c). This step was done semi-manually on the low-quality dataset by drawing a line that represents the starting point of the ROI and the direction of the cylinder base (script 04). Each ROI has a height of 1 mm and a radius of 0.5 mm and is labeled as “zone” in the following sections. The unified pore segmentation, corresponding ROIs, and the high-quality dataset were subsequently used

(script 05) to reconstruct the local pore geometries into high-resolution stereolithography (STL) surface meshes for the 84 zones.

To ensure high quality, the meshes were processed using MeshLab (PyMeshLab 2023.12.post2t library) (script 06). Processing included surface reconstruction [424] using a screened Poisson approach (depth = 8, scale = 1.1), and standard operations which included removal of duplicate and zero-area elements, merging of close vertices, elimination of non-manifold configurations (e.g., T-vertices and non-manifold edges) and self-intersections, as well as reclosing the resulting holes (if present) to ensure surface continuity. Small disconnected components were discarded using size-based filtering (20% of the zone bounding box) and, finally, meshes were checked for watertightness (Figure 4.2.2d).

Geometric and morphological quantification

Following mesh reconstruction, geometric and morphological parameters were computed directly from the mesh to ensure consistency with any downstream computer simulations (not included in this study). Porosity was calculated as the ratio of pore mesh volume to the total volume of the cylindrical ROI. A wavefront-based approach [425] was used to skeletonize the mesh and produce the centerlines that represent the pore network (Figure 4.2.2e). Local pore radii were estimated by casting perpendicular rays from each skeleton anchor to the mesh surface. A ray-triangle intersection test was performed, and the apparent pore radius was computed as the distance between the anchor and the first intersection with the mesh surface.

Porosity and pore size alone are insufficient to characterize fluid and particle transport in porous media. A critical prerequisite is percolation, defined as the presence of at least one continuous pore pathway from the caudal to the cranial surface and enabling through-flow categorized as “percolating pores”. Accordingly, vertices at the cranial-most and caudal-most extents (Figure 4.2.2e, top and bottom, respectively) were identified using a 2% tolerance (0.02 mm). Pore path lengths were then determined by computing all unique paths within the skeletonized graph that connected these two vertex groups using a depth-first search initiated from branch endpoints (Script 07). Absence of percolation suggests that the sample was not permeable at that zone.

Scripts 00 to 07 can be executed independently for each sample or executed

using Papermill (Scripts A to D), which enables parameterization and batch execution of the pipeline across multiple samples as explained in the user manual.

Porosity-dependent mechanical and transport properties estimation

In geology and material sciences, tortuosity characterizes the path of fluid flow through porous media with complex microstructures, thereby influencing mass transport [426–429]. Geometric tortuosity quantifies the deviation of pore pathways from a straight line, reflecting the extent to which they twist, turn, or branch, thereby increasing the effective path length relative to the zone thickness. It is commonly quantified as the ratio of the actual path length a molecule or fluid must traverse within the porous network to the straight-line thickness of the material (Equation 4.1). By definition, this ratio is always greater than or equal to 1, with a value of 1 representing an ideal straight path and non-percolating zones would have an undefined (infinite) tortuosity. In our case, the actual path length was defined as the average percolating pore centerline length, while the straight-line path was taken as the zone height to calculate tortuosity τ :

$$\tau = \frac{L}{H} \quad (4.1)$$

with L being average pore centerline length, and H being the zone height. The Kozeny-Carman equation is widely used to predict permeability in porous media and has been proven to be accurate for the BEP of the IVD [242]. Although it exists in several forms, all are derived from principles of classical Navier-Stokes fluid mechanics. When tortuosity is explicitly considered, the equation can be expressed as follows:

$$k = \frac{\phi^3}{c(1 - \phi)^2 S^2 \tau^2} \quad (4.2)$$

where k is the intrinsic permeability, ϕ is the porosity, and c is the Kozeny-Carman dimensionless constant. S is the specific surface area of the pores defined as the ratio of the surface area of the solid-pore interface (A_i) to the volume of the solid phase (V_s):

$$S = \frac{A_i}{V_s} \quad (4.3)$$

The volume of the solid phase can be calculated as the difference between the ROI volume and the volume of the pores. The Kozeny-Carman dimensionless constant reflects the influence of pore shape and arrangement on flow resistance. It is commonly assigned a value of 5 in studies of bone tissue engineering [430–432]. The intrinsic permeability is expressed in units of area (m^2 in International System of Units (SI)) but is more widely reported in Darcy (D), where $1 \text{ D} \approx 0.987 \times 10^{-12} \text{ m}^2$ [433, 434]. One Darcy corresponds to the permeability of a 1 cm^2 cross-section, 1 cm in length, that permits a flow of $1 \text{ cm}^3/\text{s}$ of a fluid with a viscosity of 1 cP under a pressure gradient of 1 atm/cm .

In this study, mechanical and transport properties were predicted from morphology using established empirical models. Porosity significantly influences all properties of the bone. Density (ρ) decreases linearly with increasing porosity, while elasticity (Young's modulus E), strength (compressive strength σ_u), and shear behavior (Shear modulus G) exhibit exponential decay, consistent with the Spriggs equation [380–383]. In addition, the three moduli depend on both density and strain rate ($\dot{\epsilon}$) of the measurement protocol, as exemplified in the equations below (Table 4.2.1).

$$\frac{\rho}{\rho_0} = 1 - \phi \quad (4.4)$$

$$E = A_E \rho^{a_E} \dot{\epsilon}^{b_E} + B_E \quad (4.5)$$

$$\sigma_u = A_\sigma \rho^{a_\sigma} \dot{\epsilon}^{b_\sigma} + B_\sigma \quad (4.6)$$

$$G = A_G \rho^{a_G} \dot{\epsilon}^{b_G} + B_G \quad (4.7)$$

In these equations, A , a , b , and B are empirical constants characterizing the relationship of each modulus with density and strain rate. ρ_0 ($=2.2 \text{ g/cm}^3$) denotes the density of bone without porosity derived from cortical bone, which typically has a porosity around 5% and is approximately an order of magnitude lower than that of the BEP [402, 435–437].

The BEP is described as a layer of densely fused trabeculae [172, 371, 438, 439] and commonly assigned homogeneous and isotropic material properties as is the case for trabecular bone in the literature [371]. Accordingly,

trabecular bone empirical relationships were applied at the mesoscale on the BEP zones. The relationship between porosity and density is consistent across multiple studies in the literature. An explicit equation for shear modulus (G) has been reported in only one study and was adopted here [440]. For Young's modulus (E) and compressive strength (σ_u), multiple studies propose differing parameters (summarized in Table 4.2.1); therefore, 3D regression models were created using nonlinear curve fitting to arrive at consensus relationships to predict the corresponding moduli.

4.2.3 Statistical analysis

The statistical methods were developed in consultation with the KU Leuven Biostatistics and Statistical Bioinformatics Centre. The dataset had a three-level hierarchical structure representing 84 zone observations, from 28 VB punches nested within 4 subjects (Figure 4.2.1). To account for subject-specific variability and the hierarchical structure of the data, nested random-intercept linear mixed-effects models (LMMs) were used with restricted maximum likelihood (REML). The models were implemented in R 4.5.3 (R Core Team, 2026) using the `lme4` package [446] and the full reproducible code is available in the GitHub repository [447]. Each measured value (tortuosity, porosity, and mean pore radius) was included as the dependent variable and zone was included as a fixed effect with two levels: central and peripheral. Because multiple zone measurements were obtained from each punch and multiple punches were sampled from each subject, random intercepts were included for subjects and for punches nested within the subject. Fixed effects were tested using Satterthwaite's method, and the estimated effect size, standard error (SE), 95% confidence interval (CI), and p -value were reported for the fixed effects.

Statistical power for the tortuosity, porosity, and mean pore radius analysis was estimated using a Monte Carlo simulation approach [446] based on the fitted LMM. For each simulated dataset, the significance of the zone effect was assessed by refitting the model using REML and obtaining p -values via Satterthwaite's method. Power for the observed experimental design was estimated as the proportion of simulations ($n = 500$) in which a significant zone effect was detected ($p < 0.05$). Normality was assessed for sufficiently powered models, as tests of normality are unreliable in underpowered ones. Power curves were then generated by repeating this procedure across expanded designs varying the number of subjects under balanced and unbalanced BEP sampling while preserving the observed

Table 4.2.1: Reported mathematical relationships between geometric properties and trabecular bone mechanical properties. E : Young's modulus; ρ : density; σ_u : compressive strength; $\dot{\epsilon}$: strain rate.

Prop.	Ref.	Equation type	Model	Corr. coeff.	Sample origin
Elasticity, E (MPa)	Carter [441]	Power function ($E = a\rho^b\dot{\epsilon}^c$)	$E = 3790\rho^3\dot{\epsilon}^{0.06}$	–	Human tibia
	Linde [442]	Power function ($E = a\rho^b\dot{\epsilon}^c$)	$E = 2232\rho^{1.56}\dot{\epsilon}^{0.047}$	0.84	Human tibia
	Linde [442]	Linear density ($E = (a + b\rho)\dot{\epsilon}^c$)	$E = (-321 + 2164\rho)\dot{\epsilon}^{0.047}$	0.84	Human tibia
	Linde [442]	Quadratic density ($E = a\rho^2\dot{\epsilon}^c$)	$E = 3101\rho^2\dot{\epsilon}^{0.044}$	0.82	Human tibia
	Linde [442]	Cubic density ($E = a\rho^3\dot{\epsilon}^c$)	$E = 6093\rho^3\dot{\epsilon}^{0.034}$	0.66	Human tibia
	Linde [442]	Linear strain rate ($E = \rho^b(a + c\dot{\epsilon})$)	$E = \rho^{1.43}(1534 + 103\dot{\epsilon})$	0.89	Human tibia
	Linde [442]	Carter and Hayes type ($E = a\rho^3\dot{\epsilon}^{0.06}$)	$E = 6227\rho^3\dot{\epsilon}^{0.06}$	0.66	Human tibia
Strength, σ_u (MPa)	Enns-Bray [443]	Power function ($E = a\rho^bDA^c\dot{\epsilon}^d$)	$E = 20290\rho^{0.63}DA^{0.0099}\dot{\epsilon}^{-0.55}$	0.79	Bovine femurs and tibiae
	Carter [444]	Power function ($\sigma_u = a\rho^b\dot{\epsilon}^c$)	$\sigma_u = 68\rho^2\dot{\epsilon}^{0.06}$	–	Human tibia and bovine femurs
	Linde [442]	Power function ($\sigma_u = a\rho^b\dot{\epsilon}^c$)	$\sigma_u = 40.2\rho^{1.65}\dot{\epsilon}^{0.073}$	0.93	Human tibia
	Linde [442]	Linear density ($\sigma_u = (a + b\rho)\dot{\epsilon}^c$)	$\sigma_u = (-5.8 + 37.1\rho)\dot{\epsilon}^{0.073}$	0.93	Human tibia
	Linde [442]	Quadratic density ($\sigma_u = a\rho^2\dot{\epsilon}^c$)	$\sigma_u = 52.1\rho^2\dot{\epsilon}^{0.070}$	0.92	Human tibia
	Linde [442]	Linear strain rate ($\sigma_u = \rho^b(a + c\dot{\epsilon})$)	$\sigma_u = \rho^{1.53}(25.0 + 2.26\dot{\epsilon})$	0.93	Human tibia
	Linde [442]	Power function ($\sigma_u = a\rho^2\dot{\epsilon}^{0.06}$)	$\sigma_u = 51.5\rho^2\dot{\epsilon}^{0.06}$	0.92	Human tibia
	Enns-Bray [443]	Power function ($\sigma_u = a\rho^bDA^c\dot{\epsilon}^d$)	$\sigma_u = 24.1\rho^{1.07}DA^{0.83}\dot{\epsilon}^{0.11}$	0.87	Bovine femurs and tibiae*
	Shim [445]	Power function ($\sigma_u = a\rho^b + c\dot{\epsilon}^d$)	$\sigma_u = 5.09\rho^{2.35} + 0.545\dot{\epsilon}^{0.06}$	–	Human vertebrae
	Kasra [440]	Power function ($G = a\rho^b\dot{\epsilon}^c$)	$G = 595.5\rho^{1.08}\dot{\epsilon}^{0.07}$	0.84	Sheep vertebrae

*DA is the degree of anisotropy, assumed to be 1.35 by the authors.

within-subject hierarchical structure. The number of subjects required to achieve at least 80% power was identified, providing guidance for planning larger future studies.

4.2.4 Verification using a ground-truth object at the macro scale

To verify the accuracy of the workflow described above, an object with known ground truth properties was used. A triply periodic minimal surface (TPMS) scaffold with a primitive geometry (Figure 4.A.1) defined by the equation $\cos(x) + \cos(y) + \cos(z) = 0$ was 3D printed and scanned at a voxel resolution of 4 μm . The scaffold was calcium phosphate (CaP)-based, 3D printed via vat polymerization in a layer-by-layer manner while exposed to ultraviolet (UV) light to initiate resin polymerization. The scaffold measured 6 mm in both diameter and height, and had a unit cell size of 2.19 mm, and a designed porosity of 77.5% and an as-manufactured porosity of $66.6\% \pm 5.5\%$, as previously described by Bouakaz et al. [448]. The manufactured macrochannel diameters were measured in CTAnalyzer (CTAn, version 1.20.8.0, Bruker microCT, Kontich, Belgium) using the built-in measuring tool by drawing linear distances across the pores, where the largest channels measured 2 mm.

In this context, it is important to distinguish between two types of voids present in the printed scaffold. The intended scaffold pores, being the macrochannels defined by the TPMS design, have characteristic diameters at the millimeter scale. Manufacturing variability can introduce additional pores at the micrometer scale, for example, through surface roughness or local defects. These micropores are not part of the functional scaffold architecture and cannot be reliably measured by manual methods. As a result, the verification focused on the macrochannels, which are the relevant structural features and can be measured consistently. This verification supports the geometric performance of the workflow under controlled conditions. However, it does not fully establish segmentation accuracy in heterogeneous biological bony endplate tissue.

4.3 Results

4.3.1 The semi-automated workflow was verified against ground-truth objects

The workflow reported a maximum channel diameter of 1.9 mm, corresponding to an accuracy of 95% compared to the results obtained using established tools such as CTAn. The calculated porosity was 71%, representing a porosity accuracy of 93.4% relative to the designed value. This value lies within the as-manufactured range and differs by only 0.8 standard deviations from the reported mean.

4.3.2 Porosity varies between central and peripheral regions, while pore geometry remains constant

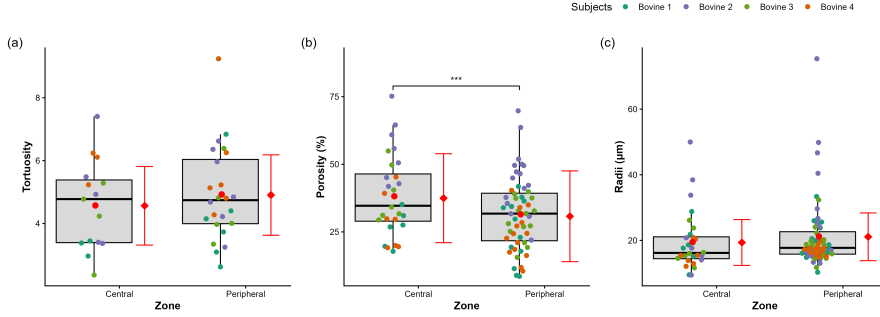


Figure 4.3.1: Boxplot of the raw data showing the IQR, mean (red circle), median (horizontal line), and the $1.5 \times \text{IQR}$ (whiskers) of the regional morphological properties in the BEP. The red diamonds and error bars represent model-based estimates from LMMs. Regional differences between central and peripheral zones in tortuosity (a), porosity (b), and mean pore radius (c). Central and peripheral zones are not independent observations and their statistical significance is based on the LMM. $P < 0.001$ is indicated by ***.

Percolation was observed in 39 of the 84 samples. Among the evaluated regions, 54% of central zones and 43% of peripheral zones exhibited percolation. Zones without percolation had undefined tortuosity and were excluded from subsequent tortuosity analysis (Figure 4.A.2).

The estimated effect of the zone on tortuosity (Figure 4.3.1a) was not statistically significant ($p = 0.441$). The model effect difference was -0.3403

(95% CI: -1.2519 to 0.5712 , SE of the difference = 0.4369), corresponding to mean tortuosity values of 4.5650 ± 0.51 (SE) in the central zone and 4.9053 ± 0.46 (SE) in the peripheral zone. The zone explained only 1.6% of the total variability, subject-to-subject variability accounted for 23.1%, differences between VB punches within the same subject accounted for 0%, and residual variance accounted for the remaining 75.3%.

Porosity (Figure 4.3.1b) differed significantly between zones ($p < 0.001$). The model effect difference was 0.0669 (95% CI: 0.0311 to 0.1026), with a low standard error of the difference (SE = 0.0179) indicating high precision. The estimated mean porosity was 0.3746 ± 0.05 (SE) in the central zone and 0.3077 ± 0.05 (SE) in the peripheral zone. The zone explained only 4.6% of the total variability, differences between punches within the same subject accounted for 46.8%, subject-to-subject variability accounted for 21.6%, and residual variance accounted for the remaining 27.0%. The distribution of standardized residuals indicated that the porosity data satisfied the assumption of normality.

Skeletonization of the BEP zones revealed a highly complex pore network. The extracted centerlines contained an average of 4,810 anchors per sample, with a minimum of 575 anchors in any given zone. Each anchor represents a local geometric inflection point, indicating a change in pore direction or radius. The effect of zone on mean pore radius (Figure 4.3.1c) was not statistically significant ($p = 0.2$). The model effect difference was $-1.728 \mu\text{m}$ (95% CI: -4.4 to $0.94 \mu\text{m}$, SE of the difference = $1.33 \mu\text{m}$), corresponding to estimated mean radius of $19.37 \pm 2.49 \mu\text{m}$ (SE) in the central zone and $17.64 \pm 2.37 \mu\text{m}$ (SE) in the peripheral zone. The zone explained only 0.6% of the total variability, differences between punches within the same subject accounted for 58.2%, subject-to-subject variability accounted for 10.3%, and residual variance accounted for the remaining 30.8%.

Under the observed effect size and variance structure, and based on 500 simulations at $\alpha = 0.05$, the estimated tortuosity statistical power of the current design was 0.14 (95% CI 0.11–0.18), for porosity it was 0.96 (95% CI 0.94–0.97) and for the mean pore radius it was 0.25 (95% CI 0.21–0.29).

In simulations varying the number of subjects while preserving the observed within-subject sampling structure, for unbalanced designs, with an average of 7 BEPs per subject (Figure 4.A.3) similar to the current design, power increased gradually until it reached 0.80 with 55 subjects for tortuosity and 0.88 with 22 subjects for mean pore radius. Balanced designs would require

fewer subjects, for example, with exactly 7 BEPs per subject, power reached 0.85 with 30 subjects for tortuosity and 0.82 with 20 subjects for mean pore radius (Figure 4.A.4).

4.3.3 Not all BEP pores are interconnected, which affects predicted permeability

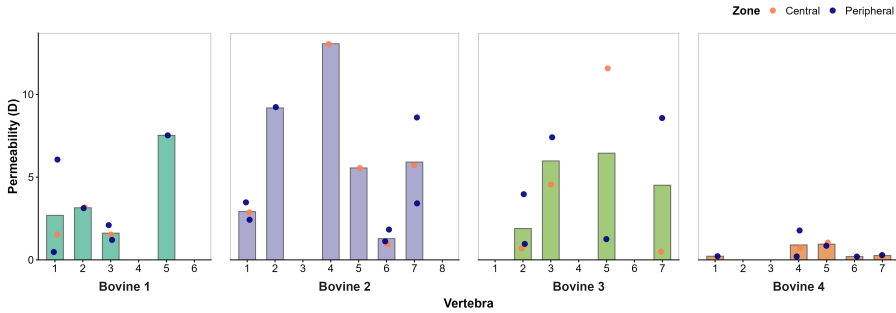


Figure 4.3.2: Differences in intrinsic permeability of the BEP at each punch. The average permeability of each BEP in each punch in each subject is represented by a grey bar with navy blue dots representing peripheral zone values and orange dots representing central zone values. The x-axis values represent identifiers assigned to the punches and do not correspond to the vertebral level.

Considering only percolating pores, the predicted mean intrinsic permeability was $3.58 \text{ D} \pm 3.99 \text{ D}$ at the central zones and $3.18 \text{ D} \pm 3.03 \text{ D}$ for the peripheral zones (Figure 4.3.2).

4.3.4 Inferred density and mechanical properties of the BEP are zone-dependent

To predict mechanical properties from these morphological measures, multiple mathematical models were used in this study. To consolidate these results into a single predictive framework, 3D regression models for Young's modulus and compressive strength were generated by fitting continuous surfaces over the density–strain rate space (Figure 4.A.5). These surfaces provide continuous predictions of Young's modulus E and compressive strength σ_u as functions of both density and strain rate, with coefficients of determination (R^2) of 0.95 and 0.99, respectively.

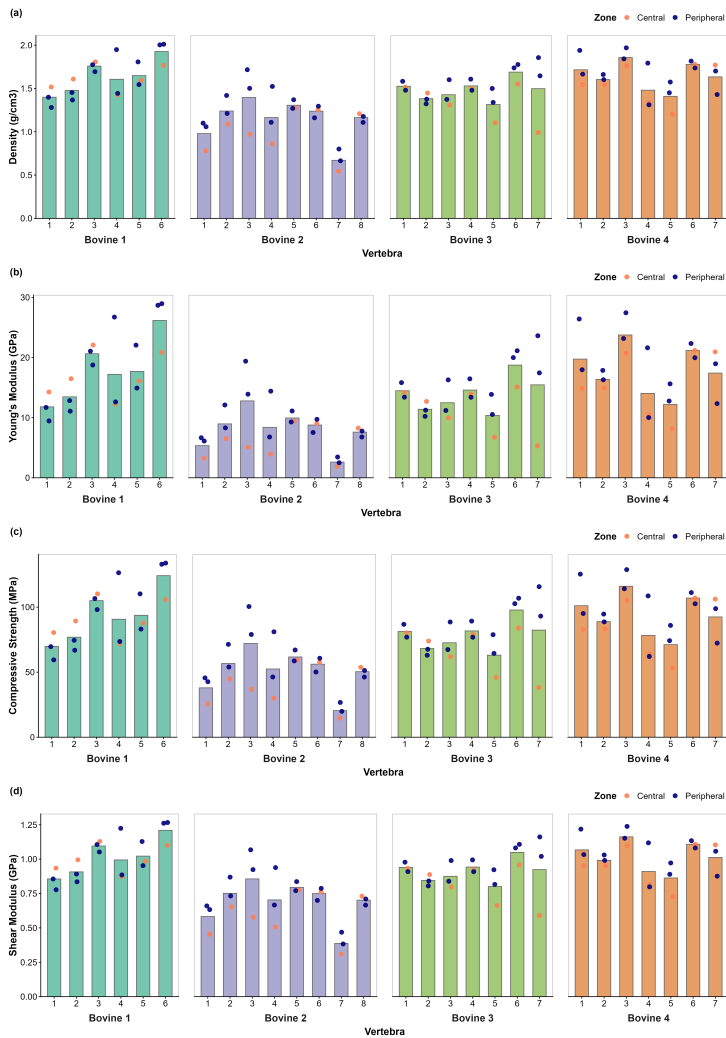


Figure 4.3.3: Density (a) of the BEP at each punch and the predicted mechanical properties: Young's modulus (b), compressive strength (c), and shear modulus (d). The average value of the density or mechanical property for each punch (numbers on x-axis) in each subject (Bovine 1–4) is represented by a grey bar with navy blue dots representing peripheral zones and orange dots representing central zones. The x-axis values represent punch identifiers and do not correspond to the vertebral level.

$$E = 4388.03\rho^{2.65}\varepsilon^{-0.08} + 1010.68 \quad (4.8)$$

$$\sigma_u = 35.33\rho^{1.87}\varepsilon^{0.07} + 3.4 \quad (4.9)$$

The predicted mean central and peripheral densities of the BEP were 1.36 ± 0.33 g/cm³ and 1.51 ± 0.29 g/cm³, respectively (Figure 4.3.3a). The predicted Young's modulus was 12.09 ± 5.9 GPa for the central zone and 15.04 ± 6.45 GPa for the peripheral zone (Figure 4.3.3b), while the predicted compressive strength was 69.07 ± 26.67 MPa for the central zone and 81.74 ± 26.49 MPa for the peripheral zones (Figure 4.3.3c), and the predicted shear modulus was 0.83 ± 0.21 GPa for the central zone and 0.93 ± 0.19 GPa for the peripheral zone (Figure 4.3.3d).

4.4 Discussion

This study introduces a semi-automated pipeline for quantitative characterization of BEP microarchitecture, providing a workflow to quantify porosity and pore morphology in BEP samples, generate 3D mesh structures, and predict their mechanical and transport properties based on relationships amalgamated from the literature. This enables reproducible assessment across anatomical zones and provides a robust foundation for accurate modeling and simulation in vertebral applications. The workflow uses μ CT scans of Hexabrix-stained VB punches to enhance contrast between bone and surrounding soft tissues. The tool was verified using a ground truth TPMS scaffold with a primitive geometry, achieving 95% and 93.4% accuracies for radius and porosity measurements, respectively, comparable to the standard tools in CTAn. These results reflect the tool's performance on the intended geometric features rather than defects or manufacturing-induced micropores. They suggest that the workflow reliably captures the macrostructural characteristics of a complex porous scaffold, which aligns with the scale and type of features quantified in the biological samples.

BEP pores vary widely in the literature, from a few micrometers up to several hundred micrometers. In humans, the most commonly reported average pore diameter ranges from approximately 20 to 60 μ m [168, 204, 449–451]. Data for bovine BEPs are more limited, with reported pore diameters ranging from 15 to 84 μ m [452]. One study directly compared bovine and human BEPs using imaging measurements and found the average diameters to be

33 μm and 58 μm , respectively [449]. In the present study, we differentiated between central and peripheral zones, and the average pore diameters of bovine BEPs were 38.74 μm and 35.28 μm in the central and peripheral zones, respectively. No significant difference was observed between the zones, which is consistent with the reported values.

Not all pores within the zones were true percolating pores, as some were disconnected blind pores that terminated within the tissue, or lateral pores that terminated at the zone sides. This could be the result of the specific zone selected for analysis, as previously mentioned, and they were excluded from the tortuosity and permeability calculations. Percolation was detected in less than half of the samples and varied between zones, with 54% of central zones and 43% of peripheral zones exhibiting connectivity. Tortuosity did not differ significantly between central and peripheral zones, but there was a noticeable subject-to-subject variability (23.1%), whereas differences between punches within the same subject were negligible. This suggests that inter-subject differences have a stronger influence on tortuosity than regional pore network geometries. This may be partly due to the exclusion of zones where tortuosity was undefined, but given that the study was underpowered for this parameter, the results should be interpreted with caution pending confirmation in larger cohorts.

Porosity, in contrast, exhibited significant spatial variation ($p < 0.001$), with central zones showing higher mean porosity than peripheral zones. Although zone location accounted for only 4.6% of the variability, differences between punches within the same subject contributed 46.8%, and subject-to-subject variability accounted for 21.6%. Since this study was sufficiently powered for this parameter, the observed variation in porosity can be confidently attributed to both intra-subject and inter-subject heterogeneity as dominant contributors.

Analysis of mean pore radius further emphasized the importance of intra-subject variability. Although the mean pore radius did not differ significantly between central and peripheral zones, differences between punches within the same subject accounted for 58.3% of the variability. Inter-subject differences were comparatively smaller (10.3%), indicating that the local geometry of individual pores could be largely determined by the vertebral level. These results should also be interpreted with caution pending confirmation in larger cohorts given that the study was underpowered for this parameter. The high number of average anchor points in the skeletonized BEPs (representing local geometric inflection points, indicating changes in pore direction

or radius) also supports the notion that the BEP is not a homogeneous structure but instead exhibits considerable spatial and morphological variability throughout. Collectively, these results suggest that the BEP exhibits both spatial heterogeneity (central vs peripheral regions) and pronounced inter-sample and inter-subject differences dominating porosity and pore size characteristics, while tortuosity is influenced more by inter-subject variability.

As previously mentioned, one of the possible applications of the presented pipeline is increasing the level of sample-specificity in numerical simulations currently used to interpret experimentally obtained organ culture results [367]. The presented pipeline can predict mechanical and transport properties based on the obtained morphological parameters, which in the case of porosity have been shown here to vary significantly across and within subjects. Considering only the percolating zones, the intrinsic permeability of the BEP was predicted to be higher at the central zones compared to the peripheral zones. The predicted density of the BEP, $1.36 \text{ g/cm}^3 \pm 0.33 \text{ g/cm}^3$ and $1.51 \text{ g/cm}^3 \pm 0.29 \text{ g/cm}^3$ for the central and peripheral zones, respectively, align with values reported in the literature, $0.75\text{--}2.1 \text{ g/cm}^3$ [453–456].

The bovine model was selected in this study because it is widely used in IVD organ culture and endplate-related experimental workflows, it is readily available as fresh tissue, and it allows reproducible sample collection with controlled processing [78, 398–401]. These practical and methodological advantages were important for the imaging-to-quantification pipeline. At the same time, we acknowledge that bovine coccygeal segments are not the primary load-bearing spinal levels and are exposed to a different mechanical environment than human lumbar vertebrae. Because endplate microarchitecture adapts to mechanical demand, differences in loading history may influence porosity, connectivity, and pore orientation, and therefore the absolute values of predicted mechanical and transport properties. Accordingly, the present results should be interpreted as specific to the bovine BEP samples analyzed in this study. This, however, does not take away from the main objective of this study, which is demonstrating the semi-automated pipeline for analysis of μCT images of the BEP, and the illustration of how the proposed pipeline can be applied within organ culture workflows to derive sample-specific mechanical and transport properties to inform specimen-specific loading or fluid flow simulations.

Biological segmentation remains challenging due to partial-volume effects,

heterogeneous mineralization, and less distinct tissue interfaces. The TPMS-based verification performed in this study primarily demonstrates the robustness of the pipeline for detecting geometric segmentation under the assumption of uniform tissue mineralization. Limited biological replicates ($n = 4$) reduced the statistical power to detect zone effects for tortuosity and mean pore radius, implying that the absence of statistical significance does not rule out biological zone effects on these parameters. Future work should therefore integrate this imaging pipeline with direct mechanical and permeability measurements on biological specimens, using human lumbar sections if feasible, to validate and refine the approach. Additionally, combining this pipeline with computational models of the CEP and the nucleus pulposus could enable a more comprehensive assessment of the mechanical and transport properties of the IVD as a whole.

The BEP plays a critical dual role in both mechanical load regulation and nutrient transport to the IVD, functions that depend strongly on its pore geometry and connectivity. Even small increases in porosity can substantially reduce local bone strength and elasticity while also influencing solute diffusion. Accurate characterization of this microarchitecture is therefore essential for predicting mechanical competence, permeability, and disc health. The semi-automated processing pipeline presented here enables efficient, reproducible characterization of BEP microarchitecture. This pipeline provides sample-specific morphological values that can be used as inputs for computational simulations of vertebral BEP function. The segmented BEP mesh can be directly imported into a computational workflow where the quantified morphological, mechanical, and transport properties replace the idealized or literature-averaged parameters that are currently typically used. Grounding these numerical models in empirically derived structural parameters would enable more accurate predictions of stress transfer, strain localization, and load-bearing capacity by capturing the true heterogeneity of the BEP microstructure in mechanical simulations. Additionally, the predicted permeability and measured pore architecture would reliably inform solute diffusion and fluid flow models, thereby improving computational predictions of nutrient transport to the IVD in transport simulations. Disc organ culture under bioreactor conditions would particularly benefit from integrating this tool into the experimental workflow. Incorporating sample-specific characterization allows investigators to explicitly account for the variability in inferred properties of the BEP, which is experimentally difficult to fully separate from the CEP [367]. Hence, this workflow represents a step toward more realistic *in vitro* and *in silico* modeling of vertebral endplate functions.

CHAPTER — 5

Cytokine-Mediated Responses Across Endplate Subregions in Intervertebral Disc Multi-tissue Explants

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The endplate (EP) is essential for maintaining intervertebral disc (IVD) homeostasis by regulating nutrient transport to the nucleus pulposus (NP). Structural or biochemical alterations in the EP, including its non-calcified cartilage endplate (ncCEP), calcified cartilage endplate (cCEP), and bony endplate (BEP) sublayers, can impair this function and contribute to disc degeneration and low back pain (LBP). While most studies focus on the NP and annulus fibrosus (AF), increasing evidence suggests that early EP degeneration is a key initiator of disc pathology.

This study combined *in silico* modeling with a novel *ex vivo* multi-tissue culture system to investigate cytokine-driven degeneration across the intact IVD–vertebral interface. A previously validated signal-transduction network model predicted that degenerative environmental conditions alone are insufficient to induce chondrocyte degeneration without cytokine stimulation. To experimentally corroborate this, intact bovine explants comprising NP, AF, cartilaginous endplate (CEP), and BEP were maintained in a composite culture system that preserves native tissue interfaces, an approach not previously reported. Explants were cultured for 14 days under standard, degenerative, and cytokine-stimulated conditions using tumor necrosis factor (TNF) or interleukin-1 beta (IL-1 β). Tissue responses were assessed using computed tomography (CT), glycosaminoglycan (GAG)-to-deoxyribonucleic acid (DNA) ratios, extracellular GAG release, histological grading, quantitative cell density analysis, and immunohistochemistry (IHC).

In silico predictions were confirmed experimentally, as degenerative conditions alone did not induce significant changes classically associated with degeneration, whereas cytokine exposure consistently triggered these changes. TNF stimulation resulted in increased extracellular GAG release and pronounced chondrocyte loss, while IL-1 β primarily altered the extracellular matrix (ECM) state with comparatively lower GAG release. Histological analyses revealed reduced proteoglycan staining in the CEP and NP/AF tissues under cytokine conditions without complete structural disruption. IHC revealed significant changes in the expression of anabolic and catabolic markers in the CEP within 14 days, whereas no significant changes were observed in the NP/AF.

These findings suggest that cytokine signaling is a critical driver of EP-associated degeneration and reveal distinct, tissue-specific responses to TNF and IL-1 β . The development of a multi-tissue *ex vivo* culture system that preserves the IVD–vertebral interface provides a new platform for studying early degenerative mechanisms and enables more physiologically relevant

investigation of cross-tissue interactions during disc degeneration.

5.1 Introduction

LBP affects approximately 80% of individuals in Western countries during their lifetime, accounting for nearly five million disability-adjusted life years (DALYs) in young adults, making it the leading cause of morbidity worldwide [457–459]. Among the various components of the spine, the EP has received comparatively little attention in the scientific literature [460], despite its critical role in maintaining the health of the IVD. Whilst calcification-induced reduction in EP permeability leads to decreased nutrient supply to the disc [461, 462], matrix degradation and microfissures within the EPs facilitate infiltration of blood vessels and nerve fibers from the subchondral bone into the otherwise immune-privileged IVD [457]. This makes EP changes the principal contributing factors in LBP [463, 464].

The EP is a layered composite structure consisting of the BEP, a semiporous region of thickened cancellous bone that is part of the vertebral body (VB), and the CEP. The CEP is subdivided into the ncCEP region, associated with the IVD, and the cCEP region, which serves as a transition between the VB and IVD. As a thin layer of hyaline-like cartilage, the CEP separates the IVD from the VB [460, 465, 466], acting both as a mechanical barrier that prevents the NP from bulging into the VB and proteoglycans from leaching out [467], and is the principal conduit for nutrient and waste transport to sustain the avascular NP [460, 467].

Cytokines, particularly TNF and IL-1 β [468–470], are key mediators of catabolic processes in cartilage and IVD and are upregulated in degenerating tissue. These cytokines are produced by resident chondrocytes within the CEP, and the NP and AF cells throughout the IVD [471, 472], and drive matrix degradation via stimulation of matrix-degrading enzymes. In the IVD, elevated TNF and IL-1 β correlate with increased degeneration, whereas healthy tissues show low levels of IL-1 β , which is balanced by expression of its natural antagonist IL-1Ra, whilst TNF shows minimal expression. IL-1 β and TNF have been implicated in disc degeneration and herniation, promoting catabolic activity, neurotrophic factor expression, and altered matrix synthesis [469, 472–475]. However, within the native human IVD, IL-1 β appears to play a greater role due to limited TNF receptor expression *in vivo* [468, 476]. Yet, the majority of studies to date have focused on

the response of NP cells and tissues to these cytokines [477–482], with some studies on AF cells and tissues [480, 482–484], but limited work to date has investigated the response and roles of these cytokines within the EPs [460, 485, 486]. In particular, the specific regulatory roles of TNF and IL-1 β within the EP subregions remain poorly defined, with no studies to date investigating intact multi-tissue explants. Notably, most EP experimental models rely on isolated cell cultures or single-tissue explants, which fail to capture the structural and functional coupling between the IVD, CEP, and BEP. This isolation does not adequately represent its *in vivo* function. Interactions across this multi-tissue interface are likely to play a central role in the initiation and progression of degeneration, particularly in response to cytokine stimuli. However, to date, no studies have investigated cytokine-driven EP degeneration in an intact multi-tissue IVD–VB system that preserves these native interfaces.

Therefore, this study developed a novel *ex vivo* explant culture model that maintains the IVD–VB architecture, including NP/AF, CEP, and BEP. This platform enables, for the first time, the investigation of spatially resolved, cytokine-mediated degenerative responses across the EP subregions within an integrated multi-tissue environment.

5.2 Methods

5.2.1 Predictive computational model of chondrocyte degeneration with and without cytokines

Chondrocyte degeneration was assessed *in silico* in degenerative media with and without cytokines using a previously validated *in silico* signal-transduction network model developed for cartilage degeneration in osteoarthritis [127, 128]. The resulting chondrocyte states were compared against reference profiles representing healthy and degenerative phenotypes. The model integrated coupled gene regulatory and protein signaling subnetworks, comprising 74 normalized parameters (0 to 1 scale) that collectively represent approximately 300 direct and indirect biochemical interactions governing cellular behavior. Model outputs included gene-level (slow) and protein-level (fast) activities, with global activity defined as their product [127, 128, 487].

The analysis was performed using a Monte Carlo approach with 10,000 random initializations, each of which was run until a stable steady state was reached. Of these steady states, 11 were classified as healthy and subsequently used as the initial conditions for the simulations. These 11 healthy initial states were perturbed by clamping selected nodes to values reflecting *in vitro* conditions for 1,000-time steps. After the clamp was released, the model was simulated until a new steady state was reached. To account for the stochasticity of the model, each perturbation was performed in 100 independent runs for each of the 11 initial states, resulting in 1100 runs for each environment. Although degenerative conditions differ from healthy conditions in both osmolarity and pH [488], the latter was not incorporated as a variable in the *in silico* model because there is insufficient quantitative experimental data to reliably parameterize and validate its effects on the signaling network. Nevertheless, pH was included in the subsequent *in vitro* experiments to address this knowledge gap and provide evidence that may support the incorporation of pH into future *in silico* models. Therefore, degenerative conditions without cytokines were simulated as low osmolarity conditions, captured in the model through overexpression of transient receptor potential cation channel subfamily V member 4 (TRPV4), based on in-house experiments and literature reports [489–491]. Degenerative conditions with cytokines additionally included the overexpression of the generic cytokine variable in the model.

Following clamp release, the system evolved to a new steady state and was classified by comparison against predefined healthy and degenerate (hypertrophic-like disease) reference attractor states. The degenerate states are defined as having high global activity of RUNX2, COL-X, matrix metalloproteinase 13 (MMP13), and IHH, along with low global activity of SRY-box transcription factor 9 (SOX9), NKX3.2, COL-II, aggrecan (ACAN), and proteoglycans, whereas healthy states exhibited the opposite activity profile as previously reported by our group [127, 128]. Simulation code is available at <http://www.biomech.ulg.ac.be/software/>.

5.2.2 Bovine disc model

The bovine IVD is increasingly recognized as a valuable model for studying the mechanobiology, cell biology, and pathogenesis of human IVD degeneration, owing to anatomical and biochemical similarities in cell phenotype, composition, and mechanical properties [492]. Research indicates that bovine CEP closely resembles that of humans in composition and organization,

including proteoglycan distribution and gene expression profiles [493], in addition to responses to cytokines, particularly TNF and IL-1 β [494–496]. In view of these similarities and the ethical and practical limitations of obtaining healthy, intact human VB–IVD samples, bovine tails were used to study the cytokine effects on the CEP over a 14-day experiment in either standard disc culture media or media modulated to mimic the degenerate environment [488] (Figure 5.2.1).

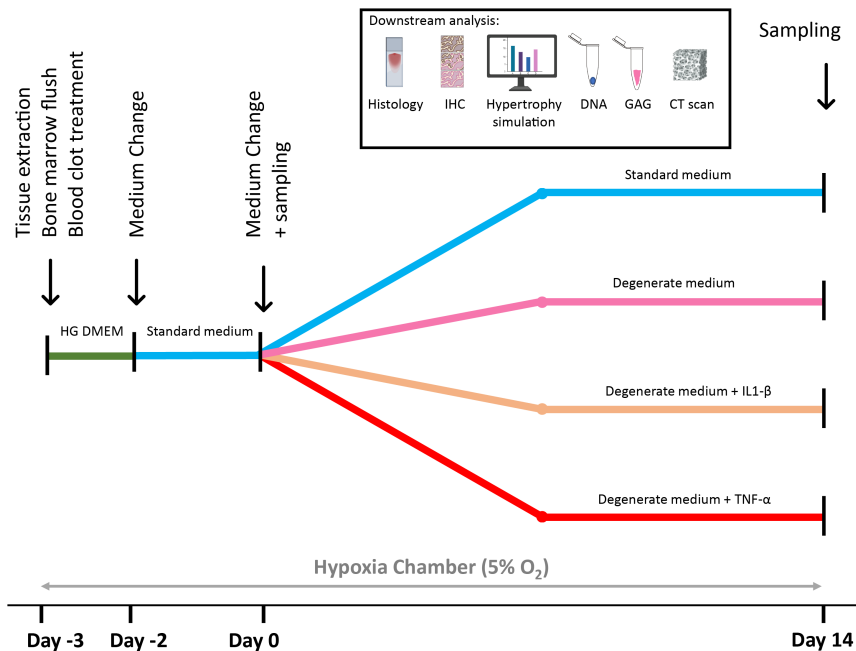


Figure 5.2.1: Chronological overview of the experimental procedures from the tissue extractions at day –3 to the sampling and storing at days 0 and 14, and the downstream analysis performed subsequently. Standard and degenerate media are made of LG-DMEM supplemented with FCS, with cytokine-treated groups denoted by “+ TNF” or “+ IL-1 β ”. More details for the media composition can be found in Table 5.2.2.

5.2.3 Tissues/cells extraction and isolation

The tissue harvesting protocol was adapted from published methodology [497, 498] where three fresh whole-length bovine tails were acquired from the local abattoir (Bramall & Son, Sheffield). The tails were acquired without skin to reduce the chance of contamination and immediately sterilized with betadine solution and placed under the laminar flow to be processed.

Muscles, ligaments, and outer connective tissues were dissected to expose the vertebrae and IVDs, and around 10 intact IVDs with $8.4 \text{ mm} \pm 2.6 \text{ mm}$ of adjacent vertebrae on each side were excised from the middle section of each tail. These IVDs were then washed 3–6 times with phosphate-buffered saline (PBS) to remove the bone marrow and any blood clots. The vertebral–IVD explants were then cut using a veterinarian oscillating bone saw (NS-1011 Oscillating Bone Saw Medical from Wuhu Ruijin Medical Instrument and Device Co., Ltd.). A total of 91 samples were harvested across 3 bovine tails (Table 5.2.1). Samples were randomly assigned to the different experimental groups (Appendix Table 5.A.1). The IVD tissue side of each of the samples was then dipped in sterile 3% w/v agarose prepared in PBS to reduce GAGs depletion and tissue swelling [499].

Table 5.2.1: Number of explants obtained per bovine stratified per environment. At day 0 (3 days post-harvesting) and standard, degenerate, degenerate + IL-1 β , or degenerate + TNF at day 14.

Day	Treatment	Test	Bovine 1	Bovine 2	Bovine 3
0	Day 0	GAG/DNA	3	3	–
		CT–histology	3	3	–
14	Standard	GAG/DNA	4	4	3
		CT–histology	3	3	3
	Degenerate	GAG/DNA	3	3	3
		CT–histology	3	3	3
	Degenerate + IL-1 β	GAG/DNA	4	4	3
		CT–histology	3	4	3
	Degenerate + TNF	GAG/DNA	3	4	3
		CT–histology	3	4	3
	Total: 91		32	35	24

5.2.4 Culture conditions

Whereas the *in silico* model compared healthy and degenerative environments by altering osmolarity, the experimental study compared standard and degenerative culture conditions. This approach was chosen because tissue culture studies commonly use standard culture conditions as the reference group [495, 496] rather than attempting to recreate a fully healthy intervertebral disc environment, facilitating comparison with existing literature. Furthermore, the objective of the experimental work was to evaluate tissue-level responses rather than isolated cell behaviour. Sample avail-

ability was limited, which precluded the inclusion of an additional healthy experimental group.

The basal media for all cultures was Dulbecco's Modified Eagle Medium (DMEM) (Gibco), supplemented with 10% v/v FCS (Gibco: A5256701). During the initial 24 hours, all samples were placed in a 5% oxygen hypoxia glove box (Don Whitley Hypoxia station) with standard high-glucose medium (4.5 g/L). This step allowed the cells and tissues to recover from mechanical disruption during harvesting and sudden loss of native nutrient supply. Subsequently, the medium was switched to standard low-glucose (1 g/L) DMEM to prevent high-glucose-induced damage to CEP cells [500] and maintain a more physiological glucose level. Culture under low glucose and 5% O₂ was maintained for 48 hours before the samples were allocated randomly to experimental conditions. Following the initial 3 day pre-culture to enable tissues to recover from extraction, this was defined as 'day 0' of the experiment and $n = 6$ samples from bovine 1 and bovine 2 were taken for downstream analysis as "day 0 controls" as described later, while all remaining samples were split into 4 groups: standard medium, degenerate medium, degenerate + IL-1 β medium, and degenerate + TNF medium (Table 5.2.2). The key difference between the standard and the degenerate media is the pH levels that were imparted with the differences in the Sodium Bicarbonate concentrations [488]. Both standard and degenerate media had an osmolarity (OSM) of 325mOsm/kg, whilst the pH of the standard medium was 7.2 and of the degenerate media was 6.8, as reported recently for NP media [488]. All cultures were performed in 6-well plates filled with 10 mL of the designated medium, and swelling was minimized via the application of the agarose 'dip'. All media were stored and refreshed three times per week until day 14, after which time all remaining samples were collected for downstream analysis.

5.2.5 Downstream analysis

At day 0 and at day 14, samples were split into 2 groups; one group was fixed using 4% w/v formaldehyde (Sigma Aldrich 1.04003) and stored at room temperature prior to imaging using a micro-computed tomography (μ CT) scanner. Following CT analysis, samples were ethylenediaminetetraacetic acid (EDTA) decalcified in 20% EDTA/Tris-buffered saline (TBS) and embedded in paraffin wax and processed for histology and IHC to study the microscopic anatomy of biological tissues. Samples of the second group were separated into their component tissue parts (VB, CEP, IVD) and

Table 5.2.2: Tissue culture media used throughout the experiment, where high-glucose was used for freshly harvested samples from day -3 till day -2 , and standard media applied from day -2 to day 0, and standard, degenerate, and degenerate with cytokines were used from day 0 till day 14. DMEM.

HIGH-GLUCOSE MEDIUM		
Component	Amount (Final concentration)	Supplier and Product Code
high-glucose Dulbecco's Modified Eagle Medium (HG-DMEM)	(500 mL) As supplied	Gibco: 10569010
Pen/Strep	(50 U/mL)	Gibco: 15070-063
Amphotericin B	(2.5 μ g/mL)	Sigma: A2942
L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate	(50 μ g/mL)	Sigma: A5960
FCS	10% v/v	Gibco: A5256701
STANDARD MEDIUM		
Component	Amount (Final concentration)	Supplier and Product Code
LG-DMEM	(500 mL) As supplied	Gibco: 11885084
Pen/Strep	(50 U/mL)	Gibco: 15070-063
Amphotericin B	(2.5 μ g/mL)	Sigma: A2942
l-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate	(50 μ g/mL)	Sigma: A5960
FCS	10% v/v	Gibco: A5256701
DEGENERATE MEDIA		
Component	Amount (Final concentration)	Supplier and Product Code
LG-DMEM powder	4.99 g per 500 mL	Gibco: 31600-083
Sodium bicarbonate	0.213 g per 500 mL	Sigma: S-6014
Pen/Strep	(50 U/mL)	Gibco: 15070-063
Amphotericin B	(2.5 μ g/mL)	Sigma: A2942
l-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate	(25 μ g/mL)	Sigma: A5960
FCS	10% v/v	Gibco: A5256701
L-Glutamine	1% v/v	Gibco: 25030-024
IL-1 β	1 ng/mL (for degenerate + IL-1 β medium)	Peprtech (200-01B-10UG)
TNF	1 ng/mL (for degenerate + TNF medium)	Peprtech (300-01A-50UG)

snap frozen in liquid nitrogen, then stored at -80°C for GAG and DNA quantification. Additionally, the spent media from the technical replicates were pooled for each bovine subject and stored at -80°C to calculate the GAGs released by the cells into the media.

CT scanning

Although it is difficult to display the structure of the CEP, NP, and AF on CT scans due to its poor contrast in soft tissues [501], the high resolution provided by the Phoenix Nanotom[®] by Baker Hughes (Waygate Technologies), in addition to short three-dimensional (3D) scanning time, enabled a detailed examination of the bone tissue integrity and the detection of any boundary artifacts. To better visualize the non-calcified cartilage in the explants, Hexabrix was used as a contrast agent since it interacts electrostatically with the negatively charged GAGs present in cartilage and IVD [502, 503]. The formaldehyde-fixed samples were submerged and incubated in 40% Hexabrix for 48 hours at room temperature, enabling imaging of the IVD [504]. The samples were then wrapped with parafilm and fitted into holders to ensure they were fixed in place while being imaged in the CT scanner using in-house optimized settings (Table 5.2.3), for osteochondral tissues with a resolution of $9\text{ }\mu\text{m}$. The exposure time was 500 ms, and 1800 images were acquired over 360° . The free and open source software, 3D Slicer 5.0.3 [505, 506], was used to visualize the scans and analyze them. Defects in the CEP-BEP interface were noted, and the maximum thicknesses of the BEP, CEP, and soft tissues (comprising both NP and AF) were calculated in each sample.

Although the endplates, especially the bony endplate regions, have been defined in different ways in the literature [507], standardized definitions were adopted for CT-based segmentation. The soft tissues, NP/AF, were identified as the medium CT intensity regions with smooth texture at one extremity of the explant. The CEP was divided into ncCEP, characterized by very low CT intensity between bone and soft tissue, and cCEP, the highest intensity layer separating the IVD from the VB; these two regions were separated by the tidemark, and the cCEP was separated from the BEP by the cement line. The BEP and subchondral trabecular bone (STB) showed similar intensities, but STB exhibited larger, marrow-filled pores. The epiphyseal growth plate, present only in younger bovines and humans, appeared as an uncalcified layer separating STB from metaphyseal trabecular bone (MTB). This clear definition allowed consistent measurement of

maximal thickness for each compartment (Figure 5.2.2). For subsequent analyses, “soft tissues” (Figure 5.2.2 tissue “a”) included NP, and AF, “CEP” included cCEP and ncCEP (Figure 5.2.2 tissues “b” and “c” respectively), whereas the VB region comprised BEP, STB, growth plate, and MTB (Figure 5.2.2 tissues “d”, “e”, “f”, and “g” respectively).

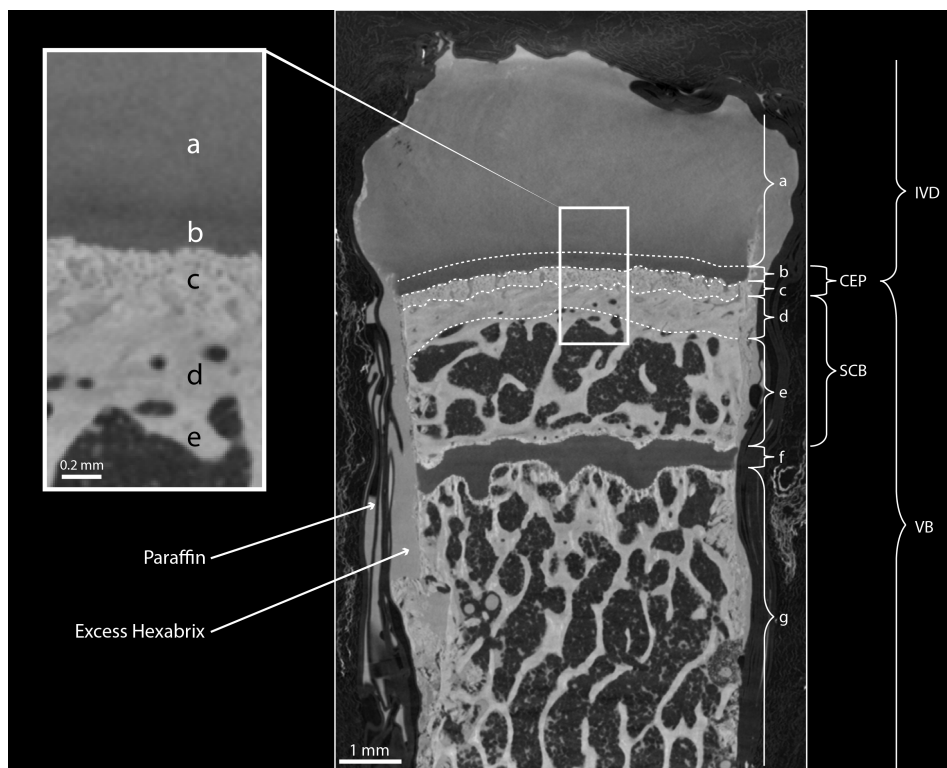


Figure 5.2.2: A labeled sample of the scanned explant in the μ CT where lowercase letters represent the different tissues (a) NP/AF, (b) ncCEP, (c) cCEP, (d) BEP, (e) STB, (f) growth plate, and (g) MTB. Scale bar = 1 mm.

Glycosaminoglycan (GAG) quantification

Downstream, the frozen samples were digested in papain solution from papaya (Sigma Aldrich P3125) for 12 hours at 60°C. The papain digestion solution was made using 0.31 mg/mL dithiothreitol (DTT) (Thermo Scientific R0861) and 1 U/mL of buffer solution that contained 20 mM sodium phosphate monobasic (Sigma Aldrich S9638), 20mM sodium phosphate dibasic (Sigma Aldrich S0876), and 1 mM EDTA·2H₂O. The digested samples, in addition to the collected media, were then used separately to quantify

Table 5.2.3: In-house optimized settings of μ CT scanner for osteochondral tissues standardized and used for all the explant samples.

X-ray Source Parameters		Acquisition Parameters		Reconstruction Parameters	
Voltage	60 kV	exposure time	0.5 s	bhc*	8
Current	170 μ A	voxel size	9 μ m		inline median
filter	0.2 mm Aluminum	fast scan	yes	filter	region of interest (ROI) CT filter
target	Diamond-coated Tungsten				filter Volume

*bhc: Beam Hardening Correction

GAG using the dimethylmethylene blue (DMMB) (Sigma Aldrich 341088) assay [508] with known concentrations of standard bovine cartilage chondroitin sulphate C solution (Sigma Aldrich C6737). The absorbance of the samples and the standards was measured at 525 and 595 using an enzyme-linked immunosorbent assay (ELISA) multimode plate reader (TECAN Infinite® 200 PRO). The difference of absorbance was used to construct the standard curve [509] and the coefficient of determination (R^2) was calculated to assess the linear regression line, which was 0.995 for both the GAGs in the tissues and GAGs in the media. Media of samples collected from each subject were pooled by condition prior to the quantitative analysis.

Deoxyribonucleic acid (DNA) quantification

To enable normalization to DNA content, the papain-digested samples used for GAG assessment were used to quantify DNA using the PicoGreen™ assay (Quant-iT™ PicoGreen™ dsDNA Assay Kits and dsDNA Reagents, Catalog number P7589, Thermo Fisher Scientific) following the instructions of the manufacturer. The fluorescence wavelengths were measured at excitation of 480 nm and emission of 520 nm on the same ELISA multimode plate reader. The standard curve was created using known concentrations of λ -DNA stock solution, and the R^2 was calculated, which was 0.998 for DNA. The GAG/DNA ratio was then determined.

Histological examination

Fixed samples were decalcified in 20% EDTA (Sigma Aldrich ED2SS) for three months, with 2–3 solution changes per week. Samples were then embedded in paraffin (VWR International 10048502) and sectioned at 5 μm using a Microtome HM355S (Thermo Fisher Scientific). Sections were mounted on Superfrost Microscope Slides (AG00008032E01MNZ20, Thermo Fisher Scientific) for histological staining. Four different staining techniques were used to assess the structure and composition of the explants. The slides were dewaxed using Histo-Clear (National Diagnostic HS-200) and methanol before being dipped in their respective stain and counterstains: Alcian blue (AB)/Nuclear Fast Red, safranin O (SafO)/Fast Green, hematoxylin and eosin (H&E), and Masson's trichrome (MTC). For all stainings, sections were first deparaffinized in Histo-Clear and rehydrated through graded alcohols to distilled water. For Alcian Blue staining, sections were incubated in 0.5% Alcian Blue solution (A3157 Sigma) at pH 1 for 60 minutes, rinsed, and counterstained with Nuclear Fast Red (642425 Klinipath) for 5 minutes. For Safranin O/Fast Green staining, sections were stained with hematoxylin for 1 minute, then acid alcohol for 5 seconds. Subsequently, sections were stained with Fast Green (Klinipath; 80051; color index 42053) for 5 minutes and 1% acetic acid for 30 seconds, followed by Safranin O staining (Klinipath 640780) for 10 minutes. For H&E staining, sections were stained with hematoxylin (Sigma Aldrich H9627) for 5 minutes, washed under running water, and counterstained with eosin (Clin-Tech 640381) for 7 minutes. For Masson's Trichrome staining, sections were refixed with Bouin's solution (Polysciences 16045) at 60°C for 1 hour, stained sequentially with Weigert's Iron Hematoxylin (Sigma HT1079) for 10 minutes, Biebrich Scarlet red (Sigma HT151) for 5 minutes, a 1:1 mixture of phosphotungstic (Sigma HT152) phosphomolybdic acid and (Sigma HT153) for 10 minute, and Aniline Blue (Sigma, B8563) for 5 minutes, followed by differentiation in 1% acetic acid for 1 minute. After staining, all sections were rinsed between steps in dH₂O, and after staining was performed, then dehydrated through graded ethanol, Histo-Clear, and mounted with Pertex (Histolab 00801-EX).

All samples were processed and stained on the same day using the same staining solution batches to ensure consistent conditions and comparable results. All samples were imaged using a brightfield microscope (Olympus IX83, Olympus Corporation, Tokyo, Japan) equipped with an Olympus DP73 digital camera and Olympus cellSens Dimension image acquisition software (version 1.18).

Stained area and staining intensities in AB, Safo, and MTC images were quantified using Fiji ImageJ [510]. To reduce background-related false positives, AB and MTC images were color-shifted to red, and Safo images to blue. Color deconvolution [511] was then performed to digitally isolate the stains using built-in ImageJ parameters specific to each stain. The CEP was traced to define the ROI. Binary masks were generated by applying automatic thresholding to the primary stain channel, using the Huang, Rényi entropy, and Mean methods for AB, Safo, and MTC, respectively. The stained area percentage within the ROI was measured from the binary mask, while the mean color intensity was extracted directly from the primary stain channel. The full set of algorithms covering these tools is available in a public GitHub repository [512], along with a user manual.

Since no standardized degeneration scoring system exists specifically for the bovine EP, the system used (Table 5.2.4) was an adaptation based on commonly reported parameters from several human and large animal models [513–517], focusing only on the CEP and BEP regions. Each sample was assigned a score from 0 to 3 for each of six criteria related to EP degeneration, where 0 indicated no degeneration, 1 indicated early/mild degeneration, 2 indicated moderate degeneration, and 3 indicated severe degeneration, resulting in a total possible degeneration score of 18. CEP cellularity was assessed based on clustering, as well as features of neovascularization. Cellular degeneration was evaluated by identifying hallmarks of apoptosis, necrosis, senescence, and cell loss. CEP lesions were characterized by cartilage erosion, localized thinning, fissures, erosions or nodes, and avulsion from the BEP. ECM structure of the CEP was evaluated for cartilage thinning, structural disorganization, loss of boundary demarcation (tide-mark and cement lines), fibrocartilage infiltration, and calcification. BEP cellularity was evaluated based on bone marrow composition, specifically the presence of red bone marrow (RBM) versus adipocytes indicative of fatty yellow bone marrow (YBM), as well as signs of neovascularization. BEP lesions included changes in demarcation, enthesis integrity, and erosions or nodules. Finally, the ECM of the BEP structure was assessed for the presence of erosive nodules, trabecular remodeling, changes in thickness, cartilage or fibrocartilage formation, subchondral bone sclerosis, and bone remodeling, including osteophyte formation.

Table 5.2.4: Hematoxylin and eosin (H&E) degeneration scoring system based on large animal models [514–516], focusing on cellularity, lesions, and extracellular matrix (ECM) structure of CEP (a) and BEP (b).

(a)

Location		CEP	
Categories	Cellularity	Lesions	ECM Structure
Markers	Cellular proliferation (clusters), Neovascularization	Cartilage erosion, localized thinning, fissures, erosions or nodes, avulsion from BEP	Cartilage thinning, structural disorganization, boundary demarcation, fibrocartilage infiltration, and calcification.
0 absence of degeneration	Single cells within lacunae, with no signs of necrosis, apoptosis, or senescence.	None.	Uniform thickness with a distinct boundary with BEP.
1 early/mild degeneration	Few instances of multiple nuclei within lacunae, with possible signs of apoptosis and cell shrinkage.	Few areas of localized erosion.	Some localized thinning and disorganization.
2 moderate degeneration	Several of multiple nuclei are present within lacunae, but without clustering, possible signs of apoptosis, necrosis, and cellular senescence, moderate cell loss, and neovascularization.	Moderate thinning, presence of fissures/nodules, and localized avulsion from the BEP.	Moderate calcification, fibrocartilage infiltration, and structural disorganization.
3 severe degeneration	Abundant clustering of clonally derived cells, with evidence of cell loss, and possible signs of apoptotic, necrotic, and senescent cells, and extensive neovascularization.	Significant loss of thickness, large fissures, and extensive areas of avulsion from the BEP.	Extensive calcification and fibrocartilage infiltration.

(b)

Location		BEP	
Categories	Cellularity	Lesions	ECM Structure
Markers	Bone marrow alterations and neovascularization.	Demarcation, enthesis state, erosions or nodules, and osteophyte formation.	Erosive nodules, trabecular remodeling, changes in thickness, cartilage formation, sclerotic subchondral bone, cartilage or fibrocartilage, bone remodeling, and osteophyte formation.
0 absence of degeneration	Hematopoietic bone marrow. RBM instead of YBM	A distinct boundary with the CEP.	Thin subchondral bone plate with distinct vertebral trabecular elements.
1 early/mild degeneration	Localized areas of fibrovascular/fatty YBM at sites of CEP damage.	Scattered irregularities at the CEP interface.	Small node with rupture of the BEP. Limited areas of fibrocartilage infiltration into the subchondral bone, with or without a sclerotic margin.
2 moderate degeneration	Moderate-sized areas of fibrovascular and/or fatty bone marrow extending into the VB.	Moderate irregularities at the CEP boundary.	Moderately sized nodes and/or erosions, with adjacent sclerosis or fibrovascular changes in the bone marrow. Sclerosis of the subchondral bone plate.
3 severe degeneration	Extensive fibrovascular bone marrow changes.	Erosive nodules extending into the vertebra, with a BEP compression fracture.	Extensive bony sclerosis.

Images of the full CEP section were taken using the Multi-position Mosaic feature. The CEP ROI was then traced as mentioned above (section: “Histological Examination”), and its area was calculated, and the nucleus of each cell in the ROI was labeled using the multi-point tool. All nuclei were labeled, including the ones showing signs of apoptotic, necrotic, and/or senescent cells, but empty lacunae were disregarded. The count was then measured and divided by the area to get the cell density of each sample.

Immunohistochemistry

Additional sections were prepared as described above (section: “Histological Examination”) and mounted on charged slides for IHC. IHC was performed using 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma Aldrich D5905) staining following the protocol described by Binch et al. [518]. The sections were deparaffinized, rehydrated, and endogenous peroxidase activity was blocked prior to antigen retrieval. Antigen retrieval was performed using one of three methods: none, enzyme, or heat (Table 5.2.5). For enzyme retrieval, sections were incubated in TBS containing 0.1% (w/v) CaCl_2 dihydrate (Honeywell 223 506) and 0.01% (w/v) α -chymotrypsin (Sigma Aldrich 1002472729) for 30 minutes at 37°C. For heat retrieval, sections were submerged in 0.05 M Tris (Fisher T/3710/60) at pH = 9.5 preheated to 60°C, irradiated in an 800 W microwave for 5 minutes at 40% power, left to stand for 1 minute, irradiated for another 5 minutes at 20% power, and then left to cool for 15 minutes at room temperature. Following antigen retrieval, sections were washed in TBS. Non-specific antibody-protein interactions were blocked using normal serum matched to the secondary antibody species for 1 hour at room temperature in a humidified staining tray to prevent drying. Primary antibodies were applied overnight at 4°C. The sections were incubated with primary antibodies against anabolic matrix staining markers collagen type II (COL2) [519] and ACAN [520] in addition to catabolic cytokines IL-1 β and TNF, and matrix-degrading enzymes matrix metalloproteinase 3 (MMP3) and a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS4) [508–511] (Table 5.2.5). Parallel sections were incubated with species-matched immunoglobulin G (IgG) controls (rabbit, Abcam ab37415; mouse, Abcam ab18394; Goat, Abcam ab37373) at equivalent concentrations. The following day, sections were washed in TBS and incubated with secondary antibodies (Table 5.2.5) for 30 minutes. After further TBS washes, avidin–biotin complex (ABC) Elite reagent (Vector Laboratories PK-7100) was applied for 30 minutes. Sections were washed again in TBS, and DAB solution (0.08% H_2O_2 with 0.65 mg/mL DAB in TBS) was applied for 20 minutes. Sections were then washed under running cold tap water for 5 minutes, counterstained with Mayer's hematoxylin (Leica Biosystems 3801582E) for 1 minute, and blued under running tap water for another 5 minutes. Finally, sections were dehydrated in ethanol, cleared in Xylene (Sigma Aldrich 214736), and mounted with coverslips using Pertex (Leica Biosystems 3808707E). Each TBS wash was repeated 3 times for 5 minutes, and the primary and secondary antibodies were purchased from Abcam, Cambridge, UK, or Invitrogen (Table 5.2.5). Quantification of immunostaining was performed using a QuPath [521]-based semi-automatic

pipeline [522] optimized for low-cellularity tissues. To enable assessment of the CEP response and potential cross-talk with adjacent tissues, regions of interest were defined for the NP/AF, CEP, and BEP of each sample. The stain vector was automatically estimated, cells were automatically detected, and the classifier was trained using 15 representative regions to distinguish positive cells, negative cells, and false detections. Samples containing fewer than 150 cells were excluded to prevent statistical bias, and immunopositivity was expressed as the percentage of positive cells for each antigen and tissue region.

Table 5.2.5: Primary and secondary antibodies used for immunohistochemical (IHC) staining with their clonality, dilution, and antigen retrieval methods.

Target antibody	Clonality	Dilution	Antigen retrieval	Secondary antibody	Dilution
MMP3 (ab53015)	Rabbit polyclonal	1:400	Enzyme	Goat anti rabbit (ab6720)	1:500
Aggrecan (ab3778)	Mouse monoclonal	1:200	Heat	Rabbit anti mouse (ab6727)	1:500
Collagen type II (ab3092)	Mouse monoclonal	1:100	Enzyme	Rabbit anti mouse (ab6727)	1:500
ADAMTS4 (ab185722)	Rabbit polyclonal	1:200	None	Goat anti rabbit (ab6720)	1:500
IL-1 β (Invitrogen M421B)	Mouse Monoclonal	1:100	Heat	Rabbit anti mouse (ab6727)	1:500
TNF (ab6671)	Rabbit polyclonal	1:200	Heat	Goat anti rabbit (ab6720)	1:500

Statistical analysis

GraphPad Prism version 9.0.0 for Windows (GraphPad Software, Boston, Massachusetts, USA) was used to conduct normality tests and statistical analyses. A normality test was performed to determine the type of statistical tests that could be conducted on the results of each downstream analysis. GAG/DNA passed both the Shapiro-Wilk and the Kolmogorov-Smirnov normality test, and, therefore, parametric significance tests were used to assess the effects of the environments on the samples. GAG/DNA in the tissues had only one variable, which was the environment, so a one-way analysis of variance (ANOVA) was performed, while GAG/DNA released in the media had an additional variable, time, so a two-way ANOVA test was performed. After the ANOVA test, Tukey's multiple comparisons test was performed to check which environment exactly differs from the rest.

Due to the bounded nature of the degeneration scores (0 to 18) and IHC percentage data (0 to 100%), the non-parametric Kruskal-Wallis test was performed, followed by Dunn's multiple comparisons test.

5.3 Results

5.3.1 *In silico* modeling predicts cytokine requirement for degeneration

Simulating degenerative conditions in the absence of cytokines predicted a negligible transition to a degenerative phenotype, with fewer than 1% of chondrocytes affected (2/1100). In contrast, inclusion of cytokines resulted in a complete shift, with 100% of chondrocytes exhibiting a degenerative phenotype. At the protein level within the *in silico* model (Figure 5.A.1a), 25 out of 74 components were significantly different from healthy controls under degenerative conditions without cytokines, whereas 55 differed from degenerated controls. With cytokines, this pattern was reversed, with 55 components differing from healthy controls and only 1 from degenerated controls. A similar trend was observed at the gene level within the *in silico* model (Figure 5.A.1b). Without cytokines, 21 components differed significantly from healthy controls and 46 from degenerated controls. While with cytokines, 47 components differed from healthy controls, and only 1 from degenerated controls. When assessed globally (Figure 5.3.1 and Figure 5.A.1c), degenerative conditions without cytokines resulted in 35 components differing from healthy controls and 73 from degenerated controls. In contrast, with cytokines, all 74 components differed from healthy controls and none from degenerated controls.

5.3.2 Downstream analysis

CT scanning showed structural stability after 14 days of culture within *ex vivo* models

CT scans revealed that the primary difference among the three bovine subjects was the absence of a visible growth plate in the VB of bovine 2. In contrast, all samples from bovines 1 and 3 exhibited a clearly identifiable

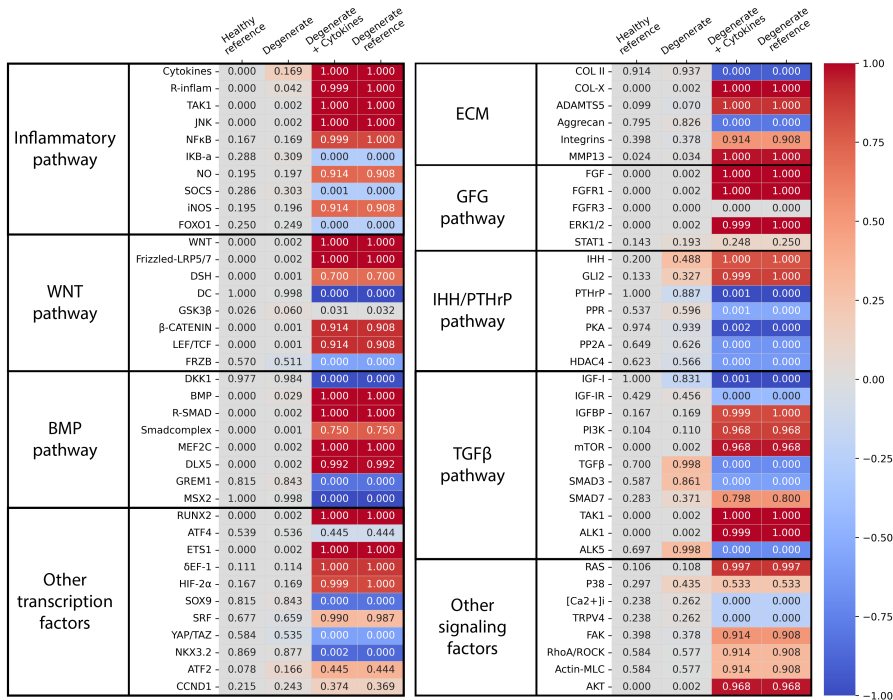


Figure 5.3.1: Normalized global activity of all the monitored components as predicted by the *in silico* model for the reference healthy chondrocytes (healthy control), chondrocytes in degenerative conditions without cytokines, chondrocytes in degenerative conditions with cytokines, and the reference degenerated chondrocytes (degenerated control). Controls have been defined in previous studies [127, 128]. The heatmap shows the deviation (in absolute values) of each component from the reference healthy environment.

growth plate, located consistently within approximately 2 mm from the BEP, with average distances of 2.32 mm for bovine 1 and 1.82 mm for bovine 3. Although some samples from bovine 2 had bone heights exceeding 11 mm, no visible growth plate was detected. This suggests that bovine 2 may have been more skeletally mature. However, structural features of the growth plate region were still distinguishable in these samples. These features were located at a similar position relative to the BEP as in bovines 1 and 3, with an estimated closed growth plate distance of 2.05 mm in bovine 2, based on anatomical landmarks (Figure 5.A.2). One sample of bovine 2 of the degenerate + IL-1 β condition had multiple Schmorl's nodes where the BEP–CEP boundary was not well defined. That sample had extreme thinning of the cartilage, and so did one sample of the same bovine of the degenerate + TNF condition, although that one did not show Schmorl's nodes, so these two samples were discarded from the analysis as the features represented donor variation rather than effects of culture conditions. All other samples showed a clear distinction between the bone, cartilage, and soft tissues. Aside from the STB, the thickness of each tissue was comparable between the samples without statistical significance, regardless of the environment ($p > 0.05$) (Figure 5.3.2). One sample of the same bovine in the degenerate + IL-1 β condition did show a small section with unusual calcification, but still had clear tissue boundaries. The thickness of each tissue for all the samples were on average, 3.9 mm for the soft tissues, 0.345 mm for the ncCEP, 0.304 mm for the cCEP, 0.701 mm for the BEP, and 2.072 mm for the STB.

GAG/DNA did not change in the tissues during culture, but TNF induced GAG release to the medium

On average, all three bovine cultures showed comparable GAG/DNA levels in the tissue on day 0, and all cultured samples for 14 days in standard medium, and degenerate groups with no significant differences in GAG/DNA seen between bovine donors (Figure 5.3.3a).

There were clear significant effects of both environment and time on the cumulative GAG/DNA secretion ($P < 0.001$ for both environment and time) (Figure 5.3.3b). Post-hoc analysis, using Tukey's multiple comparisons test, showed that at days 3, 5, and 7, none of the pairwise comparisons reached statistical significance ($P > 0.05$). From day 10 onward, the degenerate + TNF condition exhibited significantly higher cumulative GAG/DNA release compared to standard conditions ($P = 0.009$, $P = 0.001$, and $P < 0.001$ for

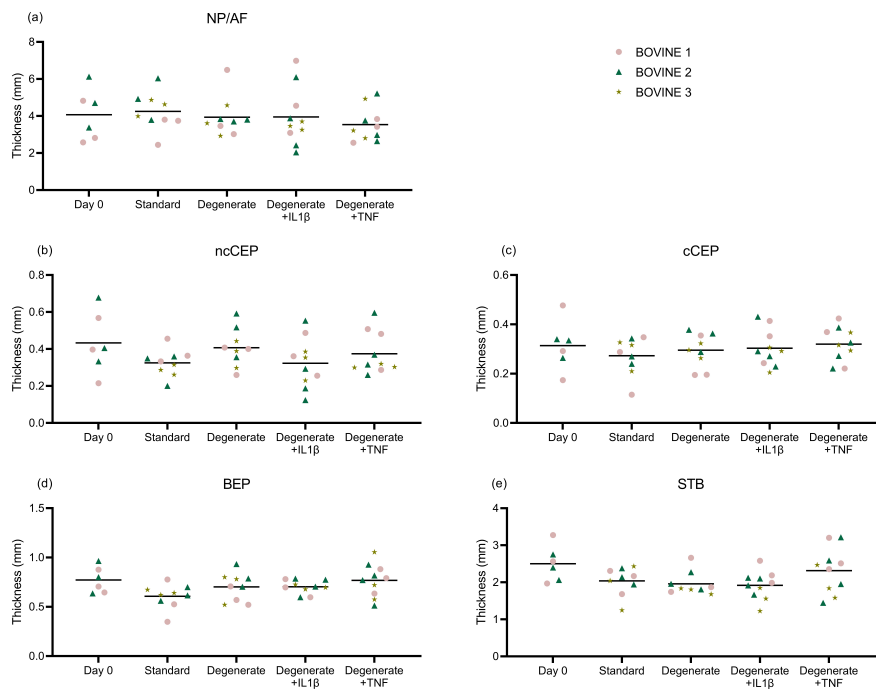


Figure 5.3.2: Thickness of (a) NP/AF, (b) ncCEP, (c) cCEP, (d) BEP, and (e) STB, in the explants as measured in the μ CT scans using 3D Slicer. Standard, degenerate, degenerate + IL-1 β , and degenerate + TNF media are all measured at day 14.

days 10, 12, and 14, respectively) while the degenerate and degenerate + IL-1 β differences remained insignificant compared to standard conditions at all measured days.

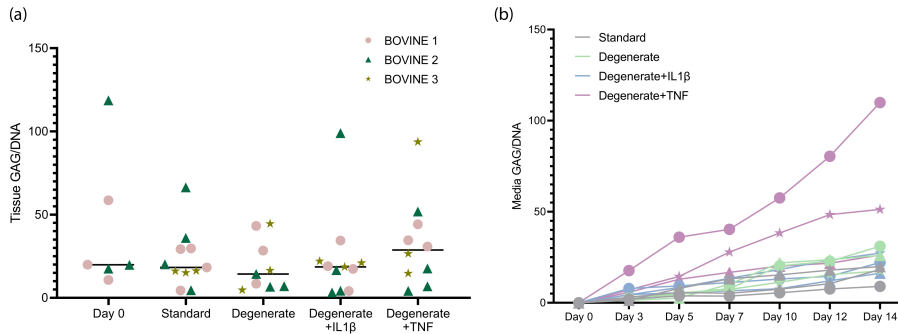


Figure 5.3.3: Quantification of (a) GAG/DNA in the tissues of the digested explants for the three bovines in each environment where standard, degenerate, degenerate + IL-1 β , and degenerate + TNF media are all measured at day 14, and (b) cumulative GAG/DNA released in each medium by all three bovine explant samples, note media samples for equivalent treatment groups were pooled at each time point for analysis. Statistical analysis was performed and significant effects of environment and time were observed for cumulative GAG/DNA release (b) where in the GAG/DNA in the degenerate + TNF media were significantly greater compared to the standard group at days 10, 12, and 14.

Histological examination showed decreased matrix staining when cultured in cytokine-simulated media

Macroscopically, AB, Safo, and MTC stainings were more intense in the samples of day 0 conditions, followed by standard, then degenerative samples. The distinction between the CEP and surrounding soft tissues was more evident under day 0 conditions, with the soft tissues exhibiting stronger AB staining than the CEP, while the CEP exhibited stronger Safo staining compared to the NP/AF regions. By day 14, this contrast was reduced. While still observable in the standard medium samples, it was notably diminished in the degenerative conditions (Figures 5.3.4a, 5.3.5a, and 5.3.6a).

The staining area and the staining intensity were quantified for both the CEP and the soft tissues (NP/AF). Concerning the CEP, quantification of the stained area for AB showed similar percentages across all conditions ($p > 0.05$) (Figure 5.3.4.bi). However, staining intensity significantly decreased in the three degenerate media treatment groups, particularly in the degenerate

+ TNF medium, while remaining comparable between the day 0 condition and the standard medium following 14 days ($p = 0.414$ for standard, $p < 0.001$ for degenerate, $p < 0.001$ for degenerate + IL-1 β , and $p < 0.001$ for degenerate + TNF compared to day 0) (Figure 5.3.4.bii). Concerning the NP/AF tissues, the stained area for AB also showed similar percentages across all conditions ($p > 0.05$) (Figure 5.3.4.ci) but the staining intensity dropped significantly in all conditions compared to day 0 ($p = 0.03$ for standard, $p = 0.001$ for degenerate, $p = 0.001$ for degenerate + IL-1 β , and $p = 0.003$ for degenerate + TNF compared to day 0) (Figure 5.3.4.cii).

In contrast, SafO exhibited a significant reduction in both stained area (Figure 5.3.5.bi) and intensity (Figure 5.3.5.bii) across all media compared to the day 0 condition, with the most pronounced decreases observed in the 3 degenerate media conditions, especially the degenerate + IL-1 β and degenerate + TNF (for stain area percentage $p = 0.019$ for standard, $p < 0.001$ for degenerate, $p < 0.001$ for degenerate + IL-1 β , and $p < 0.001$ for degenerate + TNF compared to day 0 while for stain intensity $p = 0.028$ for standard, $p = 0.001$ for degenerate, $p = 0.001$ for degenerate + IL-1 β , and $p < 0.001$ for degenerate + TNF compared to day 0). Concerning the NP/AF tissues, SafO exhibited a significant reduction in both stained area (Figure 5.3.5.ci) and intensity (Figure 5.3.5.cii) across all media compared to the day 0 (for stain area percentage $p = 0.001$ for standard, $p < 0.001$ for degenerate, $p < 0.001$ for degenerate + IL-1 β , and $p < 0.001$ for degenerate + TNF compared to day 0 while for stain intensity $p = 0.004$ for standard, $p < 0.001$ for degenerate, $p < 0.001$ for degenerate + IL-1 β , and $p < 0.001$ for degenerate + TNF compared to day 0).

MTC showed consistent stained area percentages across the day 0 conditions, standard, and degenerate media, with higher values in the cytokine conditions ($p < 0.05$ for standard, $p < 0.05$ for degenerate, $p = 0.025$ for degenerate + IL-1 β , and $p = 0.031$ for degenerate + TNF compared to day 0) (Figure 5.3.6.bi). Yet, MTC staining intensity significantly declined in all media, most notably in the degenerate + IL-1 β condition ($p = 0.05$ for standard, $p = 0.007$ for degenerate, $p < 0.001$ for degenerate + IL-1 β , and $p = 0.002$ for degenerate + TNF compared to day 0) (Figure 5.3.6.bii). Of the degenerate media, degenerate + IL-1 β showed the least AB intensity, while degenerate + TNF showed the least MTC intensity, and both degenerate + IL-1 β and degenerate + TNF equally showed the lowest intensity in SafO compared to the degenerate medium without cytokines. Concerning the NP/AF tissues, MTC showed consistent stained area percentages (Figure 5.3.6.ci) and staining intensity (Figure 5.3.6.cii) across all conditions

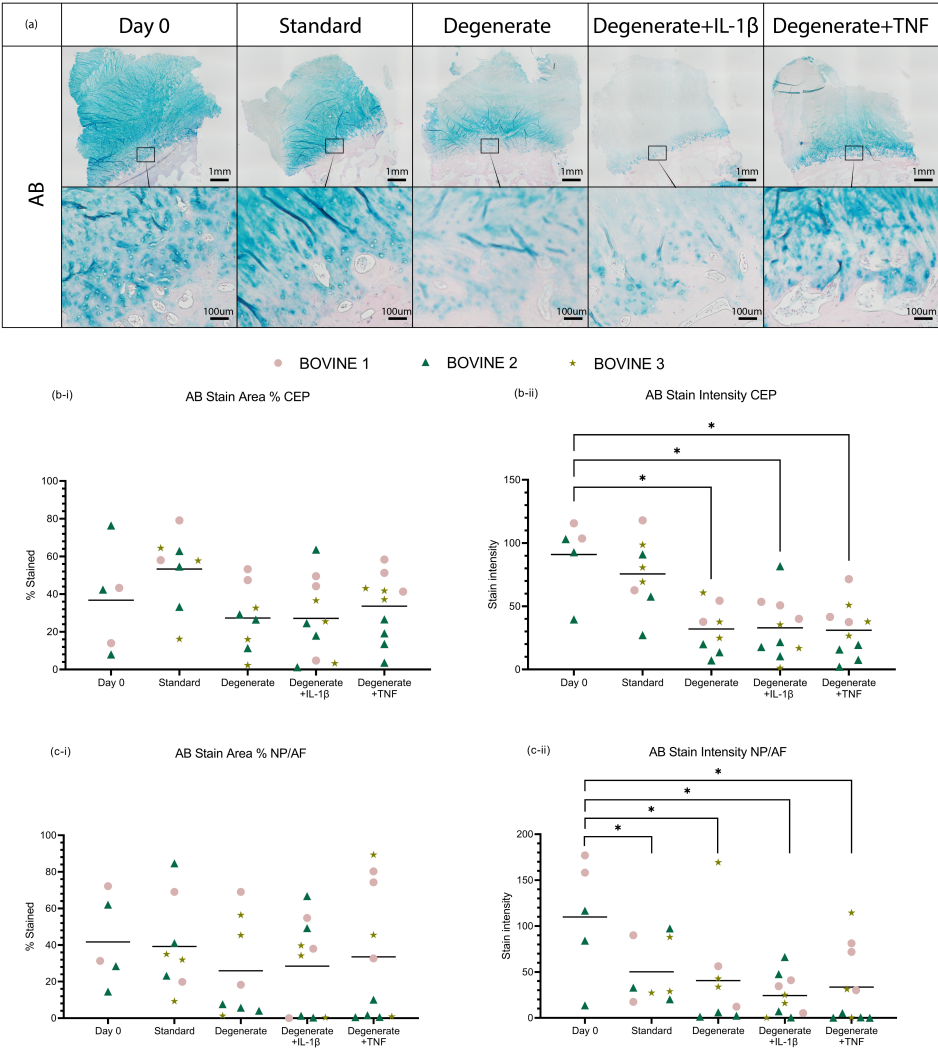


Figure 5.3.4: AB stain (a) representative samples with (i) the quantified percentage stained area and (ii) staining intensity in the (b) CEP and the (c) NP/AF between the 5 different media conditions. The box shows the location of the magnified region below each figure. Scale bar = 1 mm for the figures and 100 μ m for the magnified regions. Standard, degenerate, degenerate + IL-1 β , and degenerate + TNF media are all measured at day 14. $\ast = P < 0.05$.

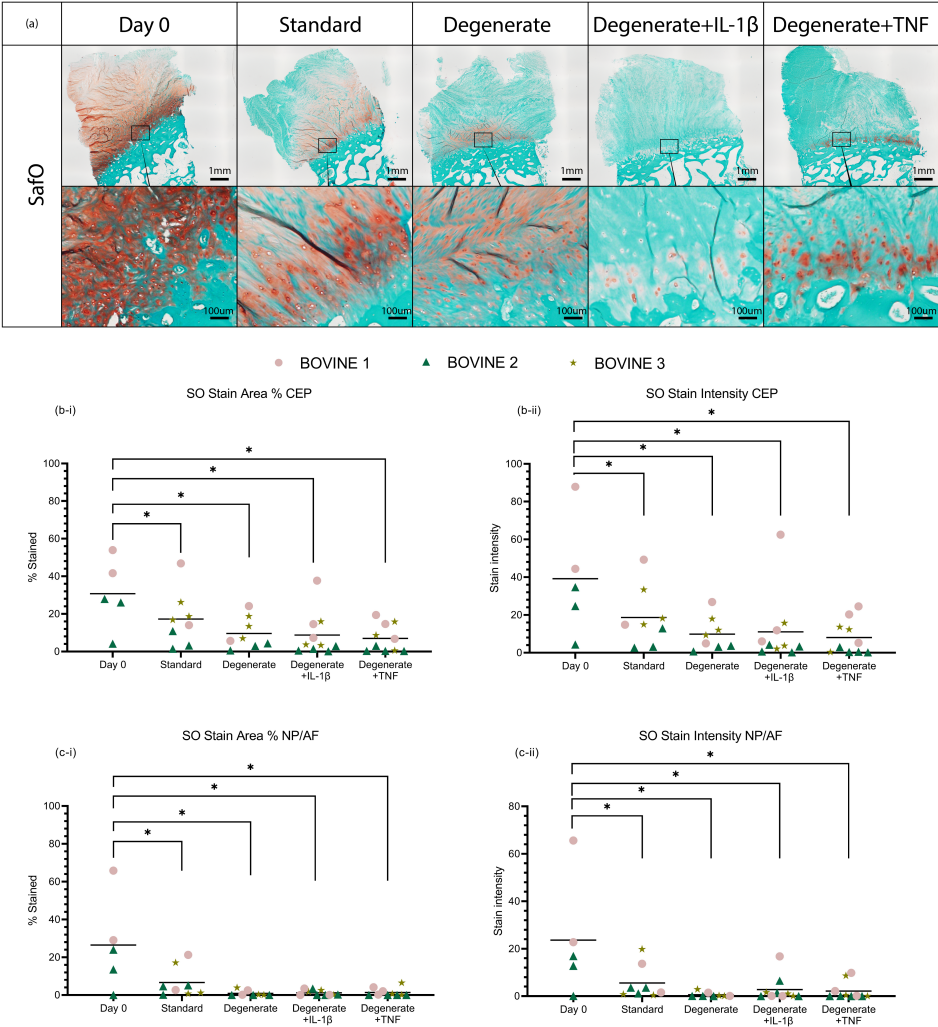


Figure 5.3.5: Safo stain (a) representative samples with (i) the quantified percentage stained area and (ii) staining intensity in the (b) CEP and the (c) NP/AF between the 5 different media conditions. The box shows the location of the magnified region below each figure. Scale bar = 1 mm for the figures and 100 μ m for the magnified regions. Standard, degenerate, degenerate + IL-1 β , and degenerate + TNF media are all measured at day 14. $\ast=P < 0.05$.

($p < 0.05$ compared to day 0).

There was no clear division between the ncCEP and the NP in AB and H&E due to their similar biochemical composition. Therefore, MTC staining was used to differentiate between the CEP and the NP since the intensity of the blue stain was relative to the collagen content, where the NP was pale blue, almost white, while the CEP showed dark staining; however, staining intensity of the CEP was indistinguishable from the AF staining intensity (Figure 5.3.7).

All the samples showed a well-organized subchondral bone with a clear cement line that divided the cartilaginous part and the osteogenic part of the sample. Tidemarks were visible in most of the explant samples, especially in H&E staining, where they appeared as a thin, wavy purple line (Figure 5.3.7a). That was clearly shown in the Safo staining, where the bone was dark blue while the cCEP was pale bluish-pink (Figure 5.3.7b).

All the samples showed uniformly distributed scattered BEP osteocytes and dense CEP chondrocytes, but only the day 0 and standard medium samples had uniform organization of CEP chondrocytes, while the degenerative samples showed more clustering of these cells (Figure 5.3.8).

They all showed an abundance of chondrocytes and osteocytes in their respective regions. Osteoclasts and Osteoblasts were present in a very small number, but appeared to be more in the degenerative samples than the day 0 conditions and standard medium samples, although this was not quantified.

Degeneration scoring of the vertebral endplate (Figure 5.3.9) revealed a clear and progressive increase across experimental conditions. Statistically significant differences were observed between all sequential groups, with scores increasing from day 0 to standard culture, from standard to degenerate conditions, and further from degenerate to cytokine-treated groups (all $p < 0.001$) (Figure 5.3.10).

Specifically, histological features of degeneration in both cytokine-induced conditions (degenerate + IL-1 β , and degenerate + TNF) were significantly higher than day 0 and standard conditions ($P < 0.001$) (Figure 5.3.10a). Histological features of degeneration in the degenerate + IL-1 β was also significantly higher than the degenerate condition without cytokines ($P < 0.05$), while degenerate + TNF did not show a significant difference compared to the degenerate condition without cytokines (Figure 5.3.10a). No significant

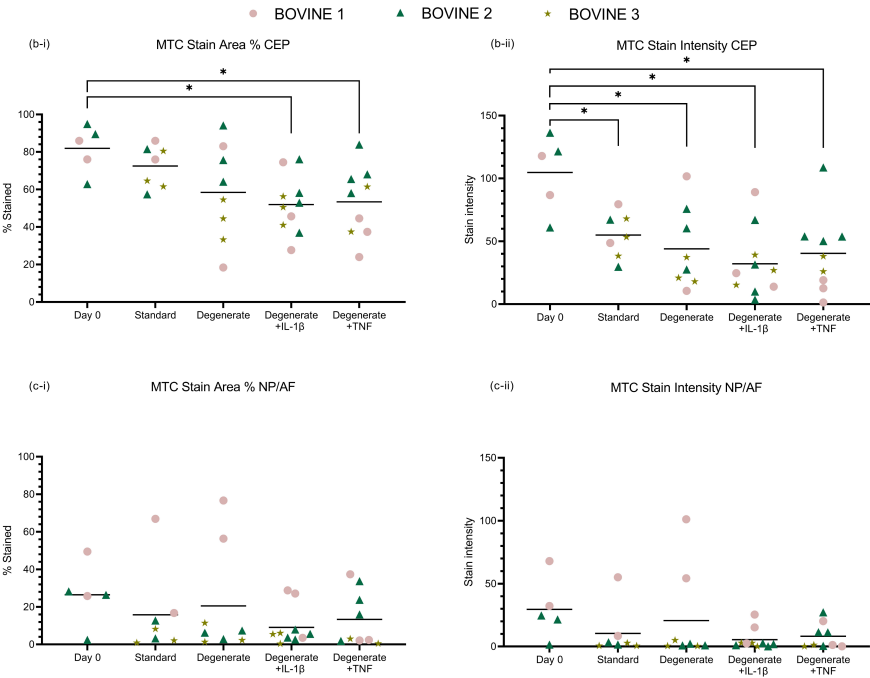
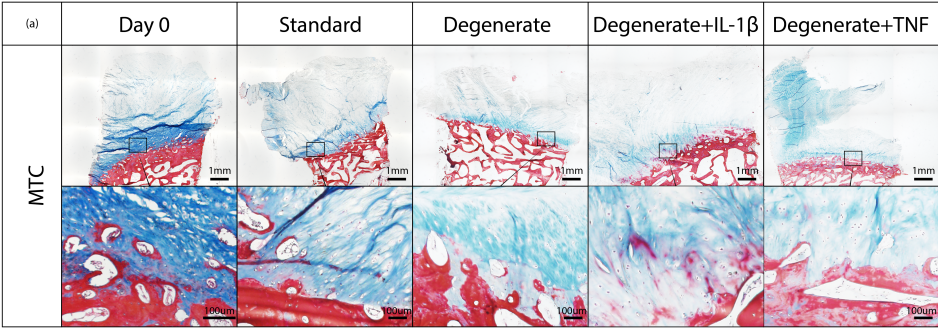


Figure 5.3.6: MTC stain (a) representative samples with (i) the quantified percentage stained area and (ii) staining intensity in the (b) CEP and the (c) NP/AF between the 5 different media conditions. The box shows the location of the magnified region below each figure. Scale bar = 1 mm for the figures and 100 μm for the magnified regions. Standard, degenerate, degenerate + IL-1 β , and degenerate + TNF media are all measured at day 14. $\ast = P < 0.05$.

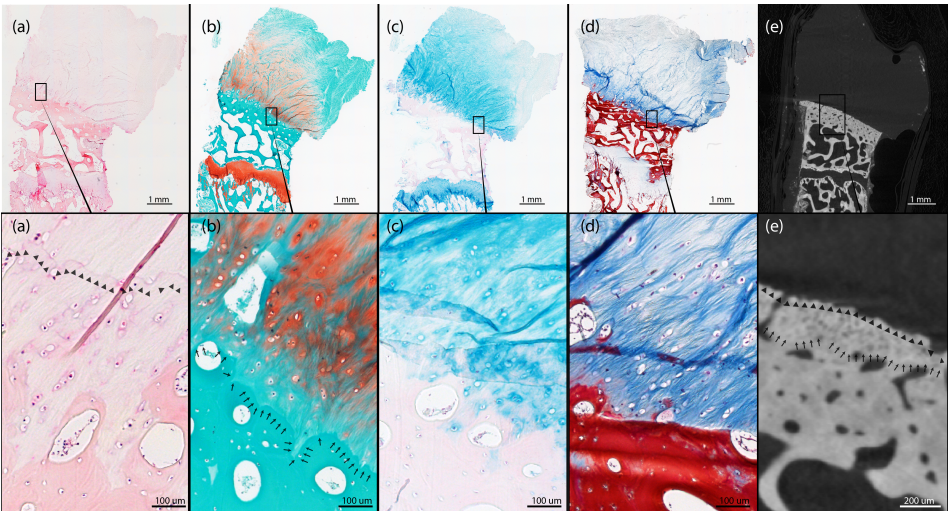


Figure 5.3.7: Histological staining of (a) H&E, (b) SafO, (c) AB, and (d) MTC in addition to (e) the μ CT scan counterpart, where $\blacktriangle$ traces the tidemark and $\uparrow$ represents the cement line. This is a sample from the standard condition.

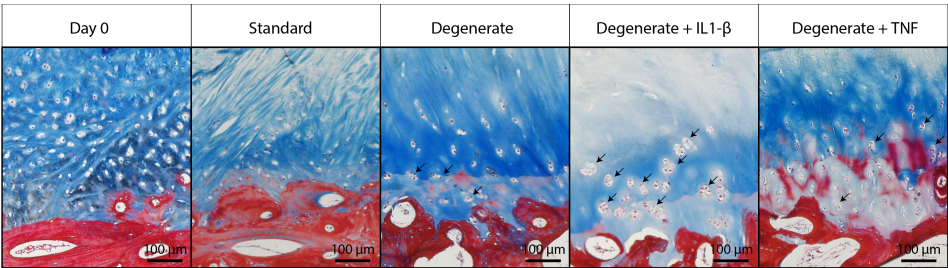


Figure 5.3.8: MTC staining showing uniformly distributed chondrocytes in day 0 and standard medium samples vs clustered chondrocytes in samples in degenerative media with and without cytokines. The arrows indicate the clustering of 3 or more chondrocytes.

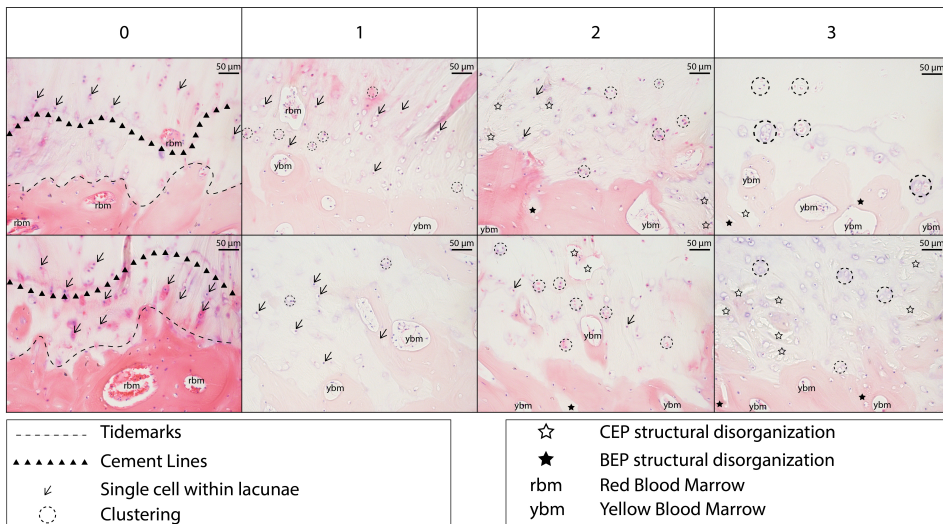


Figure 5.3.9: Two representative examples (rows) of the H&E stained CEP and BEP Sections by degeneration score from 0 to 3 (columns) based on the system presented in Table 5.2.4. Scale bar = 50 μ m.

difference was observed between the degenerate + IL-1 β and degenerate + TNF groups ($p > 0.05$) (Figure 5.3.10a), indicating comparable effects of both cytokines on endplate degeneration.

The day 0 chondrocyte cell density in the CEP averaged approximately 300 cells/mm². Relative to day 0, a significant reduction in cell density was observed in degenerate ($p = 0.014$), degenerate + IL-1 β ($p = 0.007$), and degenerate + TNF ($p < 0.001$) conditions (Figure 5.3.10b), while no significant difference was found between day 0 and standard ($p > 0.05$) (Figure 5.3.10b). Similarly, compared to standard conditions, cell density was significantly reduced in degenerate ($p = 0.038$), degenerate + IL-1 β ($p = 0.020$), and degenerate + TNF ($p < 0.001$) (Figure 5.3.10b).

No significant differences were detected among degenerate, degenerate + IL-1 β , and degenerate + TNF groups (all $p > 0.05$) (Figure 5.3.10b), indicating that although degenerative conditions and cytokine exposure reduced cell density relative to baseline and standard culture, their effects were not significantly different from each other.

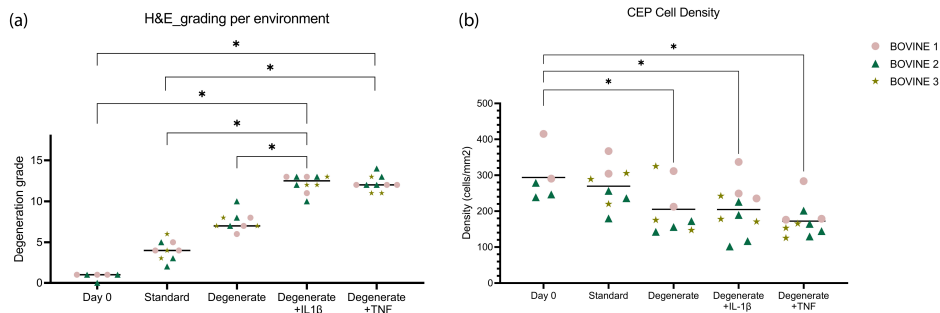


Figure 5.3.10: (a) Degeneration scoring grades for the three bovines in their different environments. (b) The chondrocyte cell density of the CEP in the three bovines in their different environments. Standard, degenerate, degenerate + IL-1 β , and degenerate + TNF media are all measured at day 14. *= $P < 0.05$.

IHC showed responses in CEP and NP/AF tissues but not in BEP

Immunohistochemical analysis (Figure 5.3.11) revealed distinct spatial patterns across anabolic, catabolic, and matrix-degrading markers. Anabolic markers were primarily localized to the CEP, with ACAN showing the highest immunopositivity in the CEP, moderate staining in the NP and AF, and low levels in the BEP, while COL2 was strongly expressed in all regions, with the highest levels in the BEP. Catabolic markers were predominantly detected in the NP/AF and CEP, with both IL-1 β and TNF showing strong immunopositivity in these regions and comparatively lower expression in the BEP. In contrast, matrix-degrading enzymes were more prominent in the BEP and NP/AF, as ADAMTS4 expression was high in these regions but low in the CEP, and MMP3 followed a similar trend, with the highest immunopositivity in the BEP, moderate levels in the NP/AF, and low expression in the CEP.

Cytokine stimulation produced highly tissue-dependent effects across anabolic and catabolic markers (Figure 5.3.12). In the CEP, regarding anabolic markers, ACAN remained the most abundant marker, showing a decreasing trend following culture, but did not reach significance between conditions ($P > 0.05$) (Figure 5.3.12a-i). For COL2, there was an increasing trend following culture where immunostaining was significantly higher in the standard conditions ($p = 0.013$) and the degenerate + IL-1 β conditions ($p = 0.0045$) compared with day 0, and was significantly higher in the degenerate + IL-1 β conditions compared with the degenerate conditions without

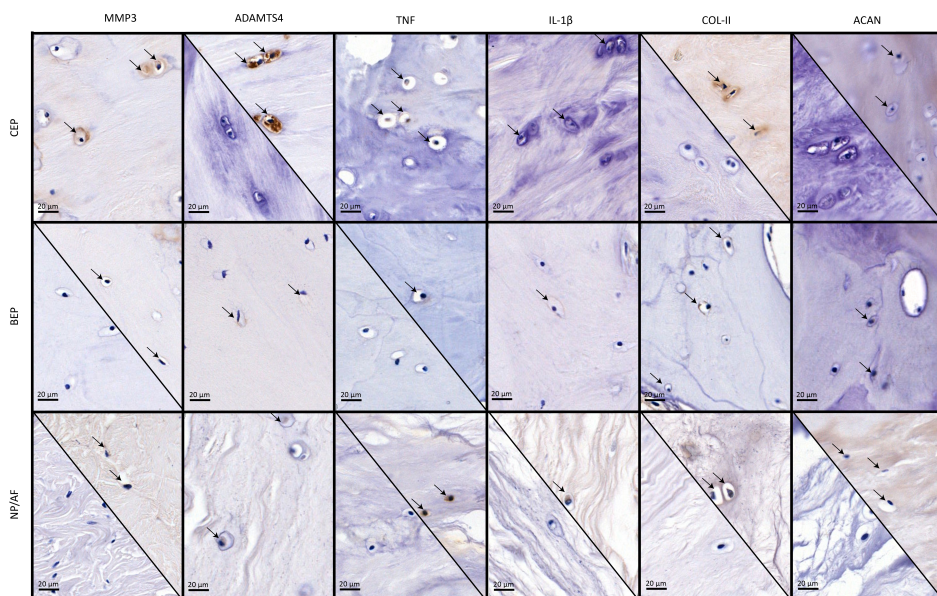


Figure 5.3.11: Immunohistochemical cell positivity and negativity of anabolic markers ACAN and COL2, catabolic markers IL-1 β and TNF, and matrix-degrading markers ADAMTS4 and MMP3 in the CEP, BEP, and NP/AF. The black arrows (→) show the positively stained cells in each tissue for each antibody. When positive and negative cells were not present within the same field of view, representative regions were shown separately within the same panel and divided by a diagonal line, with the positive results shown in the bottom triangle. Scale bars = 20 μ m.

cytokines ($P = 0.02$) (Figure 5.3.12a-ii). Among catabolic cytokines, IL-1 β immunopositivity showed a decreasing trend in all conditions compared to day 0, especially in degenerate + IL-1 β conditions ($P = 0.04$), but failed to reach significance in other conditions (Figure 5.3.12b-i). For TNF, there was a decreasing trend in immunostaining for all conditions except degenerate + TNF conditions compared to day 0, but they failed to reach significance (Figure 5.3.12b-ii). For matrix-degrading enzymes, ADAMTS4 immunostaining was significantly lower in all cytokine conditions (degenerate + IL-1 β and degenerate + TNF) compared to day 0 ($P = 0.002$ with both conditions) (Figure 5.3.12c-i). MMP3 showed an increasing trend under degenerative conditions with and without cytokines compared to day 0, but failed to reach significance (Figure 5.3.12c-ii).

In the BEP, regarding anabolic markers, ACAN decreased overall compared to day 0 but did not reach significance (Figure 5.3.13a-iii). In contrast, COL2 immunostaining increased compared to day 0, where it was significant compared to standard conditions ($P = 0.02$). There was also a significant decrease in the Degenerate + TNF condition compared to the standard condition (Figure 5.3.13a-iv). Regarding catabolic cytokines, no significant differences in IL-1 β or TNF immunostaining were detected between the experimental conditions ($p > 0.05$ for all comparisons) (Figure 5.3.13b-iii and 5.3.13b-iv, respectively). Similarly, for matrix-degrading enzymes, no significant differences in ADAMTS4 or MMP3 immunostaining (Figure 5.3.13c-iii and 5.3.13c-iv, respectively) although MMP3 showed a decreasing trend in all conditions compared to day 0.

In the NP/AF tissues, regarding anabolic markers, no significant differences were observed for ACAN or COL2 immunostaining between the experimental conditions ($p > 0.05$ for all comparisons) (Figure 5.3.14a-v). However, COL2 did show an increasing trend among all conditions compared to day 0 (Figure 5.3.14a-vi). Similarly, regarding catabolic cytokines, no significant differences in IL-1 β or TNF immunostaining were detected between the experimental conditions ($p > 0.05$ for all comparisons) (Figure 5.3.14b-v and 5.3.14b-vi respectively), but there was an increasing trend in IL-1 β in all conditions compared to day 0. Similarly, for matrix-degrading enzymes, no significant differences in ADAMTS4 or MMP3 immunostaining were observed between any experimental conditions ($p > 0.05$ for all comparisons) (Figure 5.3.14c-v and 5.3.14c-vi respectively), but there was a decreasing trend in MMP3 across all cultured conditions relative to day 0.

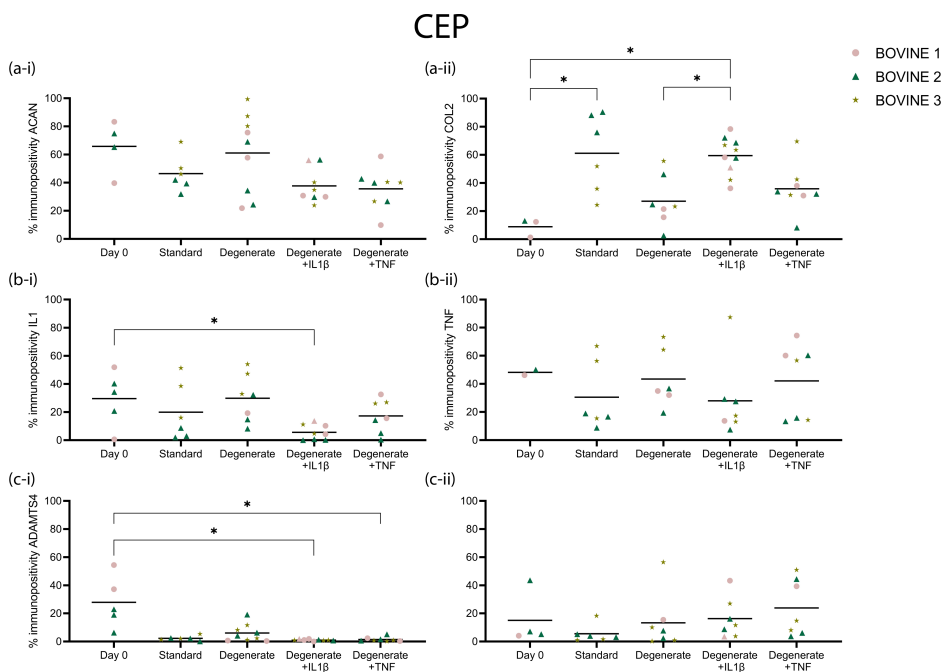


Figure 5.3.12: Immunopositivity of (a) anabolic markers ACAN and COL2, (b) catabolic markers IL-1 β and TNF, and (c) matrix-degrading markers ADAMTS4 and MMP3 in the CEP. $\ast=P < 0.05$.

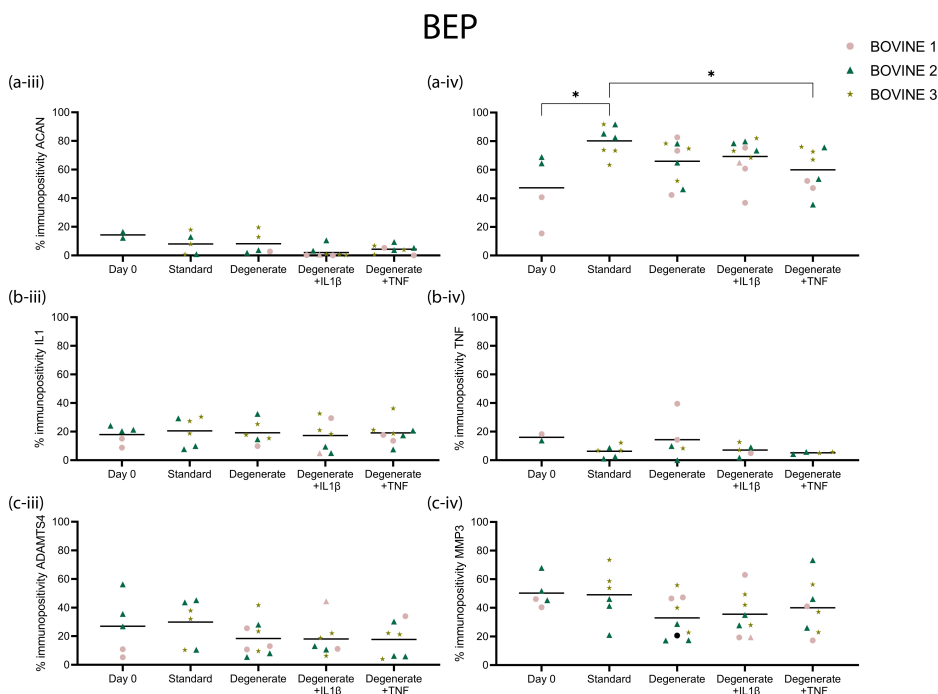


Figure 5.3.13: Immunopositivity of (a) anabolic markers ACAN and COL2, (b) catabolic markers IL-1 β and TNF, and (c) matrix-degrading markers ADAMTS4 and MMP3 in the BEP. $*=P < 0.05$.

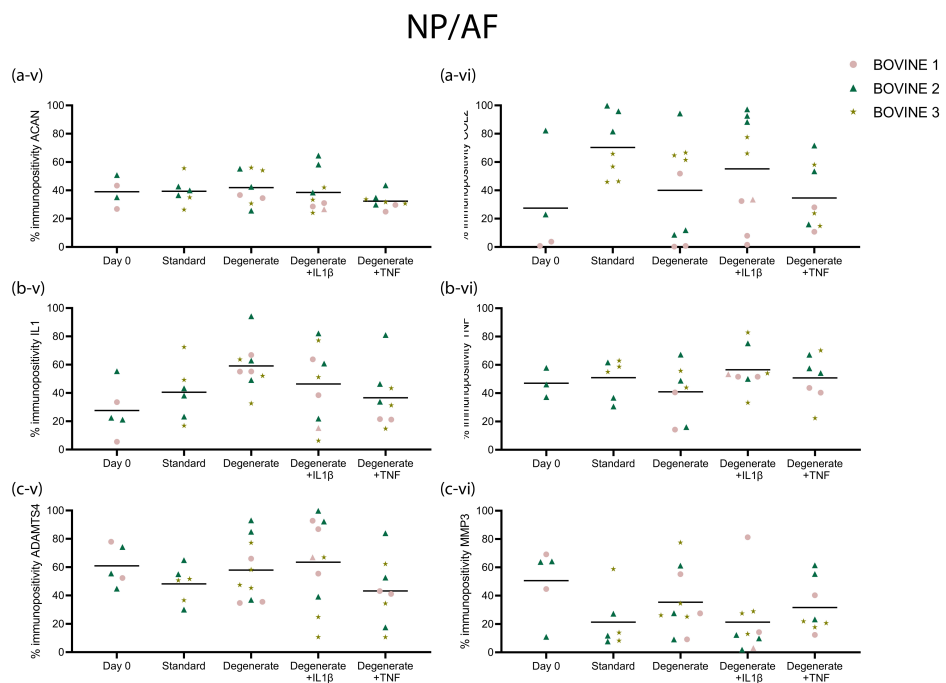


Figure 5.3.14: Immunopositivity of (a) anabolic markers ACAN and COL2, (b) catabolic markers IL-1 β and TNF, and (c) matrix-degrading markers ADAMTS4 and MMP3 in the NP/AF tissues. *= $P < 0.05$.

5.4 Discussion

In this study, we combined computational modeling with a novel multi-tissue *ex vivo* explant system to investigate the drivers of early EP degeneration. While the two approaches probe partially distinct perturbations, both converged on the same conclusion: physicochemical degenerative cues alone were insufficient to drive chondrocyte degeneration without concurrent cytokine stimulation. *In silico* simulations predicted that chondrocytes remained stable under degenerative environmental conditions, whereas the addition of cytokines led to a complete transition toward a degenerative phenotype. This prediction was corroborated experimentally, where intact bovine IVD–VB explants exposed to IL-1 β or TNF exhibited marked proteoglycan loss and reduced cellularity, while degenerative conditions alone resulted in minimal changes. These findings position cytokines as a necessary trigger for early EP degeneration.

Distinct cytokine-specific responses were observed, indicating differential roles in degeneration. TNF induced rapid and pronounced matrix degradation, as evidenced by increased GAG release and substantial reductions in proteoglycan staining. In contrast, IL-1 β produced more moderate but consistent changes, including reduced staining intensity, increased histological degeneration relative to degenerative culture without cytokines, and trends toward increased matrix remodeling activity. These findings suggest that TNF primarily drives acute and rapid catabolic responses, whereas IL-1 β contributes to sustained matrix degradation. Such differences are consistent with established roles of these cytokines in cartilage biology, where TNF is associated with rapid apoptosis and matrix breakdown [523–525], and IL-1 β with regulation of proteolytic enzymes [526–529].

Despite clear histological evidence of proteoglycan loss under cytokine stimulation, a hallmark feature of cartilage ECM breakdown [530, 531] and disc degeneration [532–535], tissue-level GAG/DNA ratios remained unchanged. This apparent discrepancy is explained by concurrent reductions in cell density, particularly in TNF-treated samples, where loss of DNA offset reductions in GAG content. At the same time, elevated GAG levels in the culture medium suggested active matrix degradation. This dissociation between retained tissue GAG and increased extracellular release is indicative of an early-stage degenerative process, in which matrix components are lost to the environment before complete depletion within the tissue [536–539] and is consistent with previous studies showing that IL-1 β and TNF

do not have a significant effect on the GAG content in the cartilage tissue [526]. Such patterns are consistent with early disc degeneration, where biochemical alterations precede structural disruption. The greater GAG release observed under TNF further supports its role as a potent catabolic driver, whereas IL-1 β appears to promote matrix turnover without equivalent matrix loss [523, 527]. This difference may reflect interactions between cytokines and matrix components, as GAGs have been reported to modulate cytokine availability, although this mechanism requires further investigation.

Degeneration exhibited a distinct spatial pattern, with the CEP showing the earliest and most pronounced changes. Proteoglycan depletion, reduced staining intensity, and altered cellular organization were most evident in the CEP, while the BEP and soft tissues (NP/AF) showed comparatively limited changes over the same time frame. Quantitative histological analysis further demonstrated that reductions in staining intensity occurred without substantial loss of stained area, indicating that matrix composition was altered prior to structural disruption. Cytokine-treated samples also showed increased chondrocyte clustering and reduced cell density, consistent with cellular stress and early degeneration. In contrast, collagen-rich structures remained relatively preserved, suggesting that early degeneration primarily affects the non-collagenous matrix. Together, these findings support a model in which degeneration in the CEP is characterized by early biochemical and cellular changes before structural breakdown becomes apparent.

A major strength of this study is the use of an intact multi-tissue IVD–VB explant model that preserves the native bone–cartilage–disc interface. Tissue thicknesses of CEP and BEP across all conditions fell within established physiological ranges reported in the literature [460, 465, 466, 540–544] confirming structural consistency across samples. This uniformity strengthens the interpretation that observed degenerative changes are attributable to experimental conditions rather than baseline anatomical variability. Unlike previous models that rely on isolated cells or simplified tissue systems, this approach maintains continuity between the NP/AF, CEP, and BEP, enabling investigation of cross-tissue signaling and transport processes in a physiologically relevant context.

Literature specifically addressing cytokine-driven degeneration of the EP remains limited [460, 465, 466, 542, 543]. Most studies have focused on Modic changes or have used isolated CEP cells and simplified systems, which do not capture the complexity of the native tissue interfaces [545, 546][547, p. 1]. Only a small number of studies have directly examined cytokine

effects on CEP tissue, primarily using diced tissue [548, 549] or digested cell cultures [550, 551], and have identified pathways such as NF- κ B activation and matrix-degrading enzyme upregulation. While these findings align with the catabolic responses observed in the present study, they lack the structural and interfacial context preserved in the current model. By contrast, the current work investigates cytokine-induced degeneration within an IVD–VB explant, providing a platform to study inter-tissue interactions and early degenerative processes *in situ*. To date, there are only two other studies that have directly addressed cytokine-induced degeneration of the CEP. One related TNF to the activation of NF- κ B pathways and a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS-5) upregulation, in diced CEP tissue [548], and the other related IL-1 β to cell activity and cartilage-related proteins [549]. The findings of both studies align with our results. Two other studies also used digested CEP cells, but did not focus on cytokines; they investigated TGF- β 3 [550] and IGF-1/CA12 [551] to enhance anabolic processes and prevent calcification.

Some limitations should be considered in this study. The culture system was static and did not incorporate mechanical loading, which plays a key role in disc physiology and EP function. Washing the samples could lead to changes compared to *in vivo* conditions. The duration of culture was limited to 14 days, restricting analysis to early degenerative changes. Only single cytokine concentrations were evaluated, and additional cytokine mediators may contribute to disease progression, although these cytokines will likely induce further cytokine secretion from the tissues [488]. Furthermore, while bovine tissue provides a relevant structural model, species-specific differences may influence cellular responses. Future studies incorporating mechanical stimulation, extended culture durations, and broader molecular analyses would further enhance the applicability of this model. It should also be noted that the *in silico* model, originally developed and validated in the context of osteoarthritic chondrocytes [127, 128, 487], simulates degenerative stress via low osmolarity, while the experimental design modulates pH as the primary degenerative cue, as the low osmolarity is modeled in both standard and degenerative media conditions. Although both parameters are characteristic of the degenerative IVD niche [499], and the directional predictions of the model were borne out experimentally, the two systems are not identical surrogates of one another. Future work should aim to integrate both osmolarity and pH perturbations within a single computational and experimental framework, and to establish whether the model's network topology adequately represents CEP chondrocyte biology, given known phenotypic differences between CEP and articular chondrocytes.

In conclusion, this study demonstrates that cytokines are key drivers of early EP degeneration, with TNF and IL-1 β exerting distinct but complementary effects on matrix degradation and remodeling. By integrating computational modeling with a structurally validated, multi-tissue explant model, this work provides new insight into the mechanisms underlying EP pathology and establishes a physiologically relevant platform for investigating therapeutic strategies targeting cytokine-driven disc degeneration.

CHAPTER — 6

Cells in Gels: Mechanical Stimulation of Cartilaginous Endplate Cells in Gel-Based Carriers

This chapter is based on published content:

Crump, Katherine B., Alminnawi, Ahmad, et al. *Exploring Mechanotransduction and Inflammation in Human Cartilaginous Endplate Cells in Blended Collagen–Agarose Hydrogels Under Cyclic Compression*. *Gels* 11.9 (2025): 736;

Crump, Katherine B., Alminnawi, Ahmad, et al. *TNF induces catabolism in human cartilaginous endplate cells in 3D agarose culture under dynamic compression*. *Scientific Reports* 15.1 (2025): 15849.

Building on the previous chapters, which established the anatomical, biological, and biomechanical importance of the endplate (EP) and developed experimental and computational tools to investigate tissue-level transport and mechanics, this chapter focuses on the mechanobiological behavior of human cartilaginous endplate (CEP) cells. Specifically, it investigates the response of cells cultured in three-dimensionals (3Ds) hydrogel systems under inflammatory and dynamic compression conditions. Intervertebral discs (IVDs) and EPs cells exhibit distinct mechanobiological responses under pro-inflammatory conditions compared to healthy conditions, indicating that degeneration alters normal mechanotransduction pathways [552–555]. Mechanical loading can also play a central role in regulating the anabolic and catabolic balance of the cellular environment [331, 556, 557]. This strong dependence on mechanotransduction makes *in vitro* investigation of the CEP particularly challenging, as it requires advanced 3D culture systems capable of reproducing physiologically relevant mechanical stimuli [558]. Consequently, there remains a limited understanding of CEP cell mechanobiology, particularly of how these responses are altered during degeneration.

As previous chapters focused mostly on the tissue level characterisation and testing, they did not allow reliable characterization of cellular responses. To address this limitation and gain a better understanding of the molecular mechanisms governing EP cell behavior, complementary studies were undertaken within the Disc4All consortium using a 3D hydrogel-based culture system specifically designed for controlled mechanical loading and cellular-level analyses. These collaborative studies combined experimental and computational approaches to investigate how carrier composition, inflammatory stimulation, and dynamic compression influence cell phenotype, matrix production, catabolic responses, mechanosensor expression, and the mechanical and transport environment experienced by the cells.

This chapter presents the essential findings from these collaborative studies while focusing on the contributions made by this doctoral candidate. In the first study, I contributed to the development and execution of the *in vitro* experiments, including the optimization of the loading platform and experimental protocols. In the second study, I contributed to the development of the *in silico* finite element modeling (FEM) used to characterize the mechanical loading and fluid transport within the hydrogel constructs. Together, these studies demonstrate how integrating experimental observations with computational modeling provides a more comprehensive understanding of CEP cell mechanobiology and supports the overarching objective of the

Disc4All project to develop mechanistic, multiscale models of intervertebral disc degeneration.

6.1 Study 1: tumor necrosis factor (TNF) induces catabolism in human CEP cells in 3D pure agarose culture under dynamic compression

6.1.1 Overview of study 1

Agarose has been the gold standard biomaterial for 3D culture of cartilaginous tissues because of its biocompatibility, its ability to maintain a rounded cell morphology indicative of a healthy cartilaginous phenotype, and its adequate strength to withstand compressive loading [558–562]. The first study investigated the mechanobiological response of human CEP cells cultured in 3D agarose hydrogels under inflammatory stimulation and dynamic compression. To elucidate the effects of pro-inflammatory cytokines on the mechanobiology of the CEP, human CEP cells were embedded in 2% agarose constructs, subjected to cyclic compressive loading resulting in a strain of up to 7%, and stimulated with TNF to evaluate the combined effects of mechanical and inflammatory cues on cellular behavior. The study demonstrated that TNF induced a time-dependent catabolic response characterized by reduced anabolic gene expression and increased secretion of proinflammatory proteins associated with intervertebral disc degeneration (IVDD), whereas the applied dynamic compression alone was insufficient to elicit a pronounced anabolic response or suppress inflammatory signaling. These findings suggested that the applied loading regime and the agarose scaffold did not provide sufficient mechanical stimulation to fully activate integrin-mediated mechanotransduction pathways.

Within this study, my contribution focused on the *in vitro* component, including the development and optimization of the experimental protocols, preparation of the hydrogel constructs, and execution of the mechanical loading experiments that formed the basis of the biological analyses.

6.1.2 *In vitro* materials and methods of study 1

Human CEP cells were isolated from IVD tissues collected from patients undergoing trauma surgery who had no history of back pain or IVD degeneration and, therefore, were considered to have a healthy phenotype. The study was approved by the ethics committee of the University Hospital of Bern, Bern, Switzerland, with informed consent obtained from patients or, when necessary, their relatives. The CEP tissue was identifiable as translucent cartilaginous tissue. Cell extraction involved a two-step enzymatic digestion where they were placed in pronase for 1 hour, then digested with collagenase II overnight. Cells were expanded until passage three or four in monolayer culture before being used for the experiments. The cells were encapsulated in 2% wt/vol agarose hydrogels. The cells were mixed with the agarose solution to achieve a final concentration of 7.5×10^6 cells/mL.

The cell-agarose mixture was pipetted into silicon molds (6 mm diameter, 3 mm height). Cell-containing hydrogels were cultured in standard medium (low-glucose Dulbecco's Modified Eagle Medium (LG-DMEM) with 10% fetal bovine serum (FBS)) for five days to allow for phenotype recovery and the formation of a pericellular matrix (PCM).

Following the recovery period, constructs were transferred to custom-made unconfined chambers within a custom-made bioreactor (Figure 6.1.1). The experimental medium was serum-free LG-DMEM supplemented with various factors (ITS+, non-essential amino acids, L-ascorbic acid 2-phosphate, and dexamethasone). The construct were subjected to four conditions over a period of 7 or 14 days, which were the control unloaded, the control dynamic compression, TNF unloaded, and TNF dynamic compression. The construct were dynamically compressed at 7% strain and supplemented with 10 ng/mL according to the assigned conditions. This strain was selected because it is within the physiological anabolic range [563, 564] ($\leq 20\%$) and did not cause cracks or breakage during initial tests. Loading occurred at a frequency of 1.5 Hz for one hour per day, five days per week, for two weeks. Each chamber contained six construct and was subjected to a total load of 1.2 N per compression cycle, resulting in an approximate force of 0.2 N experienced by each construct. The construct were collected at days 0, 7, and 14 for downstream analysis.

Cell Viability was assessed using Calcein-AM (live) and Ethidium Homodimer (dead) staining and quantified via confocal laser scanning microscopy (cLSM). Cell Metabolic Activity was evaluated using resazurin

sodium salt solution, with results normalized to deoxyribonucleic acid (DNA) content. glycosaminoglycan (GAG) and DNA were quantified in the hydrogels after papain digestion. Total GAG content was measured using 1,9-dimethylmethylene blue, and DNA content was measured using Hoechst 33258 dye. Media samples were collected at each medium exchange (every 2–3 days), and the GAG released into the medium was quantified using the same method. Gene Expression was also analyzed using quantitative polymerase chain reaction (qPCR), where ribonucleic acid (RNA) was extracted, reverse transcribed to complementary DNA (cDNA), and qPCR was performed. Gene expression was quantified for key anabolic (aggrecan (ACAN), COL2A1), catabolic (matrix metalloproteinase 3 (MMP3), interleukin-6 (IL-6)), and mechanobiological markers (transient receptor potential cation channel subfamily V member 4 (TRPV4), ITGA5, ITGB1). The relative gene expression was determined with the $2^{-\Delta\Delta C_t}$ method, normalized to the housekeeping gene 18S. Additionally, Secretome (Luminex) analysis was performed on the collected media samples to quantify 73 secreted proteins using bead-based multiplex immunoassays for absolute cytokine quantification. Phosphoprotein (Luminex) analysis was conducted on protein lysates from two donors to assess 21 phosphorylated proteins using multiplex assays for semi-quantitative measurement.

6.1.3 Results of study 1

The study results showed demonstrated a clear distinction between the strong catabolic response induced by TNF stimulation and the negligible response caused by dynamic compression in human CEP cells cultured in 2% agarose hydrogels. Cell viability remained stable throughout the 7 and 14 days of culture, showing no significant change in any condition where variability in cell viability was greater between individual donors than between the treatment conditions. Cell metabolic activity, total GAG content, and GAG content normalized to DNA content remained constant and were not significantly different across any experimental condition. DNA content also showed no significant differences between groups (Figure 6.1.2). However, GAG release into the media trended toward an increase on Day 14 in all conditions except the unloaded control.

Treatment with 10 ng/mL TNF had a significant effect on gene expression, shifting the cells toward a catabolic phenotype. TNF-stimulated constructs showed a trend toward decreased expression of genes for proteoglycans and collagens. Specifically, COL2A1 expression was significantly decreased in all

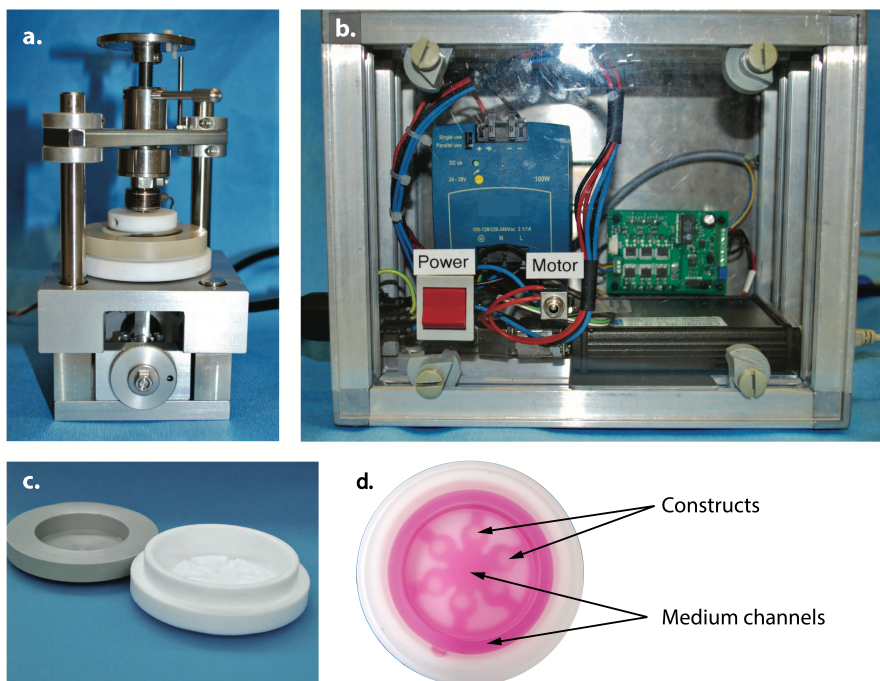


Figure 6.1.1: Experimental setup with (a) strain-controlled bioreactor, (b) power supply and controllers, and (c) custom-made chamber. (d) shows the custom-made chamber with 6 agarose constructs and media solution channels.

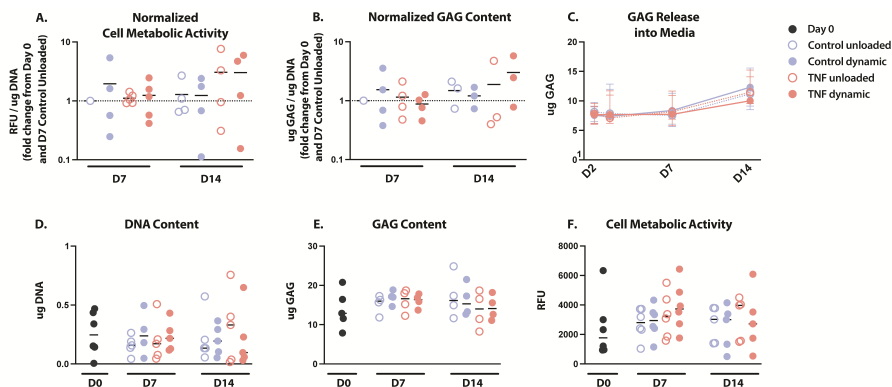


Figure 6.1.2: Cell metabolism and GAG/DNA content in human CEP cells remained constant over time. (a) Cell metabolic activity normalized to corresponding DNA content, shown as the fold change from Day 0 and the Day 7 control unloaded condition. (b) GAG content normalized to corresponding DNA content, shown as the fold change from Day 0 and the Day 7 control unloaded condition. (c) GAG content measured in the secretome throughout culture. (d) DNA content in ug of CEP cells in agarose after 7 and 14 days in culture. (e) GAG content in ug of CEP cells in agarose after 7 and 14 days in culture. (f) Cell metabolic activity of CEP cells in agarose after 7 and 14 days in culture. Shown are the medians, $n = 4-5$.

TNF-stimulated hydrogels compared to controls after 7 and 14 days. ACAN expression, which codes for proteoglycan, was significantly decreased in TNF-stimulated conditions at specific time points (Day 7 unloaded and Day 14 dynamic). Expression of the inflammatory cytokine IL-6 and the matrix metalloproteinase MMP3 was upregulated after 7 days of TNF stimulation, an effect that disappeared after 14 days. IL-6 expression was significantly increased in TNF-stimulated dynamically compressed hydrogels compared to controls. Dynamic compression at 7% strain had no significant effect on the expression of any gene, nor did time duration. Crucially, the gene expression of mechanosensors TRPV4 and integrin subunits $\alpha 5$ (ITGA5) and $\beta 1$ (ITGB1) was not significantly altered by either TNF stimulation or dynamic compression (Figures 6.1.3–6.1.4). This suggests that the cultured cells were largely unresponsive to mechanical loads.

TNF treatment exerted a much greater effect on the secretome than the duration of culture or dynamic compression. Dynamic compression had no significant effect on the secretome when compared to unloaded hydrogels. TNF significantly increased the secretion of multiple pro-inflammatory factors associated with pain, herniated discs, and degeneration. interleukin-1 beta (IL-1 β), RANTES (CCL5), IL-6, interleukin-8 (IL-8), ST2, IL-9, IL-13, IL-18, and IL-22 were all upregulated. Inflammatory adhesion molecules

A.

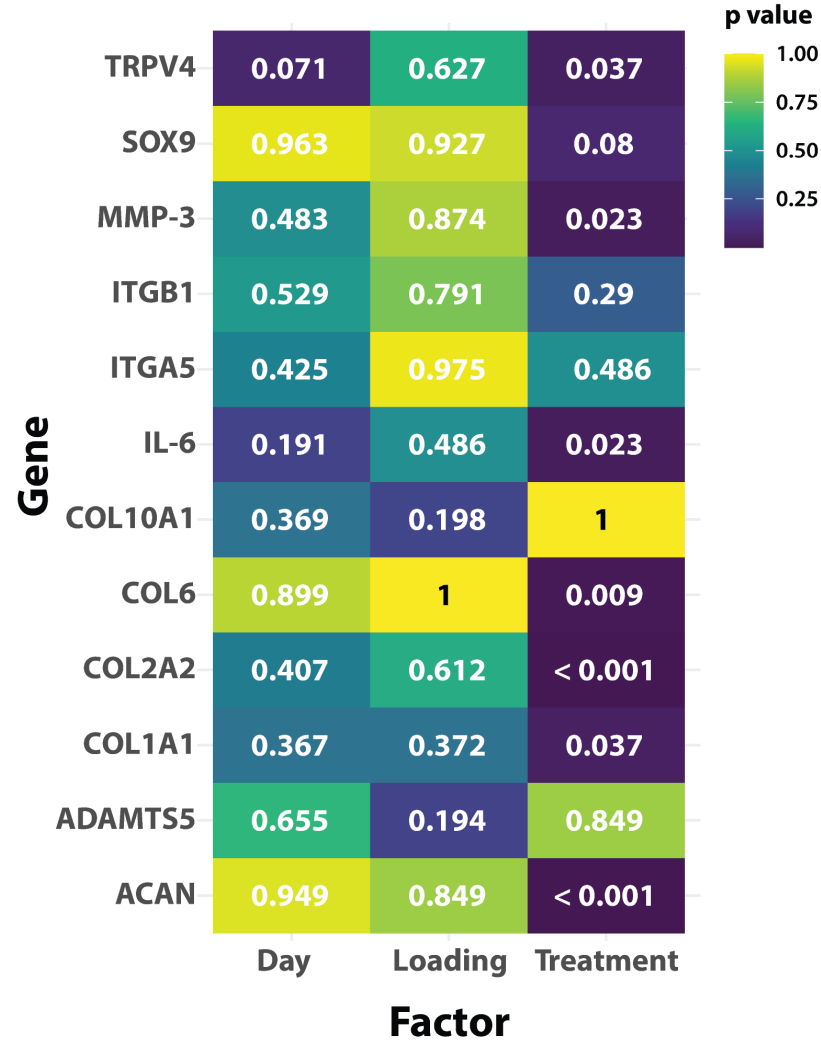


Figure 6.1.3: Heatmap of the *p* values determined through Kruskal–Wallis analysis for gene expression according to culture duration, loading, and TNF treatment in human CEP cells.

○ Control Unloaded ● Control Dynamic ○ TNF Unloaded ● TNF Dynamic

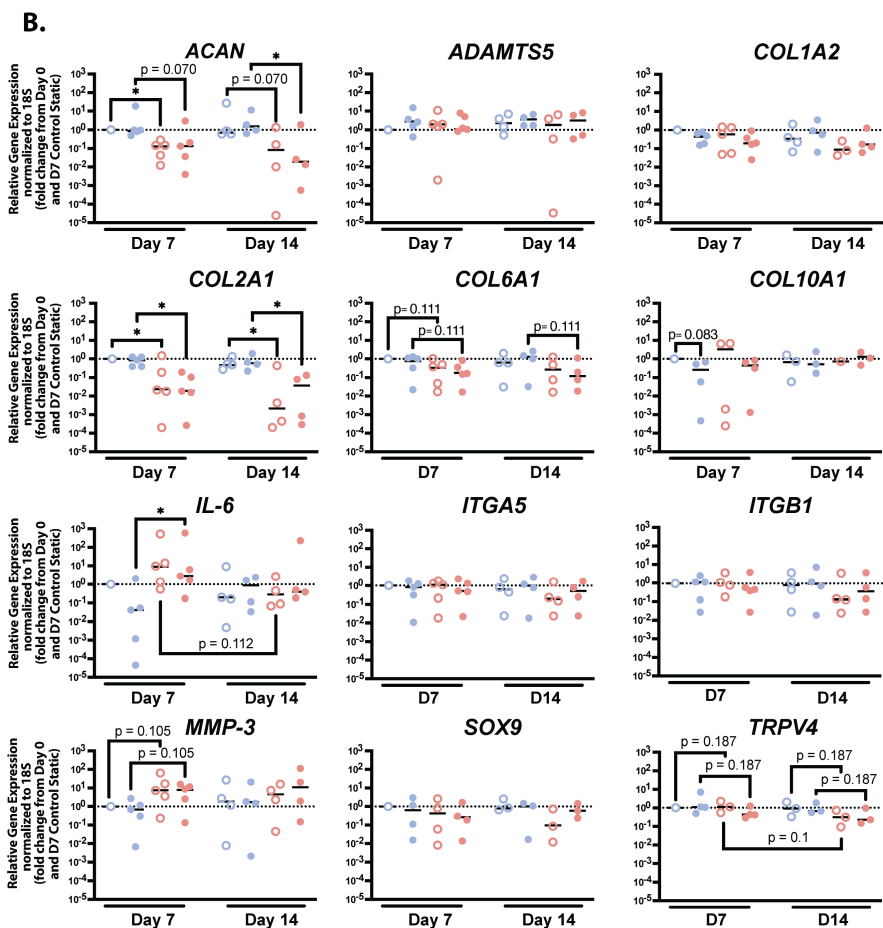


Figure 6.1.4: Relative expression of ACAN, ADAMTS-5, COL2A1, COL6A1, COL1A2, COL10A1, IL-6, ITGA5, ITGB1, MMP3, SOX9, and TRPV4, shown as fold changes relative to Day 0 and the Day 7 control unloaded condition. Data are shown as medians, $n = 4-5$. * $p < 0.05$.

ICAM-1 and VCAM-1 were highly upregulated (2.38- and 4.78-fold greater than the control, respectively) following TNF stimulation. Secretion of M-CSF and matrix metalloproteinase 9 (MMP9) was significantly increased. Beta-defensin 1 (DEFB1), an antimicrobial factor not previously studied in the IVD, was secreted 4.23x higher in TNF-treated conditions. IP10, a factor correlated to herniation, and IFN- γ were also significantly increased following TNF stimulation. Secretion of angiogenic and neurotrophic proteins, which are implicated in painful disc degeneration, significantly decreased over time in the TNF-stimulated conditions. VEGF, a key regulator of angiogenesis, showed a significantly decreased secretion after 14 days, relative to 7 days. NGF, a neurotrophic factor associated with nerve ingrowth, was increased after 7 days but returned to control levels after 14 days. This same short-term upregulation followed by a return to baseline was also seen in MMP-1 and IL-6 secretion. Interestingly, the secretion of the chemokine CCL2 (MCP-1) was downregulated in response to TNF stimulation, significantly in the unloaded hydrogels after 7 days (Figures 6.1.5–6.1.7).

6.1.4 Discussion of study 1

This study showed that TNF induced a catabolic, time-dependent response, supporting its role in IVDD. TNF downregulated anabolic genes involved in extracellular matrix (ECM) maintenance while increasing inflammatory proteins associated with pain, herniated discs, bacterial defense, and Modic change (MC), leading to reduced tissue integrity and impaired nutrient transport. Although some inflammatory and neurotrophic factors decreased over time, suggesting partial cellular adaptation to prolonged inflammation, TNF remained the primary driver of degeneration. In contrast, dynamic compression (7% strain) did not produce the expected anabolic response, likely because the 2% agarose construct was unable to effectively transmit mechanical signals to activate cell mechanosensors such as integrins. Overall, the study demonstrated that agarose is well-suited for investigating inflammatory responses in human CEP cells. However, despite being considered the “gold standard” biomaterial for 3D culture of cartilaginous tissues, agarose is bioinert and lacks the cell adhesion motifs required for integrin mediated cell ECM interactions and subsequent mechanotransduction [558]. Consequently, it is not an ideal scaffold for investigating cellular responses to mechanical loading. This highlighted the need for biomaterials that better support cell–matrix interactions, overcoming the methodological limitations of agarose. To address this, recent studies explored blended hydrogels incorporating ECM components such as type I collagen [558, 565, 566],

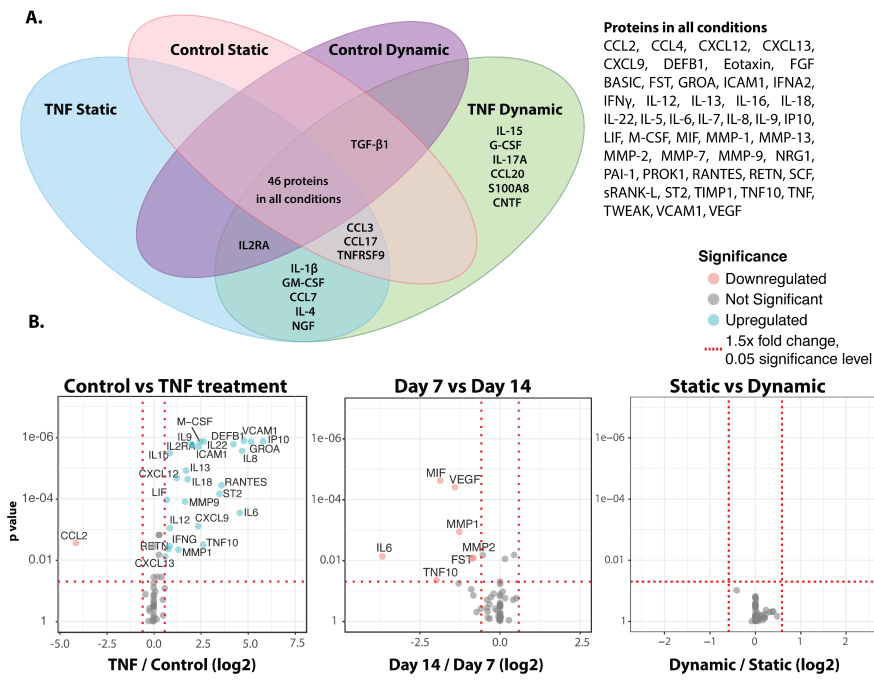


Figure 6.1.5: TNF promotes secretion of pro-inflammatory factors in human CEP cells. (A) Venn diagram showing the proteins detected in the secretome of the control static, control dynamic, TNF static, and TNF dynamic conditions. Proteins detected in all four conditions are listed next to the diagram. (B) Volcano plots comparing control versus TNF treatment, Day 7 versus Day 14, and static versus dynamic compression. Proteins were classified using a significance threshold of $p < 0.05$ and a fold-change threshold of 1.5 after normalization to the control static condition and Kruskal–Wallis analysis.

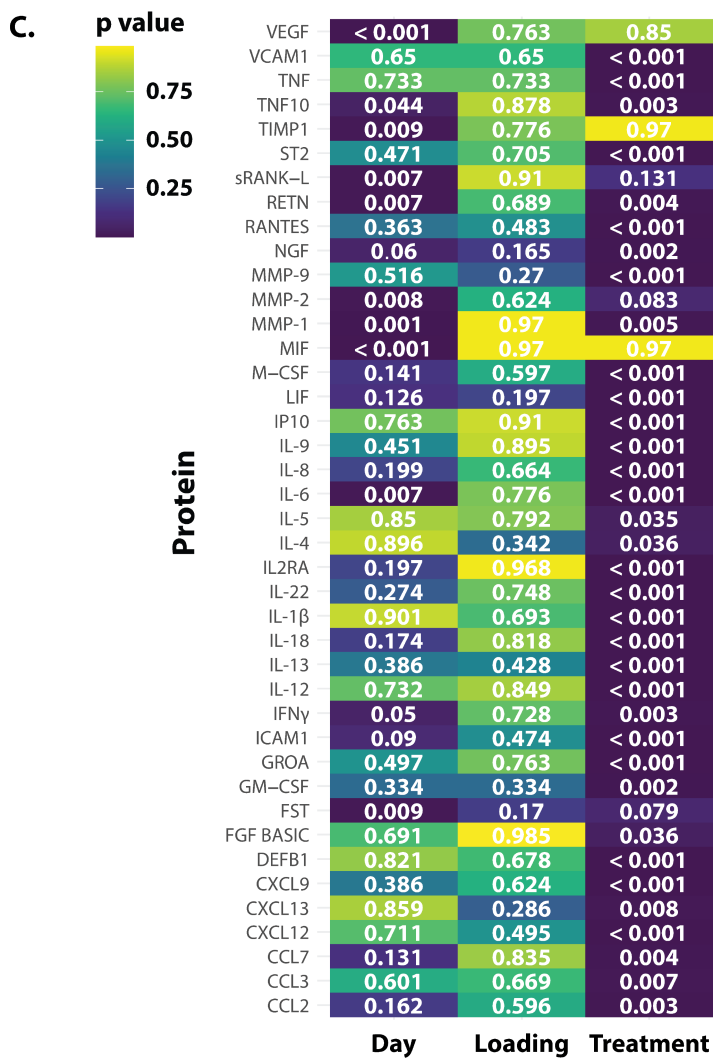


Figure 6.1.6: Heatmap of the p values determined through Kruskal–Wallis analysis for protein secretion according to culture duration, loading, and TNF treatment.

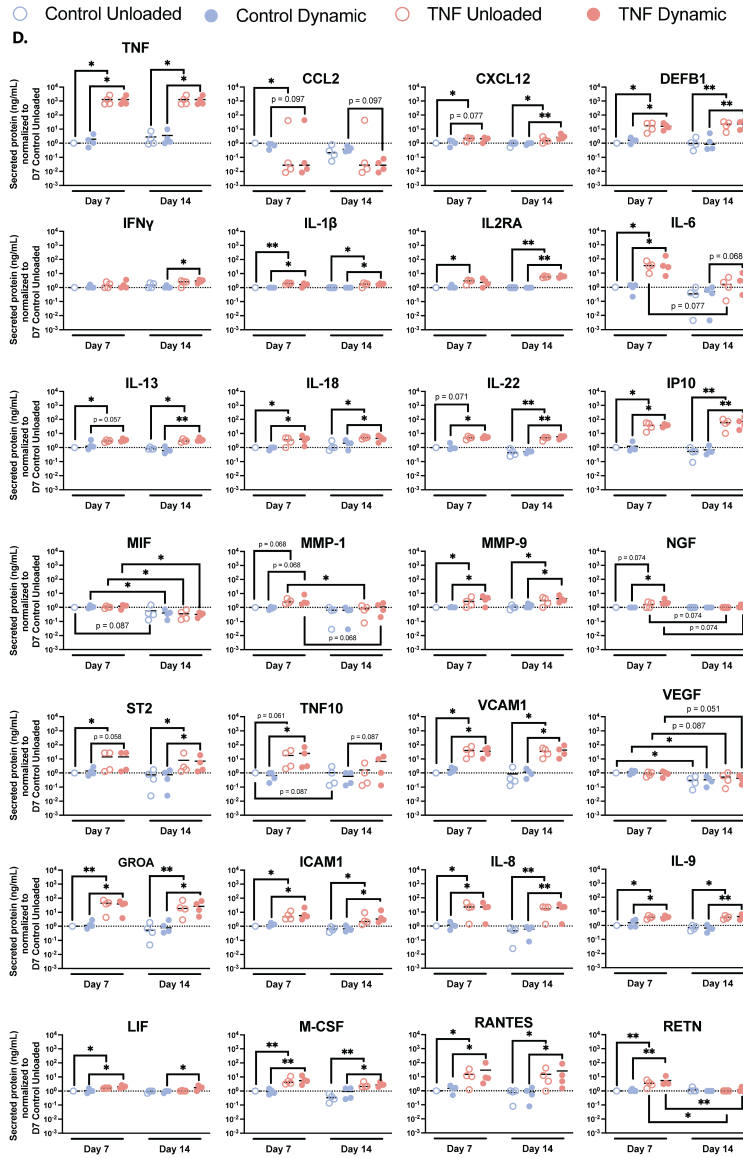


Figure 6.1.7: Secreted protein concentrations normalized to the Day 7 control unloaded condition. Proteins with statistical differences are shown, including TNF, CCL2, CXCL12, DEFB1, GROA, ICAM1, IFN- γ , IL-1 β , IL-2Ra, IL-6, IL-8, IL-9, IL-13, IL-18, IL-22, IP10, LIF, M-CSF, MIF, MMP-1, MMP9, NGF, RANTES, RETN, ST2, TNF10, VCAM1, and VEGF. Data are shown as medians, $n = 4-5$. * $p < 0.05$; ** $p < 0.01$.

laminin [566], methacrylated gelatin [567], and hyaluronic acid [568]. In particular, collagen–agarose hydrogels have been shown to enhance mechanotransduction in chondrocytes compared with agarose alone, making them a promising platform for studying the mechanobiology of CEP cells

6.2 Study 2: Exploring mechanotransduction and inflammation in human CEP cells in blended collagen–agarose hydrogels under cyclic compression

6.2.1 Overview of study 2

The second study investigated whether blended collagen–agarose hydrogels could provide a more physiologically relevant platform for studying the mechanobiology of CEP cells. Human CEP cells were cultured in agarose carriers blended with Type I collagen, as it is among the most commonly used hydrogels and the most prevalent ECM protein in mammalian tissues [565]. The cell-laden hydrogels contained 2% agarose and 2 mg/mL type I collagen, subjected to dynamic compression, and stimulated with TNF, similar to the previous study described in Section 6.1. The study demonstrated that the blended hydrogels maintained the mechanical strength of agarose while providing the biofunctionality of type I collagen, increasing cell adhesion and focal adhesion kinase (FAK) activation while sustaining high cell viability and preserving the rounded morphology of chondrocyte-like cells [558, 565]. Although the addition of collagen did not produce a significant mechanobiological response to dynamic compression under the applied loading conditions, the blended hydrogels exhibited a fourfold increase in stiffness. In addition, cells cultured inside these blended hydrogels showed enhanced expression of the cartilage marker SOX9 and the mechanosensitive ion channel TRPV4, indicating an improved culture environment compared with agarose alone. Within this study, my primary contribution was to the *in silico* component. I developed the FEM of the hydrogel constructs to characterize their mechanical behavior and fluid transport under dynamic compression, providing computational insight into the mechanical environment experienced by the embedded cells. These models complemented the experimental findings by relating scaffold properties and loading conditions to the local mechanical stimuli that regulate cellular mechanobiological

responses.

6.2.2 *In vitro* and *in silico* materials and methods of study

2

To test whether blended collagen–agarose hydrogels could provide a more physiologically relevant platform for studying the mechanobiology of CEP cells, the previously described experimental conditions were repeated using identical human CEP cells, following the same sourcing, extraction, passaging, and seeding procedures. In this experiment, the cells were encapsulated in a blended 2% agarose and 2 mg/mL type I collagen hydrogels. The constructs were again split into four groups that are the unloaded control, dynamic control, unloaded TNF, and dynamic TNF, and they were subjected to dynamic compression at 7% strain at a frequency of 1.5 Hz and/or stimulation with 10 ng/mL TNF according to their group. To have a better understanding of the mechanical environment in the gel compared to pure agarose, both blended hydrogels and 2% agarose hydrogels without cells were tested using the bioreactor under unconfined compression at 37°C. The strain was increased by 3.33% every minute up to 30% strain to determine the stiffness using a modified protocol established by Frauchiger et al. [569] where Young’s modulus (E) was calculated from the slope of the stress–strain ratio in the initial elastic region. The Height of each hydrogel was measured using Fiji and averaged per chamber to assess the changes over time. For the cell-containing constructs, cell adhesion and cytoskeletal morphology were assessed using F-actin staining (Alexa Fluor 488-conjugated phalloidin) and nuclei staining (4',6-diamidino-2-phenylindole (DAPI)) on days 0 and 14. Samples were fixed using 4% formaldehyde overnight, permeabilized using 0.1% v/v Triton in phosphate-buffered saline (PBS) for 15 minutes, and stained for 2 h in 165 nM phalloidin in PBS with 1% bovine serum albumin (BSA) and for 20 min in 1 μ g/mL DAPI diluted in 1 $\times$ PBS. Then, 3D stacked images were captured using a confocal laser scanning microscope (cLSM710). The nitric oxide (NO) radicals released into the media were quantified using a Griess reaction assay [568]. Cell viability, metabolic activity, GAG and DNA Quantification, and qPCR were performed as described earlier.

A compressible, two-dimensional (2D) axisymmetric porohyperelastic finite element (FE) model of the collagen–agarose hydrogel was created in Abaqus 2024, parameterized using a combination of experimental data and literature-reported material properties.

Permeability, Poisson's ratio, and porosity values were obtained from literature on 2–3% agarose hydrogels [570, 571], as equivalent data for collagen–agarose blends were not available. The Young's modulus was experimentally determined as described above. Boundary conditions were defined to replicate unconfined axial compression (Figure 6.2.1). Specifically, the bottom surface was constrained vertically while remaining free to expand radially, and the lateral surface was assigned a zero-pore-pressure condition to permit unrestricted fluid flow. A sinusoidal dynamic compression with a displacement amplitude of 0.21 mm at 1.5 Hz was applied to the top surface. Three hydrogel heights (3 mm, 2.5 mm, and 2 mm) were modeled to evaluate deformation behavior over the course of a 60-second simulation. reactive fluid volume flux (RVF) and reaction total fluid volume (RVT) were monitored throughout the simulation at the nodes along the lateral surface of each modeled hydrogel. The cumulative RVF and RVT values across these nodes represented the net instantaneous flow rate and the total fluid exchanged, respectively. These parameters provided insight into fluid transport dynamics, capturing how the hydrogels absorbed and expelled fluid in response to mechanical loading.

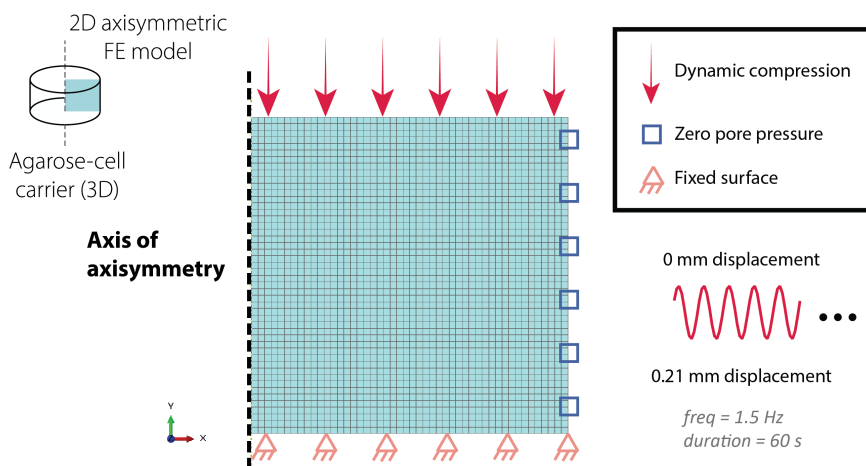


Figure 6.2.1: Boundary conditions of the axisymmetric FE model. The bottom surface was fixed in the vertical direction (orange triangle), while the lateral surface pore pressure was set to 0 (blue square). Sinusoidal dynamic compression of 0.21 mm displacement was applied to the top surface (red arrow).

6.2.3 Results of study 2

CEP cells maintained a rounded morphology (Figure 6.2.2A) and high via-

bility (Figure 6.2.3B) throughout the 14-day culture period in the blended hydrogels, with median viability ranging from 69.7% to 86.9% across all conditions. The hydrogel height decreased from approximately 3 mm to 2.5 mm after 7 days and then stabilized, with no difference between unloaded and dynamically compressed groups (Figure 6.2.3C).

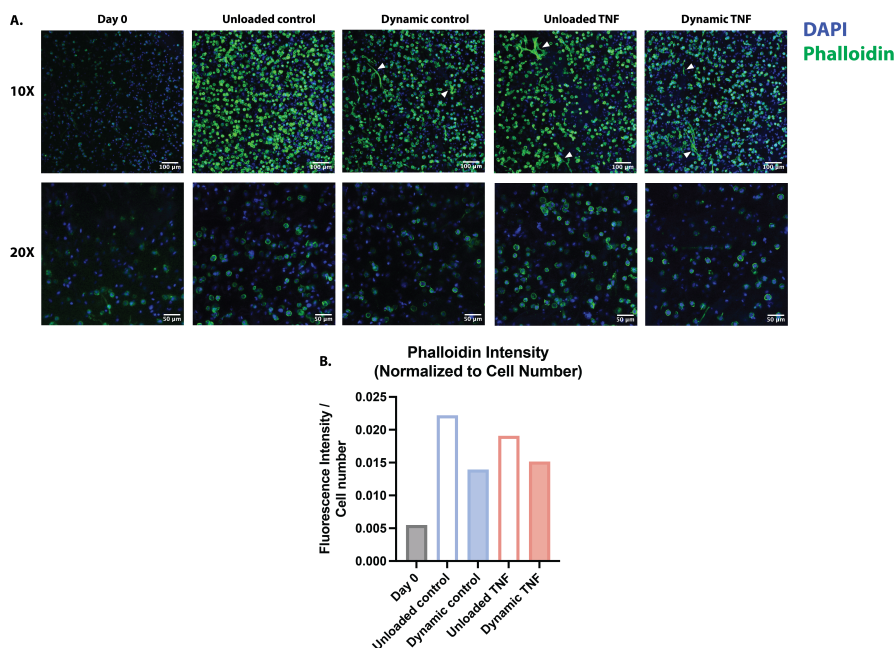


Figure 6.2.2: (A) F-actin staining with phalloidin of human CEP cells in blended collagen-agarose hydrogels at 10 $\times$ and 20 $\times$. Phalloidin is shown in green, and DAPI is shown in blue. White arrows point to elongated cells. (B) Fluorescence intensity of phalloidin relative to cell number as determined by DAPI. $n = 1$.

DNA content increased (median 1.35–1.95 $\times$) after 7 days, suggesting early proliferation, but returned toward baseline by day 14. Cell metabolism and GAG production remained stable, showing no significant differences between conditions at either time point (Figure 6.2.4).

Stimulation with 10 ng/mL TNF induced a catabolic shift in CEP cells, as indicated by reduced expression of the anabolic markers ACAN and COL2A1 after 7 days, particularly under dynamic compression. TNF also downregulated the pericellular matrix marker COL6A1 in both unloaded and dynamically compressed constructs at 7 and 14 days. NO release was significantly higher in TNF-treated unloaded constructs after 7 days compared with 14 days, while MMP3 expression remained unaffected by

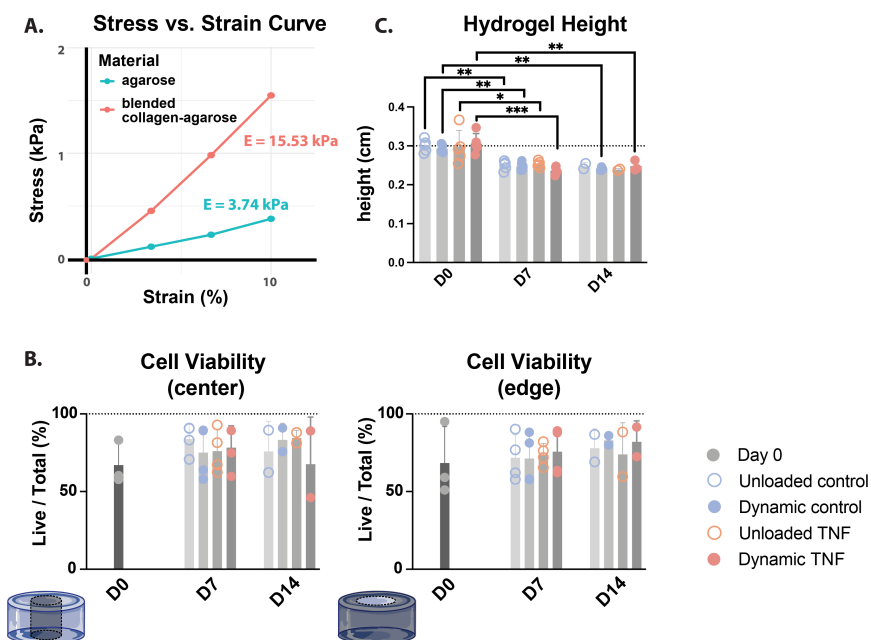


Figure 6.2.3: (A) Stress-strain curve comparing the stiffness of both 2% agarose (green) and blended 2% agarose and 2 mg/mL type I collagen hydrogels. Young's modulus (E) is calculated as the slope of the stress-strain curve. $n = 1$ (B) Cell viability of CEP-seeded blended collagen-agarose hydrogels at the center and edge of the hydrogel. (C) Heights of the hydrogels for the duration of culture. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $n = 3-4$.

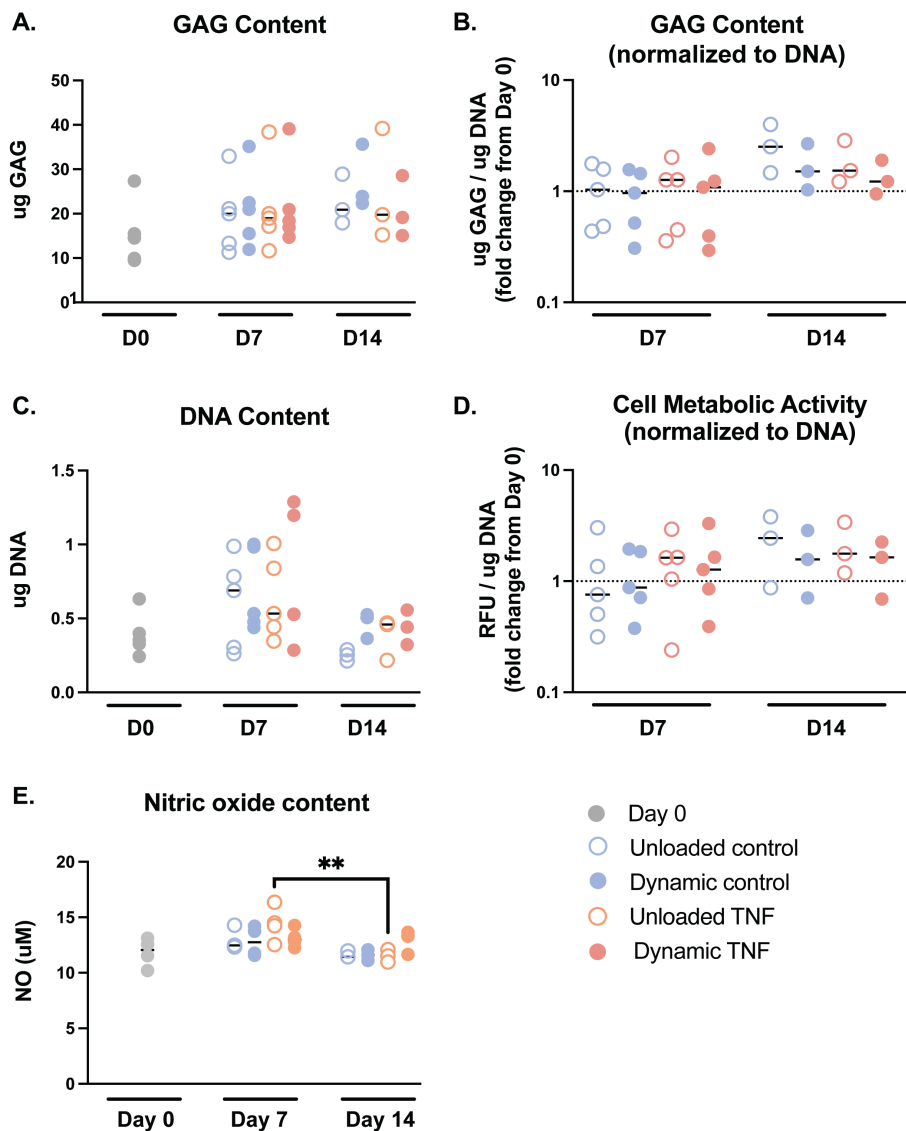


Figure 6.2.4: (A) Glycosaminoglycan (GAG) content measured in blended collagen–agarose hydrogels during culture. (B) GAG content normalized to the corresponding DNA content. (C) DNA content measured in blended collagen–agarose hydrogels during culture. (D) The cell metabolic activity of each condition was normalized to the corresponding DNA content. (E) The nitric oxide (NO) content released into the media on day 0 and after culture, $n = 3-5$. ** $p < 0.01$.

TNF or compression. The incorporation of collagen into the constructs significantly increased the expression of the transcription factor SOX9 and the mechanosensitive calcium channel TRPV4 compared to constructs with only agarose. Since TRPV4 mediates mechanotransduction and can enhance SOX9 expression, which is critical for chondrogenesis, this suggests that the blended hydrogels provide a more anabolic environment for CEP cells. In contrast, culture in pure agarose resulted in markedly lower expression of both SOX9 and TRPV4 (Figure 6.2.5).

The blended collagen–agarose hydrogels exhibited a fourfold increase in stiffness relative to 2% agarose, with a calculated Young’s modulus of 15.53 kPa compared to 3.74 kPa. Dynamic compression did not significantly alter gene expression or elicit major cellular responses in the blended constructs, similar to previous findings in pure agarose. However, baseline culture in collagen–agarose hydrogels provided clear advantages, as evidenced by consistently higher SOX9 and TRPV4 expression across all time points, indicating improved support for CEP cell phenotype and mechanosensory function.

The FE simulations confirmed that the experimentally measured maximum reaction forces (0.1052 ± 0.03378 N) aligned closely with the model predictions based on hydrogel heights ranging from 2 mm to 3 mm (Figure 6.2.6A). The 3 mm hydrogels produced a maximum reaction force of 0.08226 N, matching the lower limit of the experimental range. The 2 mm hydrogels generated a maximum force of 0.1358 N, aligning with the upper experimental limit. For the 2.5 mm hydrogels, which represented the average height after 7 and 14 days of culture, the predicted maximum reaction force was 0.1024 N, consistent with the mean experimental value.

Simulation of RVF indicated that fluid was expelled from the hydrogel during compression and subsequently reabsorbed during unloading. However, the RVT results revealed incomplete fluid recovery between successive compression cycles (Figure 6.2.6B). After one minute of simulated dynamic loading, the model predicted a net cumulative fluid loss of approximately 1.1 mm^3 , suggesting a gradual imbalance between fluid influx and efflux over repeated cycles, indicating an inability of the gel carrier to fully reabsorb the medium.

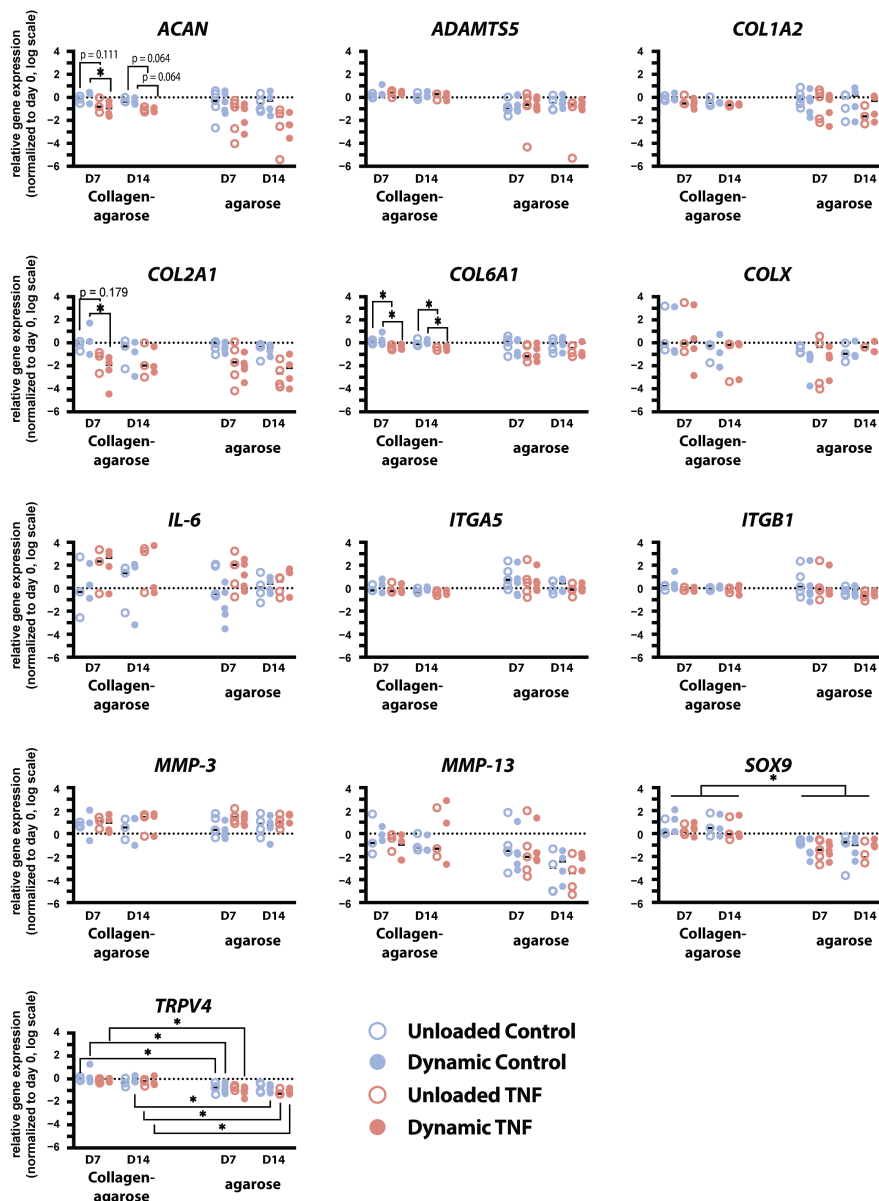


Figure 6.2.5: Relative gene expression of ACAN, ADAMTS-5, COL1A2, COL2A1, COL6A1, COL10A1, IL-6, ITGA5, ITGB1, MMP3, MMP13, SOX9, and TRPV4 in human CEP cells cultured in either blended collagen–agarose hydrogels or in 2% agarose hydrogels for 7 and 14 days. Data is normalized to 18S and the corresponding day 0 expression. * $p < 0.05$; $n = 3-5$.

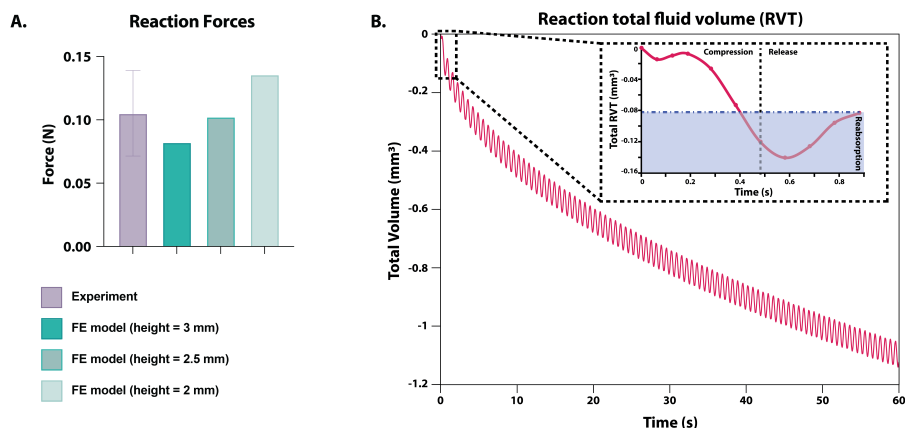


Figure 6.2.6: (A) Maximum reaction force during dynamic compression as measured experimentally (pink) or predicted in an FEM (green). Three hydrogel heights between 2 and 3 mm were modeled, while displacement remained at 0.21 mm. (B) Predicted RVT measured during one minute of loading within the FEM, shown here for the 3 mm hydrogel starting height. The box in the top right corner zooms in on the change during one cycle of compression and release, and the area in purple is the fluid that has been reabsorbed before the next cycle of compression begins.

6.2.4 Discussion of study 2

Collagen, the most abundant ECM protein in mammalian tissues, is widely used as a scaffold material for 3D culture systems. Incorporation of type I collagen introduces biofunctionality and enhances the biological relevance of *in vitro* models. In the blended collagen–agarose hydrogels, the presence of collagen can promote cell adhesion and FAK activation while preserving the rounded morphology typical of chondrocyte-like cells. The use of blended matrices enables independent tuning of the biophysical properties without altering collagen concentration.

While agarose contributed structural stability and biocompatibility, the addition of collagen, investigated in this study, improved biofunctionality and stiffness, resulting in a matrix that more closely mimics the native ECM and supports mechanotransduction signaling crucial for advanced *in vitro* modeling. The construct showed a maintenance of a rounded cellular morphology consistent with a healthy cartilaginous phenotype; however, some elongated fibers were observed in TNF-treated and dynamically compressed control hydrogels after 14 days, suggesting fibrotic-like changes associated with mechanical or inflammatory stress.

Downregulation of anabolic and PCM markers under TNF stimulation indicates that the cytokine promotes ECM degradation and impairs PCM formation. The observed increase in NO release in TNF-treated unloaded hydrogels after 7 days, compared to 14 days, further suggests a transient, time-dependent catabolic response. Interestingly, TNF exposure did not significantly affect MMP3 expression, which contrasts with findings from the agarose-only hydrogel study, implying that matrix composition influences cellular responses to inflammatory cues.

FE modeling revealed that variations in hydrogel height substantially affected the mechanical load experienced by the cells inside individual constructs within the bioreactor, potentially introducing variability across samples. The simulations showed that fluid was expelled during compression and partially reabsorbed during unloading. However, incomplete reabsorption between cycles led to cumulative fluid loss. This predicted fluid imbalance may explain the gradual reduction in hydrogel height observed experimentally over time.

The blended hydrogel demonstrated increased stiffness, bringing its mechanical behavior closer to that of native CEP tissue, which must be sufficiently rigid to withstand intradiscal pressure while remaining porous enough to facilitate solute exchange [164, 241, 572]. The measured stiffness approached the reported PCM stiffness of chondrons (17–200 kPa [573]) but remained below that of native endplate tissue. This advancement marks progress toward more physiologically representative *in vitro* models. However, until further optimization is achieved, the use of native CEP tissue may still provide the most accurate platform for studying endplate mechanobiology.

6.3 Conclusion

These two studies establish several findings that are relevant to this thesis. First, TNF consistently induced a catabolic and inflammatory response in human CEP cells, whereas the applied dynamic compression produced little measurable biological response in cells seeded either in pure agarose or collagen–agarose hydrogels. Second, the composition of the carrier strongly influenced the state of the incorporated cells: blending agarose with collagen increased hydrogel stiffness and supported higher SOX9 and TRPV4 expression, demonstrating that the matrix is not a passive carrier but an active determinant of phenotype and mechanosensory capacity that can

influence the experimental results. Third, the FE modeling showed that hydrogel height and fluid loss altered the force and transport conditions experienced during repeated loading. Therefore, nominal bioreactor inputs such as applied strain do not necessarily represent the local mechanical stimulus perceived by individual cells. These findings also reveal important limitations of cell–gel systems that are specific to the broader aims of this thesis. Isolated CEP cells are removed from their native extracellular and pericellular matrices, expanded in a monolayer, and then embedded in an artificial material with properties that remain substantially different from those of the native CEP. The gels do not reproduce the organization or interactions of the non-calcified cartilage endplate (ncCEP), calcified cartilage endplate (cCEP), bony endplate (BEP), nucleus pulposus (NP), annulus fibrosus (AF), and vertebral bone marrow. They also lack native nutrient gradients, tissue boundaries, resident cell diversity, and communication between adjacent tissues. Consequently, the weak response to compression reflects insufficient transmission or sensing of mechanical signals within the simplified culture environment. Although cell–gel *in vitro* models provide valuable experimental control and permit the effects of matrix composition, cytokines, and loading to be isolated, they cannot reproduce the coupled biological and mechanical behavior of the complete osteochondral endplate. These limitations reinforce the rationale behind the complementary approaches adopted throughout this thesis. Chapter 4 addressed the limitations of homogenized computational models by quantifying sample-specific BEP microarchitecture from micro-computed tomography (μ CT) images and using these measurements to infer local mechanical and transport properties. Chapter 5 preserved the native interfaces between the NP, AF, CEP, and BEP using intact osteochondral explants to investigate tissue-specific responses to inflammatory stimulation while maintaining the structural complexity of the EP. Building on these studies, while taking into account the limitations observed in this chapter, chapter 7 presents a custom perfusion bioreactor and computational workflow designed to impose and quantify fluid transport across intact osteochondral EP explants under controlled conditions. Collectively, these chapters progress from simplified cellular models toward increasingly physiologically relevant experimental and computational platforms that preserve native tissue architecture and transport pathways, providing a more comprehensive framework for investigating EP degeneration.

CHAPTER — 7

Design and Development of an *In Silico–In Vitro* Workflow for Testing Endplate Explant Punches

This chapter is based on a collaboration with the mechanical design engineer, Joris Bleukx. The bioreactor requirements, functional specifications, and overall design decisions were defined as part of this research. Joris translated these specifications into the engineering implementation by producing the CAD designs, selecting the electromechanical components, fabricating the custom 3D printed parts, and developing the control software.

The work presented in previous chapters demonstrated the importance of capturing structural properties of endplates under physiological and degenerative conditions, as well as the influence of mechanical loading on intervertebral disc (IVD)-derived cells. An important element of endplate functionality, as highlighted in Chapters 1 and 2, is mass transport, which remained unaddressed in the previous chapters. This chapter, therefore, focuses on the development and application of an *in silico*–*in vitro* workflow to study fluid flow, pressure gradients, and wall shear stress (WSS) across IVD endplate (EP) explants. It reviews the role of perfusion systems in bone, cartilage, and endplate research, emphasizing their importance for modeling nutrient transport, mechanotransduction, and tissue adaptation under physiologically relevant conditions. This chapter continues by describing the design of a custom perfusion bioreactor, as well as the prototype testing. The chapter then discusses the development of an automated computational fluid dynamics (CFD) pipeline, building on work from Chapter 4, that uses computed tomography (CT)-derived, sample-specific endplate geometries to estimate WSS distributions and support optimization of perfusion parameters for individual specimens. The chapter ends by providing suggestions for future experiments and design improvements. A comprehensive validation study of the developed workflow fell outside the scope of this PhD, but is included at the end of this chapter as well as in the suggestions for further work in Chapter 8.

7.1 Perfusion bioreactors in literature

Perfusion bioreactors are essential tools for studying how fluid flow affects biological tissues, replicating the mechanical and transport conditions found *in vivo*. They enable investigation of fluid-induced shear stress, a key driver of cellular mechanosensing and tissue adaptation under musculoskeletal loading [574]. In osteochondral tissues, such as the EP, WSS regulates the biological response of both cartilage and bone, although each tissue type exhibits distinct physiological thresholds.

In cancellous bone, the porous architecture of trabeculae, osteons, and lacunae is filled with interstitial fluid. Mechanical loading compresses the unmineralized matrix, driving fluid movement and generating shear stress on cell membranes. This shear stress regulates cell proliferation and stimulates the release of osteogenic growth factors, including bone morphogenetic protein (BMP) and TGF- β [575]. Dynamic loading promotes stronger

osteogenic responses than static loading [574], and *in vitro* studies have identified fluid shear stress as the principal mechanical stimulus governing both bone and cartilage cell activity [574, 576]. Osteoblast proliferation and the transition toward mineralization have been reported for WSS between 0.55 and 30 mPa [577, 578], whereas higher physiological loading conditions of approximately 0.4 to 1.2 Pa [574, 578] stimulate adaptive bone remodeling through nitric oxide (NO) and prostaglandin E2 (PGE₂) production and activation of osteogenic signaling pathways.

In cartilage research, perfusion bioreactors provide a controlled environment to study how type II collagen and extracellular matrix (ECM) respond to physiological shear forces [579, 580]. Fluid movement through the tissue generates shear stresses that influence chondrocyte proliferation, differentiation, apoptosis, and ECM synthesis, thereby maintaining cartilage structure and function [580, 581]. This interplay between mechanical and biochemical cues forms the foundation of mechanotransduction, where chondrocytes sense and respond to their fluid microenvironment. Importantly, shear stress can exert dual effects depending on its magnitude. Controlled levels between 0.05–0.21 Pa enhance mechanical and biochemical properties and have been shown to promote chondrocyte proliferation and ECM production, whereas excessive shear stress (>0.21 Pa) can lead to apoptosis, tissue degradation, and inflammatory responses [582]. Sustained WSS in the range of 1 to 2 Pa induced cartilage degeneration through increased inflammatory signaling, NO production, and reduced collagen II and aggrecan synthesis [583–585]. Thus, regulating shear levels within controlled perfusion systems is crucial for balancing beneficial and detrimental outcomes.

At a cellular level, fluid-induced shear stress modulates receptor regulation, cell morphology, and metabolic activity, influencing both chondrocyte homeostasis and differentiation [586]. Furthermore, shear stress affects the expression of matrix components such as aggrecan, collagen type II, and COMP, and alters integrin signaling, all of which are essential for maintaining tissue integrity [580].

Additionally, perfusion bioreactors can play an important role in enhancing nutrient transport and waste removal within osteochondral tissues *in vitro*, overcoming diffusion limitations of static cultures [580, 581]. Even modest flow rates can significantly improve cell survival, proliferation, and growth factor production, supporting tissue formation under physiologically relevant conditions. By allowing precise control of flow rate, oxygen tension, and shear magnitude, these systems model both normal and pathological

environments, offering insights into osteochondral tissue physiology and regenerative processes.

7.2 Perfusion experiments on the endplate

Few studies have experimentally investigated fluid flow dynamics within the IVD, particularly with respect to the role of the EP in regulating hydration and nutrient transport. Laboratory setups have been created to mimic the mechanical and physiological environment of the body. Some used full organ culture where the entire IVD would be submerged in a medium bath [587–590], others used the EP directly in a piston device [182] or constant flow rate pump [252] to generate controlled fluid flow. *In silico* techniques have also been utilized, such as analytical models [253], finite element modeling (FEM) [591–593], and CFD [242]. Yet, most studies neglect to identify and analyze each sublayer of the EP, which are the bony endplate (BEP), calcified cartilage endplate (cCEP), and non-calcified cartilage endplate (ncCEP).

Fluid transport across the EP is governed by several factors: mechanical loading, degeneration, calcification, trauma, and age [48, 164, 594]. Permeability varies spatially within the EP, with higher values in the central zone than at the periphery [164, 204], and varies temporally through diurnal cycles of fluid loss under load and recovery during rest [170, 204, 594]. Flow resistance is also directionally asymmetric, being substantially greater during outflow (exudation) than during inflow (imbibition) [252, 253], which facilitates fluid recovery in unloading periods. Together, these transport characteristics are essential for IVD health, reflecting the balance between external mechanical loads and internal osmotic pressures.

Analytical models have been developed to theoretically explain the influence of fluid-flow direction on EP effective permeability, suggesting that the ratio of porosities in the cartilaginous endplate (CEP), capillary bed, and subchondral bone could account for contradictory experimental findings in the literature, specifically between data gathered from living subjects (*in vivo*) and data gathered from isolated tissue segments studied in the lab (*ex vivo*) [595]. This mechanism is hypothesized to be caused by constrictive flow in the CEP overlying the marrow contact channels. During flow out, fluid drag forces and stresses compress the cartilage matrix into the channels, increasing resistance. Conversely, during flow in, the cartilage expands away

from the opening, decreasing resistance [252]. To test this hypothesis, a perfusion bioreactor was developed to monitor the fluid flow across EP samples with respect to vertebral level, inlet pressure, and direction of flow.

7.3 Bioreactor design and function

A custom-built bioreactor (Figure 7.3.1) was developed, and its parts were chosen to perfuse culture medium through EP samples while precisely controlling and monitoring the pressure and flow around the sample (Table 7.3.1). The system can operate in two modes. The first maintains a constant pressure difference across the sample while measuring the corresponding flow rate, and the second maintains a constant flow rate while monitoring the resulting pressure difference.

The bioreactor was designed to maintain a minimum stable pressure difference of 2 bar and sustain a flow rate between 0 and 500 $\mu\text{L}/\text{min}$. These operating ranges were selected empirically during system development based on preliminary testing, representing ranges that allow medium perfusion through explants. Real-time data on pressure, flow rate, and corresponding timestamps were continuously transferred to a desktop computer for recording and subsequent analysis. The bioreactor was engineered for compatibility to be placed in standard incubator conditions (temperature = 37°C and relative humidity $\geq 85\%$), allowing operation under physiologically relevant environmental parameters. All custom-designed components were modeled using Solid Edge 2019 (version 219.00.00.091, Siemens Digital Industries Software, Plano, TX, USA). More information on the design principles and the different parts is provided below.

Table 7.3.1: Parts used to construct the bioreactor, divided into (I) 3D-printed parts, (II) accessories, and (III) ordered components.

I. 3D-printed parts		II. Accessories	
i.	Chamber part 1	ix.	Clamps for front window
ii.	Chamber part 2	x.	Clamps for chamber
iii.	Reservoir	xi.	Cole-Parmer UNF-to-Luer female adapter
iv.	Reservoir lid	xii.	Cole-Parmer Luer-male-to-barbed adapter
v.	Front window		
vi.	Rear cover		
vii.	Flow dampener		
viii.	Flow dampener cap		

III. Ordered parts			
Component	Manufacturer	Part number	
xiii. Pressure sensor, 4 bar, media isolated	Honeywell	MIPAG1XX004BAAAX	
xiv. LEMO receptacle, 3 pin	LEMO	EGG.0B.303.CLL	
xv. LEMO receptacle, 4 pin	LEMO	EGG.0B.304.CLL	
xvi. LEMO cable plug, 4 pin	LEMO	FGG.0B.304.CLAD52Z	
xvii. LEMO cable plug, 3 pin	LEMO	FGG.0B.303.CLAD52Z	
xviii. Flow sensor	Sensirion	SLF3S-0600F	
xix. Motor controller	Nanotec	SMCI12	
xx. Stepper-motor-driven pump	Watson-Marlow	100 Series Stepper Motor Pump (exact pump head to be specified)	
xxi. Arduino	Arduino	Nano Every (ABX00033)	
xxii. Han Modular 4-slot fixed frame	HARTING	09 14 016 1711	
xxiii. Han Modular 4-slot floating frame	HARTING	09 14 016 1701	
xxiv. Han Modular D-Sub plug insert	HARTING	09 14 009 3001	
xxv. Han Modular D-Sub receptacle insert	HARTING	09 14 009 3101	
xxvi. Han Modular Universal Serial Bus (USB) patch cable insert	HARTING	09 14 001 4601	
xxvii. Han Modular DDD module plug	HARTING	09 14 017 3001	
xxviii. Han Modular DDD module receptacle	HARTING	09 14 017 3101	
xxix. Han Dummy 1	HARTING	09 14 900 1000	
xxx. Han Dummy 2	HARTING	09 14 900 2000	
xxxi. Multiport serial adapter	MOXA	UPort 1450	

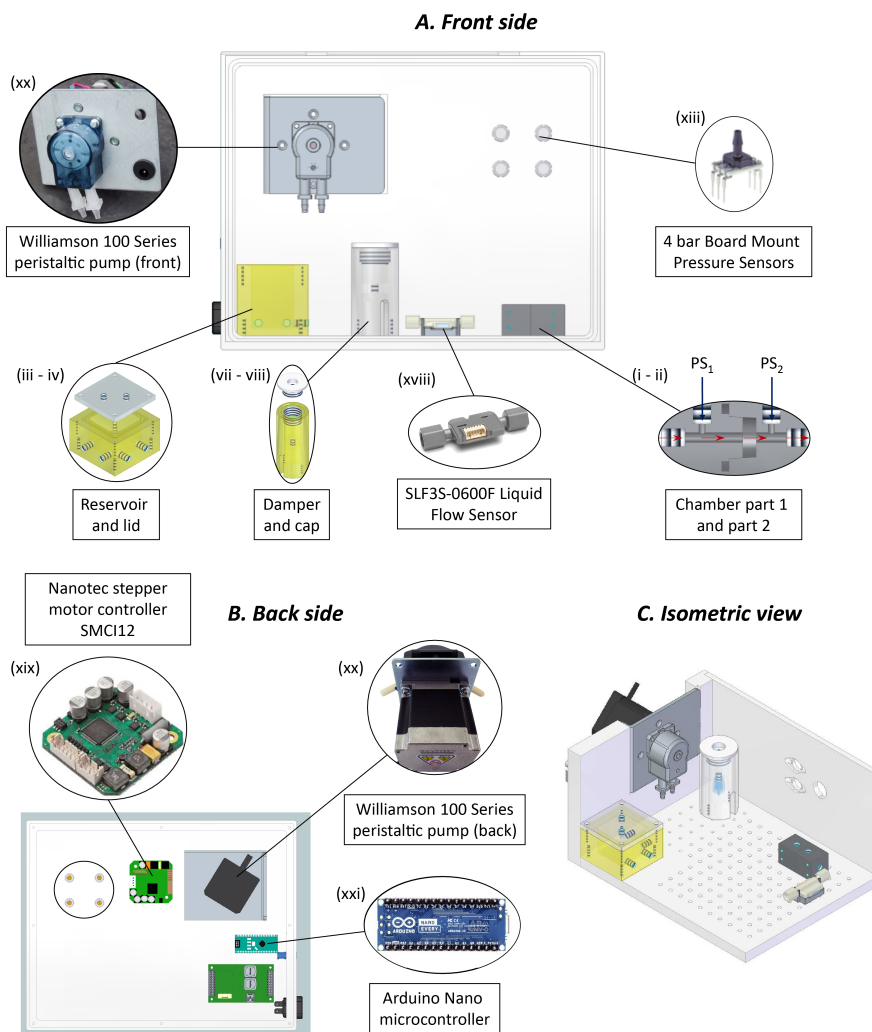


Figure 7.3.1: Custom-made perfusion bioreactor for IVD osteochondral EP (C). The sensors and the perfusion components are located on the front side (A), while the electrical components are located on the back side (B). Tubing and wiring connections were removed from the figure for clarity. Lowercase Roman numerals shown in parentheses correspond to the component numbers listed in Table 7.3.1.

7.3.1 Operating principle

The EP bioreactor operates using a simple flow-through design. A peristaltic pump draws culture medium from a reservoir and drives it through the EP sample, which is secured within a custom housing. Two pressure gauges positioned upstream and downstream of the sample continuously measure the fluid pressure. The difference between these readings, relative to atmospheric pressure, defines the pressure difference across the sample. Flow rate is measured at the outlet of the housing, after which the medium returns to the main reservoir for recirculation. A peristaltic pump was selected to ensure that the medium remained fully isolated from the internal components of the pump. However, the inherent pulsatile nature of peristaltic pumping, combined with the high hydraulic resistance of the sample, introduced fluctuations in both pressure and flow. To minimize these oscillations, a flow dampener was installed between the pump outlet and the sample inlet. The bioreactor was designed for bidirectional perfusion, allowing the flow direction through the sample to be reversed. Consequently, either connection can function as the inlet or outlet, enabling comparison of fluid transport in both directions across the EP.

7.3.2 Frame

The bioreactor components were mounted within a modular 3D-printed frame designed to securely hold all system elements in place while minimizing fabrication costs. The frame parts were produced using selective laser sintering (SLS) with polyamide, providing sufficient mechanical stability and resistance to incubator conditions.

7.3.3 Medium reservoir

The medium reservoir (Figures 7.A.1 and 7.A.2) was fabricated by milling a cavity into a polysulfone (PSU) block and sealing it with a transparent polycarbonate lid. The lid was fastened using an O-ring cord to ensure an airtight seal. Both the reservoir and lid were equipped with several threaded openings (UNF 1/4-28) to accommodate standard laboratory Luer fittings. One port on the lid was fitted with a Luer air filter, maintaining the reservoir at atmospheric pressure while preserving sterility.

Polycarbonate was selected for the lid to enable visual inspection of the medium, while PSU was used for the main body due to its superior resistance to cracking after repeated autoclaving.

7.3.4 Pump and motor drive

A Williamson 100 Series peristaltic pump was used to drive the flow of culture medium through the system. This pump type was selected for its suitability for low flow rates and its ability to maintain complete separation between the medium and the internal pump components, as it only contacts the tubing compressed by the rollers. The pump was driven by a stepper motor controlled by an SMCI-12 motor driver (Nanotec GmbH & Co. KG, Germany). The SMCI-12 was selected primarily for its availability, although any compatible motor driver capable of speed control could be used.

The motor driver was configured using NanoPro software (Nanotec), operated in speed control mode. Motor parameters such as phase current were adjusted to minimize power consumption and heat generation. The driver communicated with a desktop computer via an Recommended Standard 485 (RS-485) interface, enabling control of motor functions including start, stop, direction, and speed. The system also allowed optional logging of motor status data.

A USB-to-serial converter (MOXA UPort, configured in RS-485 4-wire mode) was connected to the motor driver via a standard 9-pin Sub-D cable. Electrical connections between the pump, pressure sensors, flow sensor, and computer followed the wiring schematics developed for the system (Figures 7.A.9, 7.A.8, and 7.A.7, respectively).

7.3.5 Flow dampener

A flow dampener (Figures 7.A.5 and 7.A.6) was incorporated into the system to reduce fluctuations in pressure and flow caused by the pulsatile operation of the peristaltic pump. The dampener consists of a vertically oriented, airtight chamber that is initially empty. During operation, medium entering from the top compresses the trapped air pocket, which acts as a cushion to smooth out pulsations in the flow. The design prevents backflow toward the pump and maintains a constant pressure on the sample once the air pocket

is compressed.

This mechanism effectively stabilized the pressure across the sample. Figure 7.3.2 illustrates the improvement in flow stability achieved with the addition of the flow damper compared to direct pump connection.

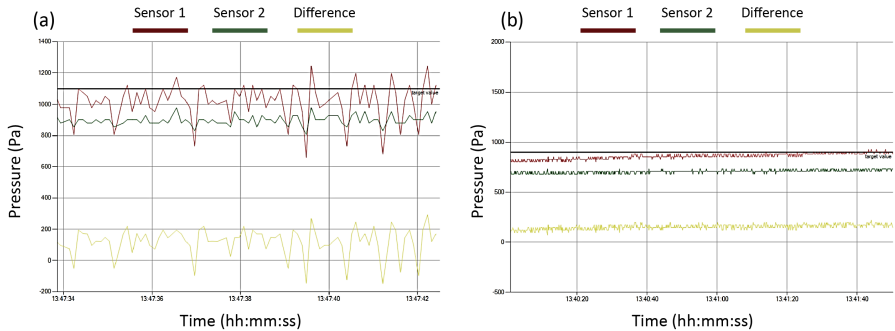


Figure 7.3.2: Stability reached in flow pressures between (a) direct connection of the pump to the chamber and (b) adding an intermediary flow damper. Pressure sensors 1 and 2 measure the pressure immediately upstream and downstream of the sample, respectively.

7.3.6 Sample chamber

EP samples are prepared as cylindrical specimens of 8 mm in diameter and 4 mm in height using a biopsy punch as previously mentioned in Chapter 3. To prevent fluid bypass along surface irregularities, each sample was enclosed in a silicone tube (inner diameter 8 mm, outer diameter 12 mm) cut to a length similar to the sample height. The sample and its silicone jacket were inserted into a tapered stainless-steel chamber (Figures 7.3.3) with a diameter of 11.6 mm. The resulting compression of the deformable silicone ensured a leak-tight interface between the sample, jacket, and chamber wall.

The chamber consisted of two stainless-steel components (Figures 7.A.3 and 7.A.4). One part contained the tapered recess accommodating the sample assembly, while the other part featured a matching protrusion that secured the sample in place and ensured proper sealing. The two parts were held together by integrated latches providing a total clamping force of approximately 50 kg. Each chamber part included a fluid inlet or outlet with a connected side channel for pressure sensor attachment. Stainless steel was selected for its durability and ability to withstand the mechanical forces required for repeated sealing and handling.

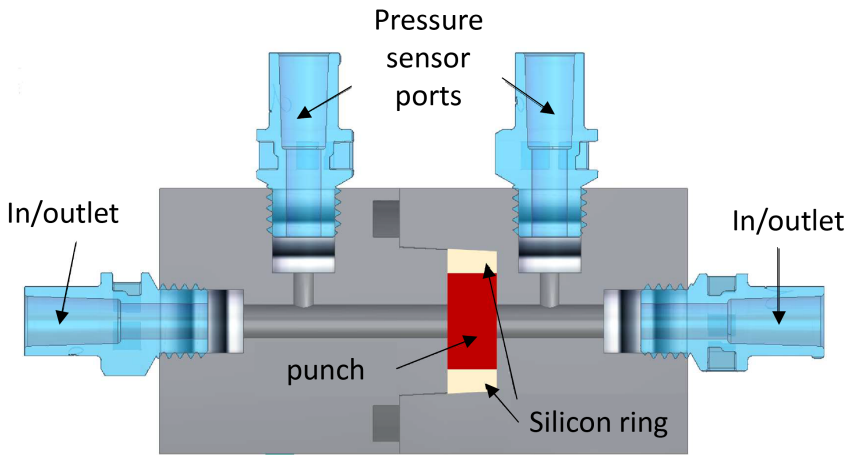


Figure 7.3.3: Osteochondral endplate sample in the assembled chamber with fittings.

7.3.7 Pressure sensors

Fluid pressure across the EP sample was measured using Honeywell analog output pressure sensors. Two sensor ranges were initially implemented to determine the appropriate measurement range for the system. The first set (Honeywell SSCDRRV100MDAA5) measured pressure differences up to 100 mbar, functioning as gauge sensors with one inlet open to air. The second set (Honeywell ABPDANV060PGAA5) measured gauge pressures up to 60 psi, approximately 4.1 bar. In practice, only the latter set was used, as no measurable flow occurred below 100 mbar pressure difference.

Both sensor types operated at 5 V and provided an analog output up to 5 V. The output signals were acquired using an Arduino Nano microcontroller, which features a 10-bit analog-to-digital converter (ADC). Given that the output range of the sensors is 0.5–4.5 V, the effective resolution was 819 discrete values over the 0–4.1 bar range, corresponding to approximately 0.005 bar per bit. This resolution was deemed sufficient for the current setup.

The pressure sensors and the Arduino were powered by a regulated 5 V external supply to ensure signal stability. The same reference voltage was applied to the analog reference input of the Arduino for consistent conversion accuracy.

7.3.8 Flow sensor

Medium flow rate was measured downstream of the EP sample using a digital liquid flow sensor (Sensirion SLF3S-0600F, Sensirion AG, Switzerland) capable of detecting flow rates up to 2 mL/min. The sensor was connected to an Arduino Nano microcontroller using dedicated inter-integrated circuit (I²C) pins (A4 for data, SDA; A5 for clock, SCL) outputs data via an I²C interface. Communication with the sensor was implemented using the Arduino Wire library. While active, the sensor continuously updates flow data in an internal buffer, which can be read via I²C. Each read operation returns nine bytes containing flow, temperature, and status information.

7.3.9 Data reading and reporting

In measurement mode, the Arduino continuously collects data from the flow sensor and the pressure sensors. The acquired data are saved as “flow rate, flow sensor temperature, flow sensor status flags, pressure sensor 1 value, and pressure sensor 2 value,” where flow sensor status flags show if there are any air bubbles in the system. The control software then converted the analog readings into pressure values using sensor-specific calibration data.

7.3.10 Tubing and connectors

To facilitate easy disconnection of the sensors during handling or sterilization, push-pull connectors were implemented between the sensors and the main electronics. LEMO 0B series connectors were selected for their reliability and availability in various pin configurations.

The fluidic circuit was assembled using standard tubing and Luer fittings commonly available from laboratory suppliers.

7.3.11 Sterilization procedures

Prior to use in cell culture experiments, the bioreactor components were sterilized to ensure a sterile environment and prevent microbial contamination. Autoclaving was not feasible due to the presence of plastic and

electronic components; therefore, gas sterilization was chosen.

The fluidic components, including the housing, tubing, and fittings, were first disassembled and cleaned with 70% ethanol to remove any residues or particulates. After air drying under a laminar flow hood, the cleaned parts were reassembled, sealed in a sterilization pouch, and sent for gas sterilization as a complete setup.

Following sterilization, the bioreactor remained sealed until use. The only component opened post-sterilization was the sample chamber, which was accessed under sterile conditions under a laminar flow hood to insert the sample. Fresh culture medium was then introduced into the reservoir by pushing it through the filter using a sterile syringe.

7.3.12 Prototype testing

A prototype of this bioreactor has been tested in a 4-day operation on one fresh EP punched out sample (from here on abbreviated as “punch”). The punch was extracted from a bovine tail as described previously. An osteochondral sample, measuring 8 mm in diameter and 4 mm in height was punched from the sample. The punched sample was then left in a petri dish with high-glucose Dulbecco’s Modified Eagle Medium (HG-DMEM) for 2 days to recover from explantation and handling stress. Prior to the experiment, the punched sample was treated for blood clots and debris by washing it with phosphate-buffered saline (PBS) several times and placing it in a heparin-streptokinase solution. Subsequently, the sample was moved to a petri dish with low-glucose Dulbecco’s Modified Eagle Medium (LG-DMEM) overnight before being fitted into the chamber of the bioreactor for 20 minutes. The entire bioreactor was placed in the incubator at standard 37°C and the signals from the pressure inlet, pressure outlet, and flow rate sensors were recorded.

Throughout the trial, air bubbles were present in the system, and the pressure difference across the sample increased steadily after the first 3 minutes while flow rates remained minimal (Figure 7.3.4). This suggests that even with the treatment for removal of blood clots and debris, the sample itself was not percolating. This could be due to location-specific morphology, as was previously shown (Chapter 4). Future experiments should incorporate sample percolation testing prior to bioreactor fitting.

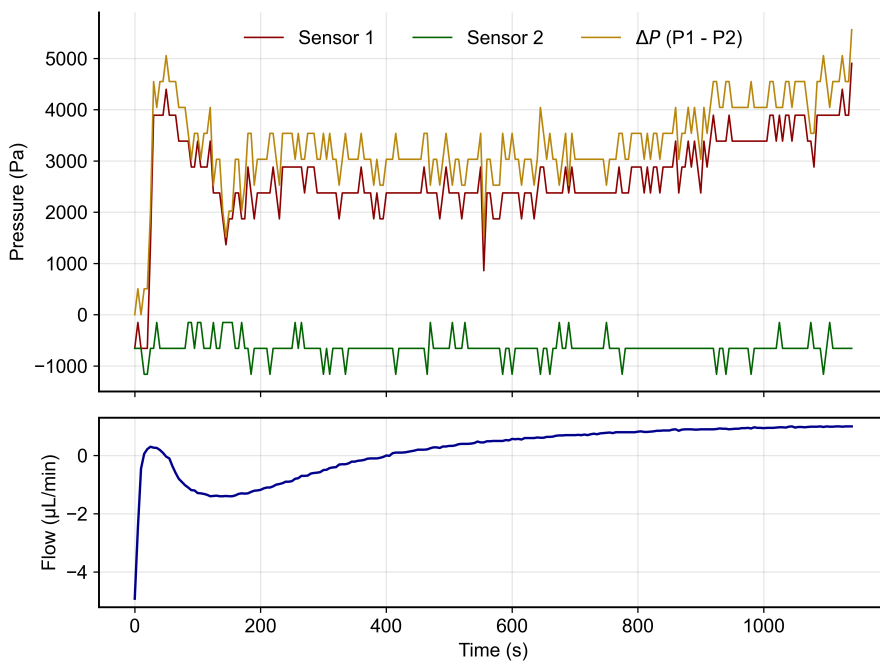


Figure 7.3.4: Pressure recorded by sensors 1 and 2, calculated pressure difference (ΔP), and flow rate during prototype testing of the perfusion bioreactor.

7.4 CFD simulation pipeline for perfusion bioreactor analysis

The mechanical environment of the EP, particularly WSS, regulates mechanotransduction pathways that influence key cellular behaviors. Replicating physiologically relevant WSS during perfusion bioreactor experiments is therefore essential for studying IVD health. Therefore, a fully automated CFD simulation pipeline was developed as a standalone extension to the *in silico* pipeline previously discussed in Chapter 4. The pipeline allows to quantify WSS and to accurately characterize the flow-induced component of the mechanical environment in the perfusion bioreactor experiments.

7.4.1 Automated CFD simulation workflow

The *in silico* tool uses the punch models generated from CT scans (Chapter 4), initial inlet and outlet pressures and velocities, fixed pressure/velocity at the inlet and/or outlet, and the fluid viscosity as inputs. It outputs the inlet and outlet velocities over time, and the WSS distributions at the final simulation time point, either at steady state or after a user-defined duration. The tool assumes laminar flow due to the small pore diameters within the punch [596], although this setting can be modified by the user. The tool is available in a public GitHub repository [597]. The pipeline (Figure 7.4.1) was developed using OpenFOAM v9 for CFD simulations [598] and ParaView 5.10.1 for post-processing and visualization [599]. This CFD simulation workflow was tested on six punches taken from the same bovine subject.

Preprocessing uses a Python script where the workflow first reads user-defined experimental parameters, including inlet/outlet tubing geometry, and generates cylindrical extensions to the punch model to represent the perfusion channels and create a continuous flow domain. These extensions are automatically positioned at the inlet and outlet surfaces and merged with the punch geometry using Boolean operations to create a continuous fluid domain. The resulting surface model is then processed and partitioned into five regions corresponding to the inlet, outlet, tubing sections, and punch wall boundaries (Figure 7.4.1e). Cell-center-based spatial thresholding is applied to separate these regions while preserving the original mesh topology. The resulting surfaces are exported in OpenFOAM-compatible American Standard Code for Information Interchange (ASCII) stereolithography (STL) format with

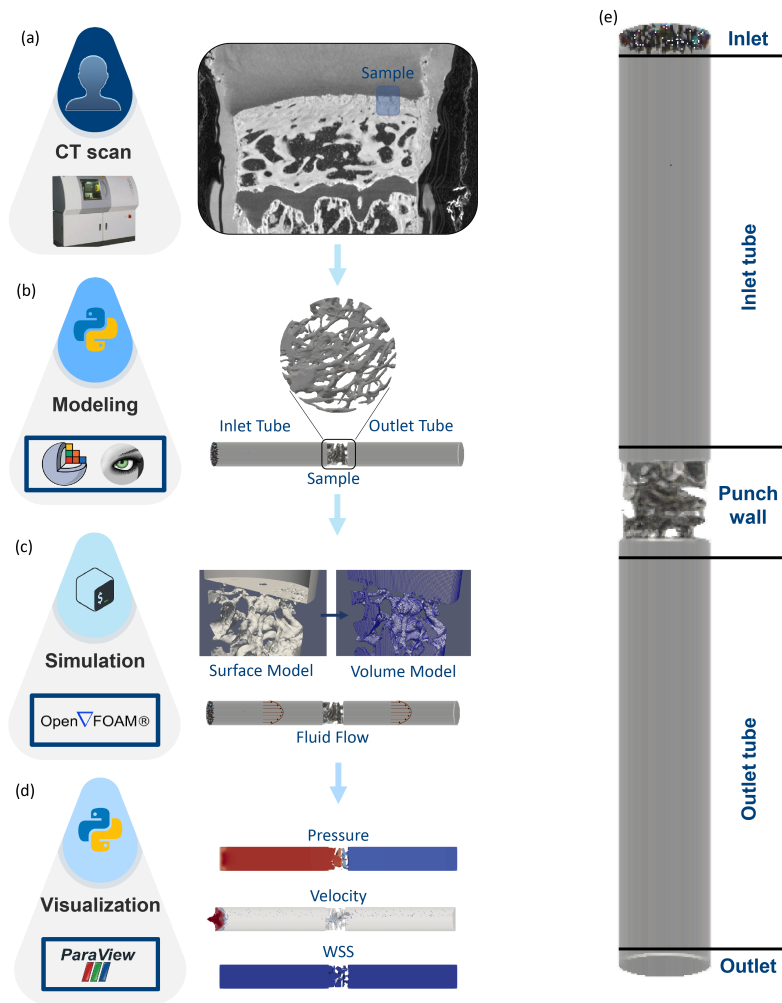


Figure 7.4.1: Overview of the workflow, illustrating the sequential steps from sample imaging (a) to morphological surface mesh generation, as described in Chapter 4, and the creation of the inlet and outlet tubing (b), followed by volumetric mesh generation and CFD simulation (c), and finally post-processing and visualization of the simulation results (d). The full model (e) is partitioned into inlet, inlet tube, punch wall, outlet, and outlet tube.

standardized naming conventions. Finally, the workflow automatically determines the internal reference point and bounding box required by snappyHexMesh and blockMesh for OpenFOAM mesh generation, storing all geometric parameters and simulation specifications for subsequent CFD analysis. This preprocessing script (script 08) was added to the same GitHub repository from Chapter 4.

A Bash script was developed to automate the complete OpenFOAM simulation workflow, minimizing manual intervention and ensuring reproducibility. The script first reads the geometric parameters generated during the preprocessing stage, including the location of an internal reference point and the dimensions of the bounding domain. These parameters are automatically transferred to the OpenFOAM configuration files, allowing each simulation case to be adapted to the corresponding specimen geometry. The script then executes the CFD workflow sequentially. Initially, a background hexahedral mesh is generated, after which the surface edges required for mesh snapping are extracted. The computational mesh is subsequently created, which refines the background mesh and conforms it to the STL geometry while preserving the inlet, outlet, wall, and tubing boundary patches, and then generates a hexahedral volumetric mesh. Steady-state incompressible flow is then simulated, using the Semi-Implicit Method for Pressure-Linked Equations (SIMPLE) algorithm, which computes the pressure, velocity, and WSS fields throughout the porous domain. Finally, checkMesh is executed to verify mesh quality, topology, and geometric consistency before post-processing. The workflow was designed to operate in parallel execution, divided over the available central processing unit (CPU) cores.

Finally, a Bash script was developed to automate the post-processing of the OpenFOAM simulation results using ParaView. The script sequentially executes a series of Python scripts that generate standardized visualizations of the computational mesh, pressure field, velocity field, and WSS distribution without requiring user interaction. Additionally, it automatically extracts quantitative CFD results. For the inlet and outlet boundaries, the script isolates the corresponding surface patches and computes the temporal evolution of area-averaged pressure and velocity. The resulting pressure and velocity profiles are exported as both graphical representations and comma-separated value (CSV) files for subsequent quantitative analysis. To quantify the mechanical stimulus experienced by the cells, the sample wall boundary is extracted at the final simulation time step, and the WSS distribution over the entire punch surface is exported as a CSV file. Operating ParaView in batch mode ensures that identical visualization settings are

applied to every specimen, improving consistency and reproducibility across simulations.

7.4.2 Results and discussion

The CFD simulations performed using the sample-specific geometries for a simulation duration of 500 s produced distinct flow characteristics for each specimen (Figure 7.4.2a). This difference in outlet velocity magnitudes between samples could be due to the difference in pore morphology, which was proven in Chapter 4. The average pore diameter ranged from approximately 0.02 to 0.04 mm across the samples (Figure 7.4.2b). However, all simulations exhibited a similar temporal profile, characterized by an initial rapid increase in outlet velocity, followed by a decrease, and then another increase throughout the remainder of the simulation. At the end of the 500 s simulation period, the calculated WSS ranged from approximately 0.4 to 0.8 MPa across the six specimens (Figure 7.4.2c). Samples with larger average pore diameters generally exhibited higher WSS values, indicating a relationship between specimen morphology and the resulting mechanical environment predicted by the CFD model.

7.5 Conclusions and future work

7.5.1 Disentangling effects of structure and WSS

As demonstrated in Chapter 4, the morphology of the EP is sample-specific and varies not only between individuals but also between specimens from the same subject. Consequently, identical bioreactor settings do not necessarily produce the same fluid flow patterns or mechanical environment across all samples. *in vivo*, structural changes associated with degeneration alter fluid transport and the resulting WSS, making tissue morphology and mechanical stimulation intrinsically coupled. By combining specimen-specific computational modeling with experimental perfusion, the proposed *in silico-in vitro* workflow provides the opportunity to disentangle these effects. The CFD simulations can be used to determine the bioreactor operating conditions required to achieve a target WSS for each specimen, thereby enabling studies that isolate the influence of tissue morphology from that of fluid-induced mechanical stimulation. Conversely, the workflow also allows

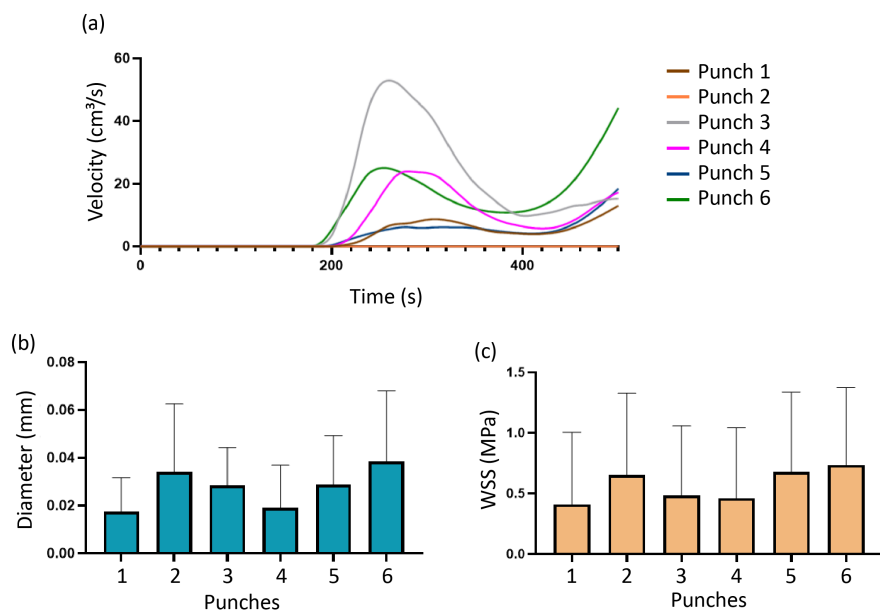


Figure 7.4.2: Sample-specific CFD results showing (a) outlet velocity over the 500-s simulation, (b) mean pore diameter, and (c) calculated WSS for the six punches at the end of the simulation.

the deliberate modulation of WSS within the same specimen geometry to investigate its direct biological effects.

The developed CFD framework aimed to provide an automated and reproducible workflow for estimating fluid-induced mechanical stimuli within porous biological tissues during perfusion culture. By integrating specimen-specific geometries with automated mesh generation, simulation, and post-processing, the framework enables quantitative assessment of WSS and flow characteristics. Due to practical and time constraints, the work in this chapter did not go beyond the initial creation and preliminary testing of the integrated workflow. Several of the next steps required to complete this study are discussed below.

7.5.2 Redesigning the bioreactor housing

An updated version of the bioreactor (Figure 7.5.1) has been designed but not yet fabricated. The new design retains the same operating principles as the current prototype but introduces improvements to the housing. The

system is divided into two main components connected via a HAN Modular connector.

The main operating chamber adds an additional layer of sterility by isolating the fluid circuit from the general incubator environment when required. Access to the internal fluid loop is provided through a removable polymethyl methacrylate (PMMA) window that seals airtight when closed, preventing contamination while allowing visual inspection. The unit connects to the power unit exclusively via a single HAN Modular connector, enabling quick attachment and detachment for handling, maintenance, or sample exchange.

The housing of the bioreactor chamber is divided into a front and a back section. The front section contains the fluid circuit and sensors, while the back section accommodates the electronic components. The separation wall between the two sections holds the peristaltic pump and includes LEMO connector sockets for attaching the flow and pressure sensors.

Dedicated slots are incorporated into the housing design to securely position the flow sensor, as well as to accommodate a modified medium reservoir and the flow dampener. This layout ensures compact integration of components while maintaining ease of assembly and accessibility for maintenance or sterilization.

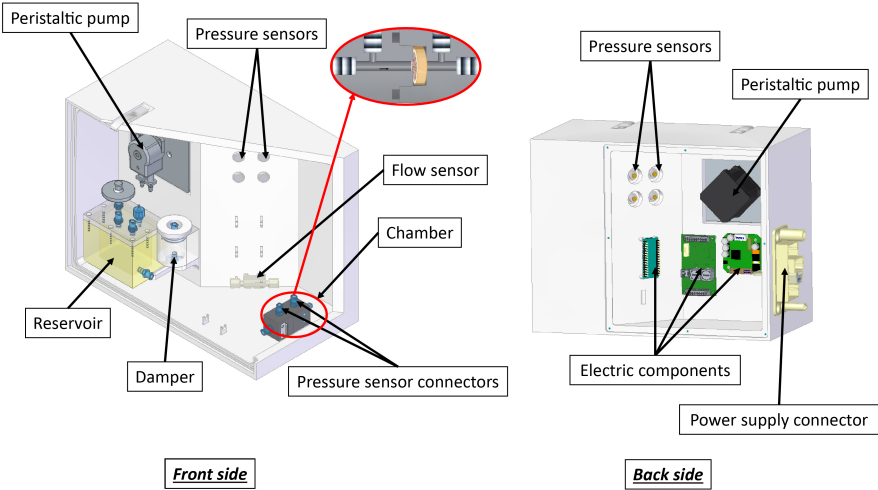


Figure 7.5.1: Improved custom-made perfusion bioreactor for IVD osteochondral endplates. Tubing and wiring connections were removed from the figure for clarity.

7.5.3 Experimental procedure

To increase the throughput of perfusion experiments, additional bioreactors could be fabricated and integrated into the existing control platform, enabling the simultaneous culture of multiple osteochondral samples under independently controlled or shared operating conditions. This would facilitate longer-term perfusion studies, for example, over one week, with sufficient sample numbers to evaluate biological responses under different mechanical loading conditions.

After sample washing and incubation in a heparin-streptokinase solution, samples should be assessed for permeability by tightly fitting them into a tube containing fluid on one side and centrifuging the assembly to determine whether fluid passes through the tissue (Figure 7.5.2). Only samples that permit fluid transport should be included in subsequent perfusion experiments along with static controls. Experimental conditions could include using different culture media, such as standard, degenerative, degenerative-supplemented-with-cytokines, and chondrogenic media, to investigate the influence of the biochemical environment on the tissue response to perfusion. Downstream analyses could include quantitative polymerase chain reaction (qPCR), glycosaminoglycan (GAG) and deoxyribonucleic acid (DNA) quantification, histological and immunohistochemistry (IHC) staining, and micro-computed tomography (μ CT) imaging to evaluate the biological and structural effects of fluid-induced mechanical stimulation under different culture conditions.

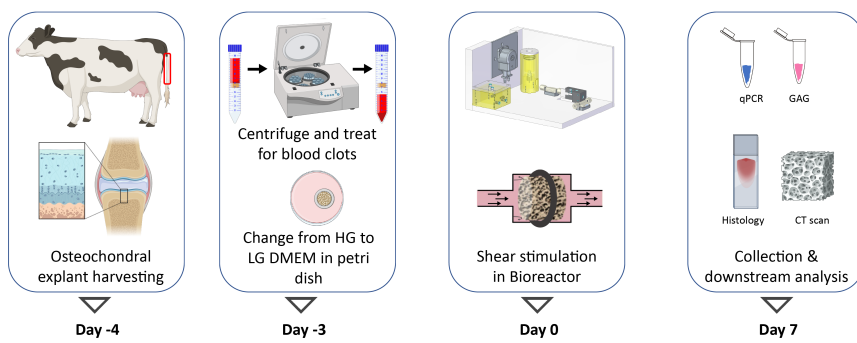


Figure 7.5.2: Suggested study design for future work to test the osteochondral perfusion bioreactor.

7.5.4 Integrating the automated CFD pipeline into the bioreactor experiment

In future work, the predicted WSS distributions can be used to optimize bioreactor operating conditions for individual specimens, allowing physiologically relevant flow-induced environments to be reproduced across morphologically diverse samples. Combined with the semi-automated modeling pipeline presented in Chapter 4, the developed CFD framework establishes an integrated approach for translating CT-derived, sample-specific tissue morphology into optimized perfusion bioreactor parameters (Figure 7.5.2), enabling customization of the perfusion regime for each individual specimen. This allows the effects of EP structure and flow-induced stimulation to be studied independently.

The platform's main limitation is that, unlike whole-disc bioreactors, it does not reproduce the coupled mechanical environment of the IVD, where cyclic loading simultaneously deforms multiple tissues, alters intradiscal pressure, and exposes cells to matrix strain, hydrostatic pressure, and fluid-induced shear stress. Furthermore, the CFD modeling pipeline implemented here assumes a rigid porous domain and therefore neglects fluid-structure interactions and deformation-induced changes in permeability. Future work could combine controlled perfusion with cyclic compression for the bioreactor part, and implement substrate mechanics capturing deformation-induced changes in permeability to further improve physiological relevance while preserving the ability to quantify specimen-specific stimulation.

CHAPTER — 8

Discussion, Limitations, and Future Directions

This chapter recapitulates the main findings of the thesis and discusses endplate degeneration as a multiscale process involving impaired transport, altered mechanobiology, structural heterogeneity, and cytokine-driven inflammation. It reflects on the strengths and limitations of the experimental and computational models used throughout the thesis, including hydrogel systems, osteochondral explants, imaging-based reconstruction, and perfusion bioreactor development. This chapter also outlines future directions for combining CT-derived modeling, CFD simulations, and perfusion experiments to link sample-specific endplate morphology with biological responses and therapeutic strategies.

8.1 General discussion

8.1.1 Overview

Intervertebral disc (IVD) degeneration is a major contributor to low back pain (LBP) and remains a central target for the development of biologically informed therapeutic strategies. The overarching aim of this thesis was to investigate mechanisms of endplate (EP) degeneration using complementary *in vitro* and *in silico* approaches. Taken together, the findings of this doctoral work support a central conclusion: the EP should be investigated further as a possible primary regulator of IVD health, with early degenerative changes that may precede those in the disc itself. They also highlight the need to study the EP within explant systems, particularly when controlled mechanical loading is introduced.

This thesis addressed EP physiology and pathology across multiple experimental and computational setups and length scales. The early chapters established the clinical relevance of disc degeneration and reviewed the physiology of the disc, with particular emphasis on immune privilege, transport dependence, and the structural and biological role of the cartilaginous endplate (CEP) (Chapters 1 and 2). The later chapters translated that framework into imaging-based reconstruction of EP microstructure (Chapter 4), osteochondral explant models (Chapter 5), cell-based hydrogel systems (Chapter 6), and development of an *in silico-in vitro* workflow including a custom perfusion bioreactor setup (Chapter 7). Together, these components provide a framework that captures key aspects of disc complexity while permitting mechanistic investigation under controlled conditions.

8.1.2 Contributions across scales

A consistent theme across the thesis is that EP function cannot be understood through a single disciplinary lens or a single length scale. The EP is simultaneously a transport barrier, a mechanically loaded interface, and a biologically active tissue. Accordingly, degeneration is best interpreted not as an isolated failure of one compartment, but as a multiphysics and multi-scale process driven by interactions among structure, transport, mechanics, and cytokine activity.

The review chapters established the conceptual basis for this interpretation. They showed that the healthy disc exists in a tightly regulated environment that restricts immune-cell access and depends on EP-mediated exchange of nutrients and metabolites. They also showed that the CEP differs from both articular cartilaginous tissues and the bony endplate (BEP) in composition, transport behavior, and functional role. By distinguishing these tissues more clearly, the thesis framed the EP as an active determinant of disc homeostasis.

The first result chapter started by providing a tool to describe the structure of the BEP. The semi-automated micro-computed tomography (μ CT) workflow showed that the BEP is structurally heterogeneous, with differences in porosity, percolation, and predicted permeability between regions and among specimens. These findings indicate that spatially uniform representations of the EP are insufficient and that local microstructure is likely to influence degeneration in both the EP and the IVD.

The following chapter stayed at the same spatial (tissue) scale and shifted focus from physiology to pathology. In the osteochondral explant model, preservation of the native vertebra–endplate–disc interface enabled observation of region-specific responses in degeneration. Within this experimental setting, physicochemical changes alone were insufficient to induce marked degeneration over the experimental window, whereas cytokine stimulation consistently produced proteoglycan loss, reduced cellularity, and early degenerative changes at the endplate–disc interface. The distinction between tumor necrosis factor (TNF) and interleukin-1 beta (IL-1 β) was also informative, with TNF appearing to induce a more acute catabolic response and IL-1 β a more moderate but sustained remodeling-related effect. Together, these results indicate that loss of immune privilege and exposure to pathological cytokine mediators are likely early components of the degenerative cascade.

The experimental work then continued with a deeper dive in cell-specific responses to mechanical loading and degenerative conditions. The hydrogel studies confirmed that TNF drives CEP cells toward a catabolic phenotype, whereas mechanical loading alone did not rescue that response in pure agarose. This negative finding was informative because it indicated that mechanobiological behavior depends not only on the loading regimen but also on the biological relevance of the matrix transmitting that load. The transition to collagen–agarose hydrogels improved biofunctionality and cell attachment, but also reinforced a broader methodological point: artificial carriers may not transmit physiologically relevant mechanical cues to EP cells, highlighting the value of explant or whole-organ culture systems that better preserve the native tissue environment.

Finally, this thesis addressed the need to further disentangle the effects of structure and flow-induced mechanical stimulation of EP cells through the development of an *in silico–in vitro* workflow. The perfusion bioreactor represents a first step towards a unified platform capable of controlling flow, pressure gradients, and biochemical conditions in osteochondral EP tissues. Together with the imaging and modeling tools, it provides the basis for an integrated workflow in which specimen-specific morphology, predicted flow behavior, and measured biological responses can be studied in parallel.

Across these chapters, a coherent picture emerges. Early EP degeneration appears to involve loss of barrier integrity, impaired transport, altered mechanobiological signaling, and pathological cytokine activation. These processes are interdependent, and the EP may act both as an initiator and as an amplifier of IVD degeneration.

8.1.3 Methodological reflections

A further contribution of this thesis lies in the progression of model systems used to study EP biology. Each model captured a different level of complexity and addressed a different class of questions.

The gel systems represented a frequently used setup for controlled mechanobiological experiments. Their principal strength was experimental tractability. At the same time, they demonstrated that simplicity comes at a biological cost. Pure agarose lacked key extracellular matrix (ECM) cues, and even the collagen–agarose system remained a simplified representation of native tissue. These models were therefore valuable for mechanistic studies, but

insufficient on their own to reproduce full EP behavior.

The osteochondral explant model addressed several of those limitations by preserving native matrix architecture and tissue interfaces. This enabled evaluation of how cytokines and physicochemical conditions affect multiple contiguous tissues within the vertebra–endplate–disc unit. Although static hypoxic culture cannot reproduce the full dynamic transport and mechanical environment of the living vertebral column, it provided a more physiologically relevant platform for studying early degenerative changes than isolated cell culture alone.

The computational modeling workflow added a complementary level of analysis by quantifying EP microstructure and inferring permeability and mechanical properties from imaging data. Its major value lies in moving beyond generic representations toward sample-specific structural characterization. The perfusion bioreactor extends this approach toward dynamic experimental validation by establishing a practical foundation for studying fluid-induced shear and transport under defined conditions.

Taken together, these methods form a staged multiscale strategy: simplified hydrogels for controlled cellular questions, explants for tissue-level biological relevance, imaging-based reconstruction for structural quantification, and bioreactor development for dynamic transport interrogation.

8.2 Limitations and outlook

Several limitations should be considered when interpreting the findings of this thesis. First, much of the experimental work relied on bovine tissues or on human cells removed from their native tissue context. Although informative, these models are not identical to the human lumbar EP in age, loading history, geometry, or degeneration profile. Likewise, isolated human CEP cells expanded in culture cannot fully recapitulate the phenotype of cells embedded within their native ECM. These differences limit direct clinical translation.

Second, the computational workflow inferred permeability and mechanical properties from microstructural measurements using relationships derived from the literature rather than direct specimen-specific testing. The reported material properties, therefore, remain predictions rather than direct

measurements and should be validated in future work.

Third, the osteochondral explant model preserved native interfaces more effectively than cell-gel systems, but it still represented an early and time-limited *ex vivo* model of degeneration. The culture period was sufficient to detect cytokine-driven matrix loss and cell depletion, but not to reproduce chronic remodeling, calcification, vascular ingrowth, or pain-associated changes characteristic of long-standing human degeneration. The imposed physicochemical environment was also simplified relative to the fluctuating conditions experienced *in vivo*.

Fourth, the hydrogel studies highlighted an important methodological constraint: *in vitro* loading is only as meaningful as the matrix through which it is transmitted. The pure agarose system likely underestimated physiological cell–matrix interactions, whereas the collagen–agarose system, although improved, remained a simplification of the native ECM of the EP. In addition, construct geometry, height changes over time, and fluid loss during repeated compression introduced uncertainty in the effective mechanical stimulus experienced by the cells.

Finally, some aspects of the thesis remain at the development stage. The perfusion bioreactor is a promising enabling technology, but the present work emphasizes design, prototyping, and initial testing rather than full biological validation across multiple experimental conditions. Similarly, integration of imaging-derived structural models with live perfusion experiments is only partially realized within the present body of work and requires a credibility assessment before it can be reliably incorporated in the integrated *in silico–in vitro* workflow.

The most immediate next step therefore could be to connect the structural, biological, and transport findings of this thesis into a closed-loop multiscale framework. This was precisely the aim of the final study (Chapter 7), of which only the first implementation steps could be performed in the scope of this thesis. In such an integrated *in silico–in vitro* workflow, individual samples would first be imaged and reconstructed in three-dimensional (3D), then tested experimentally under matched perfusion conditions, while parallel computational fluid dynamics (CFD) simulations estimate local flow fields and fluid-induced shear stresses experienced by the cells (Figure 8.2.1). Experimental measurements of volumetric flow rate and pressure drop could then be used to further validate the computational predictions.

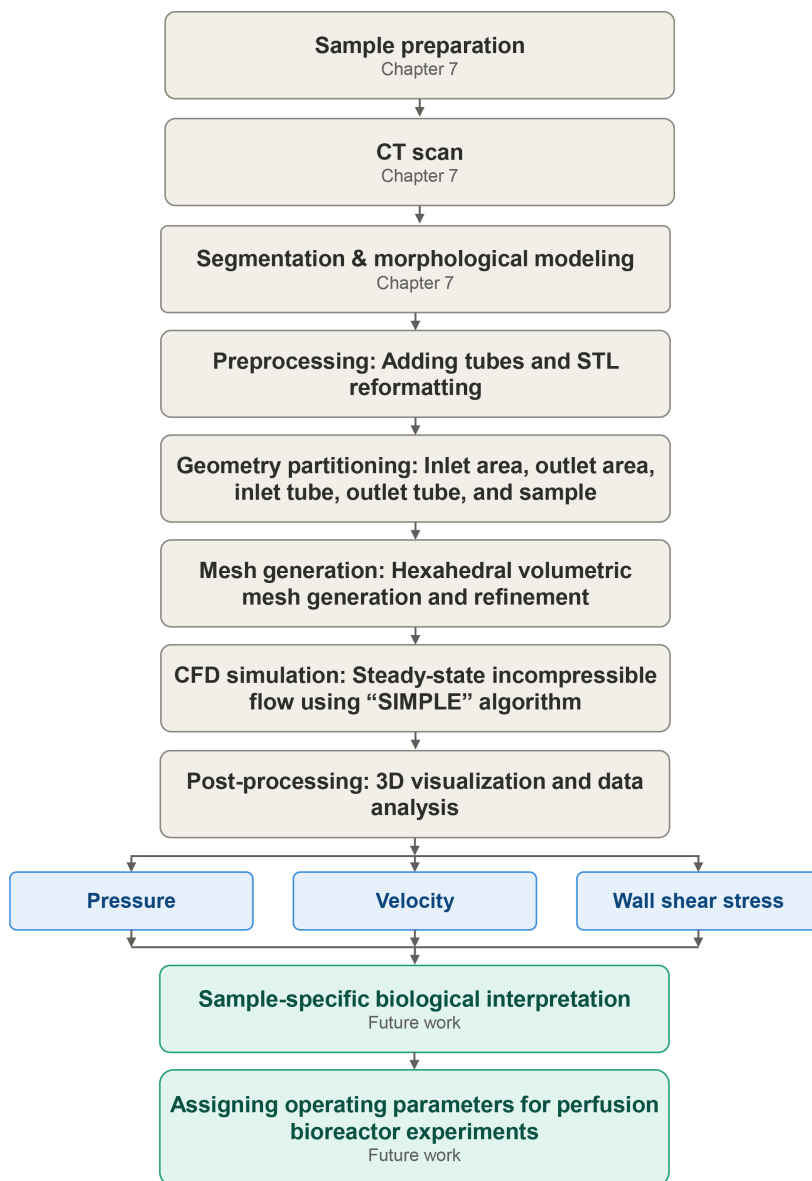


Figure 8.2.1: Overall workflow incorporating both the modeling semi-automated pipeline and the extension CFD automated pipeline with the perfusion bioreactor experiment.

This integrated workflow would enable biologically meaningful downstream analysis, including histology. This would provide a direct means of relating specimen-specific structure and transport behavior to region-specific cellular and matrix responses.

8.3 Conclusion

This thesis contributes to the research into EP degeneration as a multiscale disorder involving structural disruption, impaired transport, altered mechanobiology, and cytokine signaling. By combining literature synthesis, imaging-based computational reconstruction, osteochondral explants, cell-based hydrogel models, and perfusion bioreactor development, the work presented here developed enabling methods and technologies that would allow future work to further study the vertebra–EP–IVD unit across scales.

The principal conclusion of this thesis is that the EP should be studied in a multiscale system covering the bases of its permeability, cell behavior, and mechanical influence on the neighboring tissues.

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Chapter 4 appendix: Verification and supplementary analyses

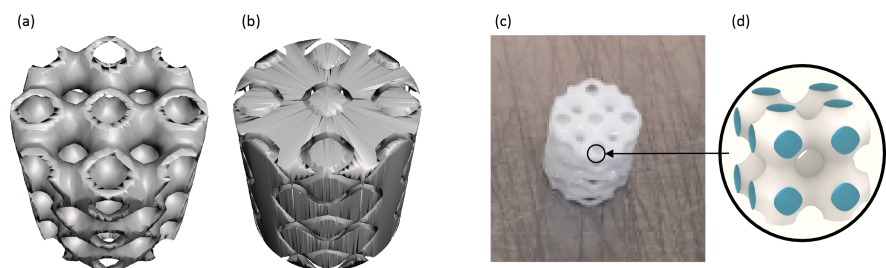


Figure 4.A.1: TPMS scaffold with primitive geometry used for verification of the workflow: designed structure defined by the equation $\cos(x) + \cos(y) + \cos(z) = 0$ (a), the negative structure illustrating interconnected macrochannels (b), the as-manufactured 3D-printed CaP-based structure (c), and corresponding repeating unit cell of the scaffold (d).

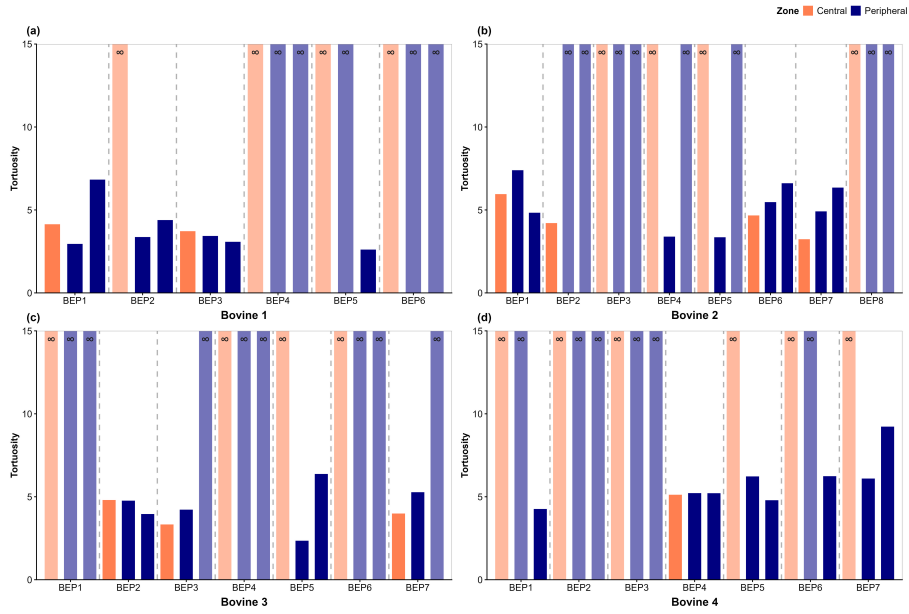


Figure 4.A.2: Difference in tortuosity between the central and the two peripheral zones in each BEP punch in bovine 1 (a), bovine 2 (b), bovine 3 (c), and bovine 4 (d). The translucent bars represent infinite tortuosity, indicating non-percolating zones. The numbers on the x-axis refer to the VB punch but do not refer to the vertebral level. The x-axis values represent arbitrary identifiers assigned to BEP punches and do not correspond to vertebral level.

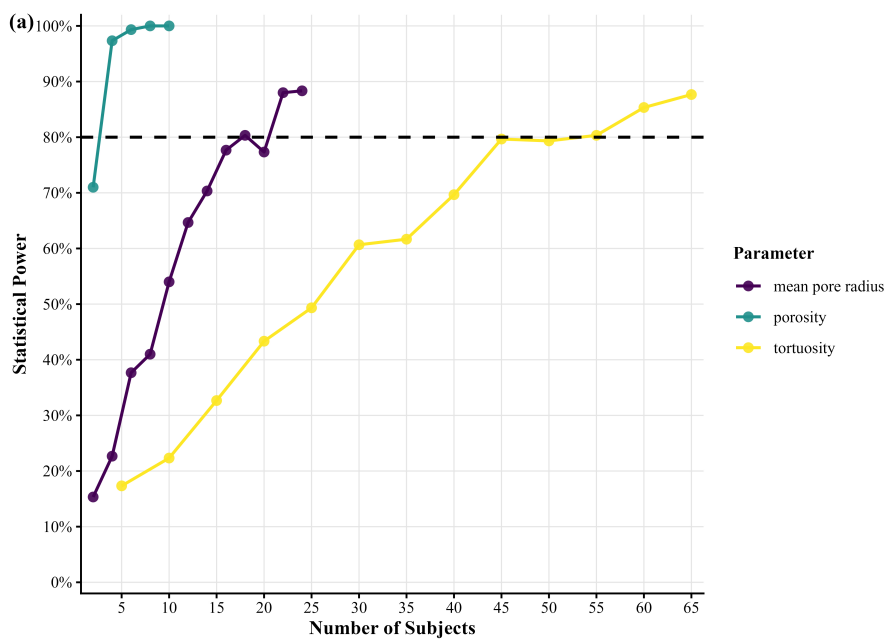


Figure 4.A.3: Power analysis for unbalanced designs: Effect of the number of biological replicates (subjects) with an average of 7 BEPs per subject on the statistical power for LMM of tortuosity (a), porosity (b), and mean pore radius (c).

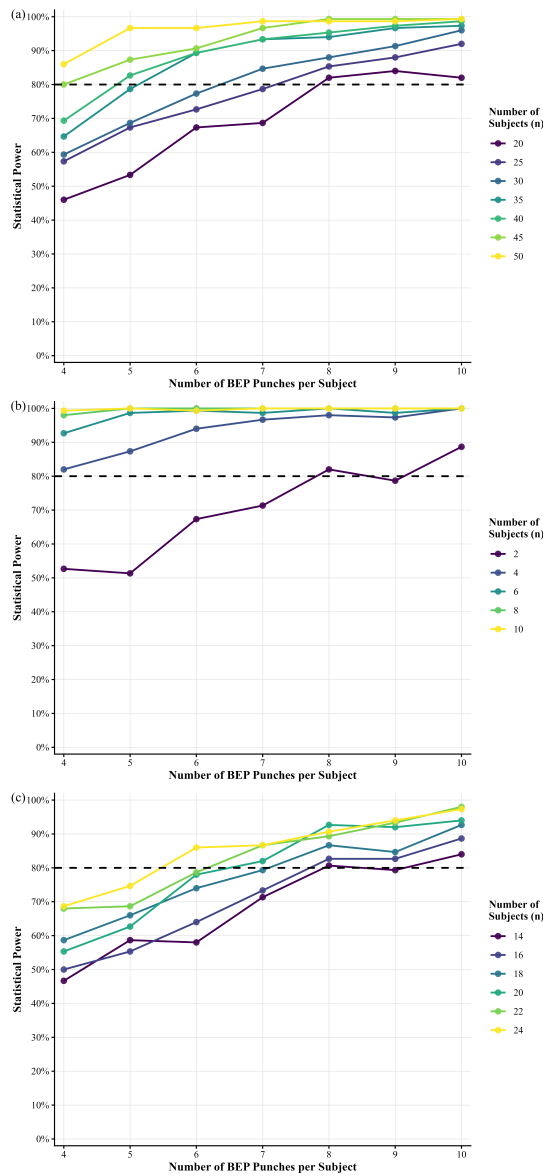


Figure 4.A.4: Power analysis for balanced designs: effect of the number of biological replicates (bovine subjects) and of BEP punches per subject on the statistical power for LMM of tortuosity (a), porosity (b), and mean pore radius (c).

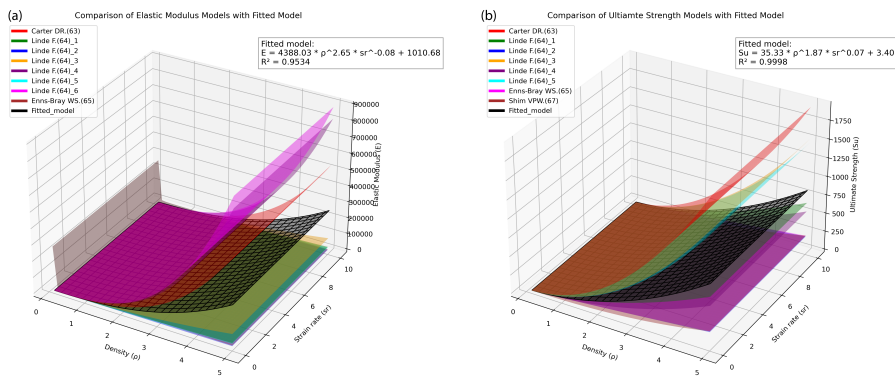


Figure 4.A.5: Three-dimensional regression models using nonlinear curve fitting of the reported mathematical relationships between geometric properties and bone mechanical properties in literature to predict the corresponding Young's modulus (a) and compressive strength modulus (b).

Chapter 5 appendix: Supplementary sample information and analyses

Table 5.A.1: Sample overview showing presence of NP, AF, or both (NP+AF) in the soft tissues (ST) of each sample stratified by bovine donor and culture medium. Day 0 (D0) signifies the start of the experiment, taking place 3 days post harvesting. Samples were maintained for 14 days in Standard or Degenerate medium, with cytokine-treated groups denoted by “+ TNF” or “+ IL-1 β ”. Sample numbers are indicated by “_#”.

Bovine	Sample medium	ST type	Bovine	Sample medium	ST type
1	D0_1	AF + NP	2	Degenerate_2	NP
1	D0_2	AF + NP	2	Degenerate_3	NP
1	D0_3	AF + NP	2	Degenerate + IL-1 β _1	NP
1	Standard_1	NP	2	Degenerate + IL-1 β _2	NP
1	Standard_2	AF	2	Degenerate + IL-1 β _3	NP
1	Standard_3	AF + NP	2	Degenerate + IL-1 β _4	NP
1	Degenerate_1	AF	2	Degenerate + TNF_1	NP
1	Degenerate_2	AF	2	Degenerate + TNF_2	AF + NP
1	Degenerate_3	AF + NP	2	Degenerate + TNF_3	AF + NP
1	Degenerate + IL-1 β _1	AF + NP	2	Degenerate + TNF_4	NP
1	Degenerate + IL-1 β _2	AF + NP	3	Standard_1	NP
1	Degenerate + IL-1 β _3	AF + NP	3	Standard_2	NP
1	Degenerate + TNF_1	AF + NP	3	Standard_3	NP
1	Degenerate + TNF_2	NP	3	Degenerate_1	NP
1	Degenerate + TNF_3	NP	3	Degenerate_2	NP
2	D0_1	NP	3	Degenerate_3	AF + NP
2	D0_2	NP	3	Degenerate + IL-1 β _1	AF + NP
2	D0_3	NP	3	Degenerate + IL-1 β _2	NP
2	Standard_1	AF + NP	3	Degenerate + IL-1 β _3	NP
2	Standard_2	NP	3	Degenerate + TNF_1	NP
2	Standard_3	NP	3	Degenerate + TNF_2	AF + NP
2	Degenerate_1	NP	3	Degenerate + TNF_3	NP

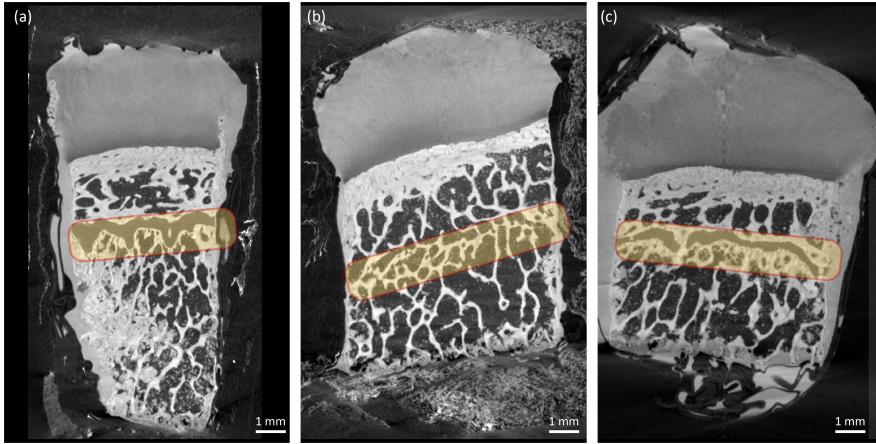


Figure 5.A.2: Representative μ CT scans showing the visible growth plates of (a) bovine 1 and (c) bovine 3 vs. the closed growth plate of (b) bovine 2. The location of the growth plate is indicated by the translucent rectangle. Scale bar = 1 mm.

Chapter 7 appendix: Technical drawings and electrical schematics

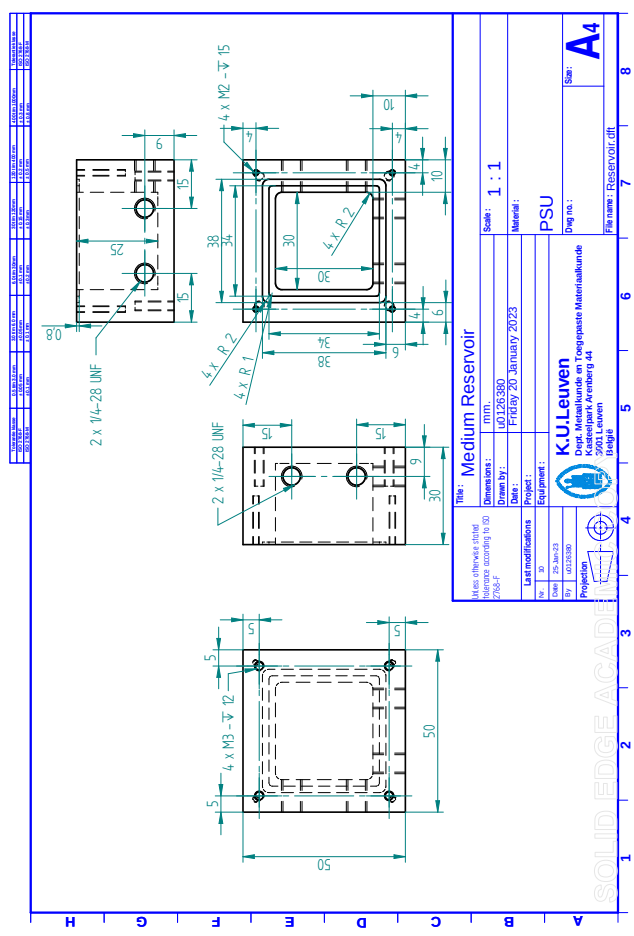


Figure 7.A.1: Technical drawing of the custom culture-medium reservoir. Dimensions are reported on the drawing.

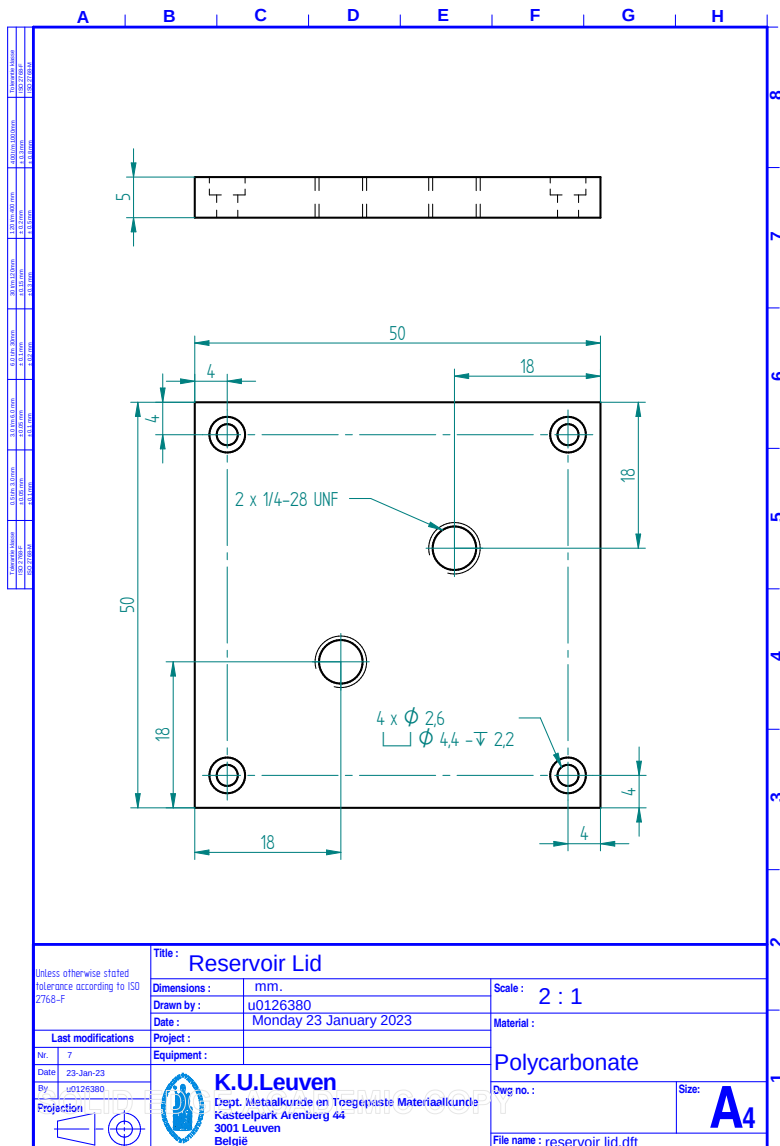


Figure 7.A.2: Technical drawing of the custom culture-medium reservoir lid. Dimensions are reported on the drawing.

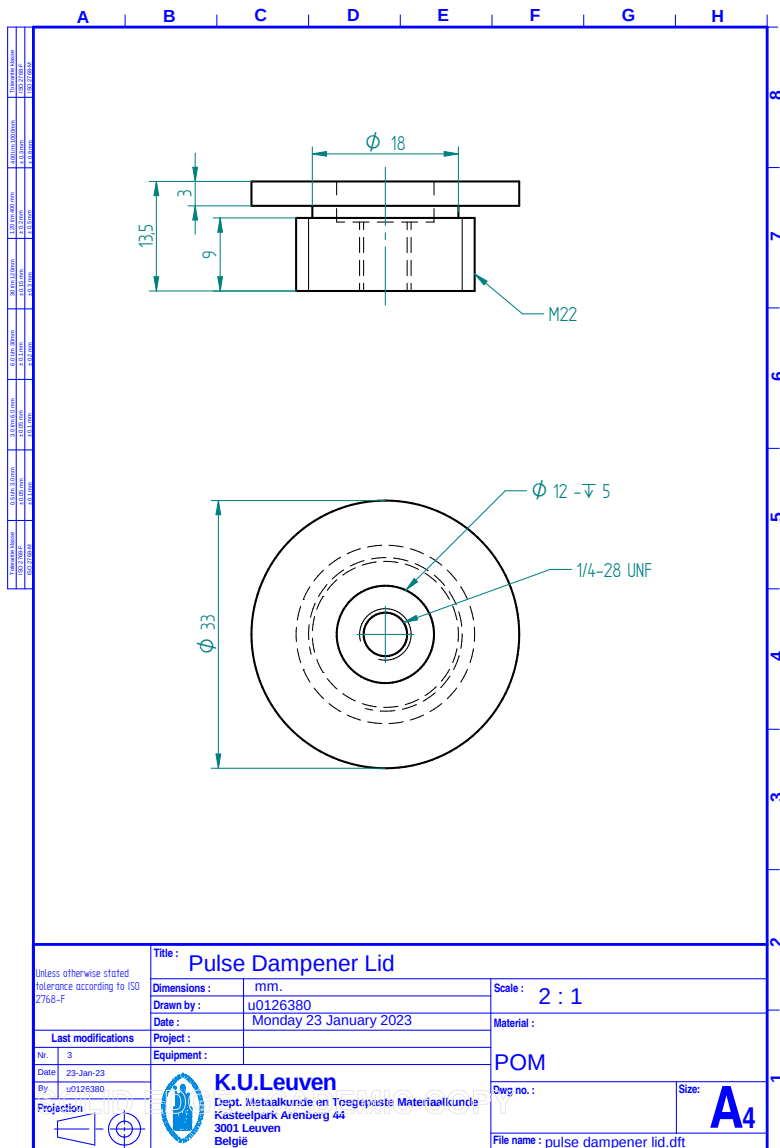


Figure 7.A.6: Technical drawing of the custom pulse-dampener lid. Dimensions are reported on the drawing.

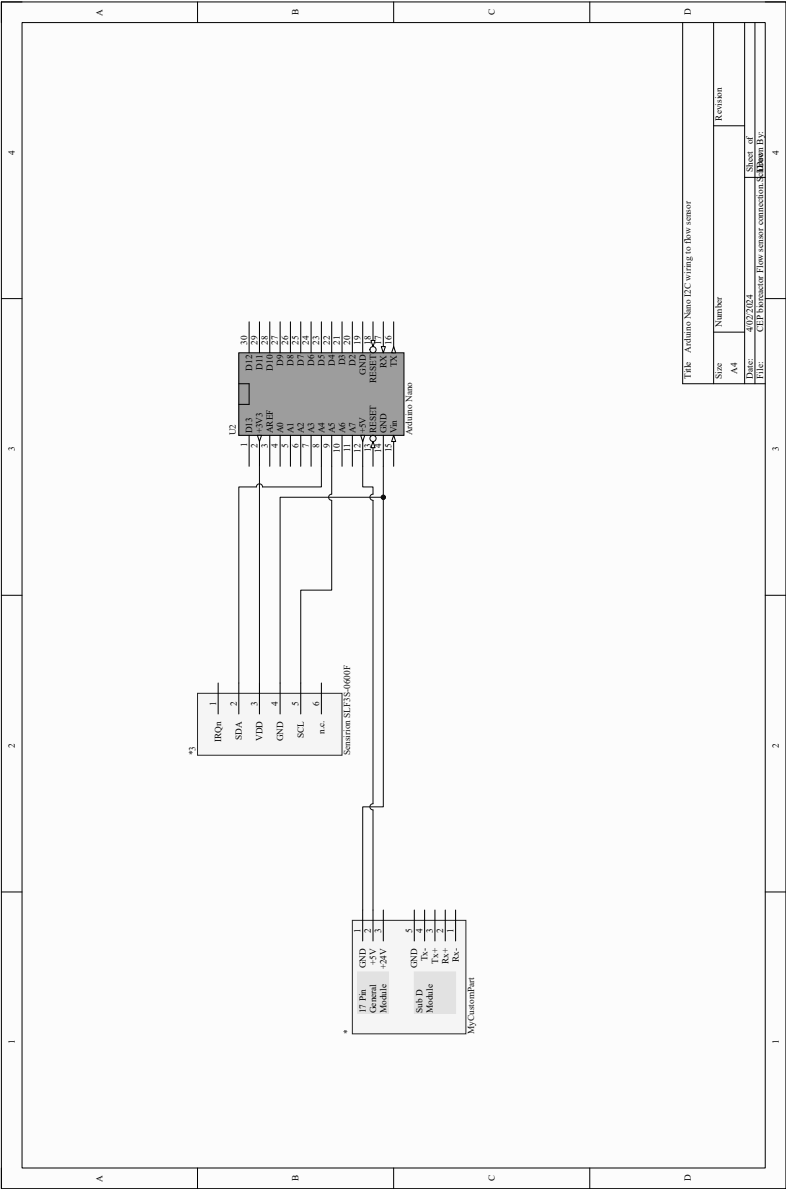


Figure 7.A.7: Electrical connection schematic for the bioreactor flow sensor and its connection to the control electronics.

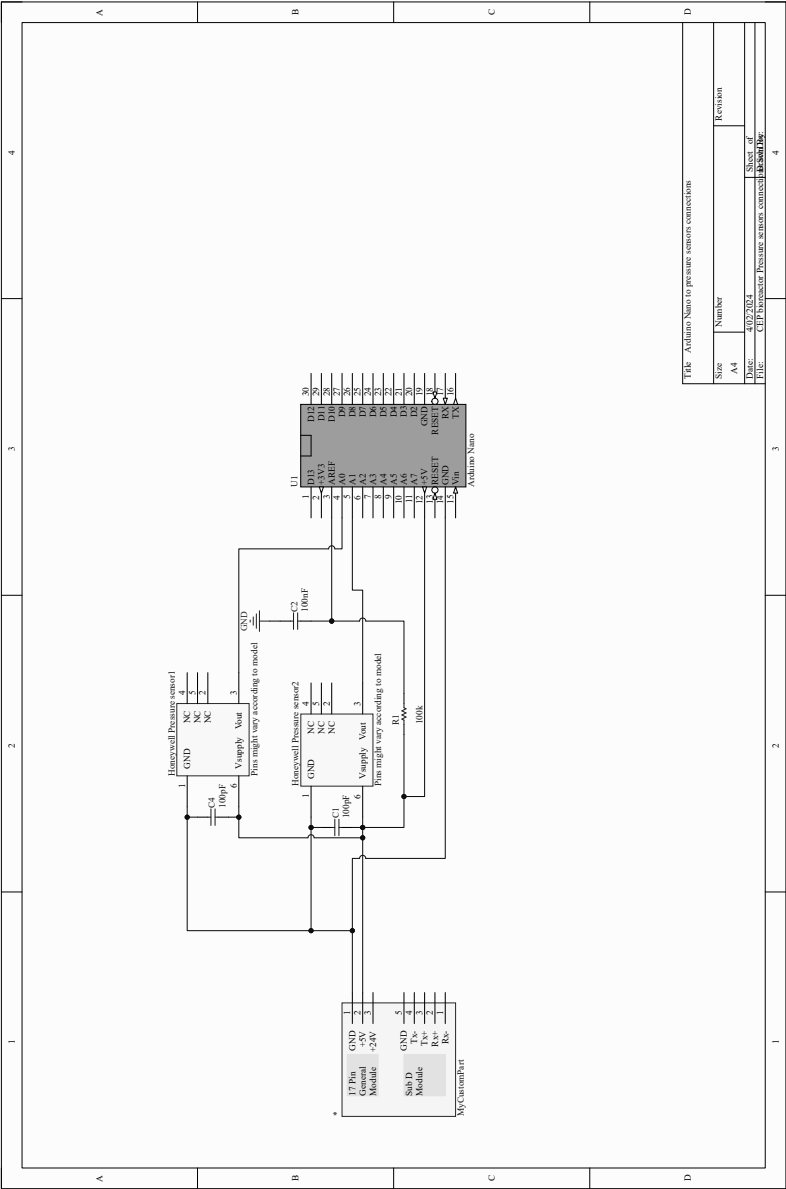


Figure 7.A.8: Electrical connection schematic for the bioreactor pressure sensors and their connection to the control electronics.

Biography: The Journey Inked

Born in Lebanon, educated in Japan, and professionally engaged across Europe, Ahmad Alminnawi is a globally minded individual with a diverse academic and professional background.



He began his journey at the Lebanese American University in Beirut, where he earned a bachelor's degree in mechanical engineering, graduating with honors. Ahmad's academic path then led him to Japan, where he was awarded the prestigious Monbukagakusho Scholarship (文部科学省奨学金) by Japan's Ministry of Education, Culture, Sports, Science and Technology. At Osaka University, he completed his master's degree in bioengineering and published his first research article, focusing on the use of smart materials to design a lower-limb orthosis. Ahmad's intellectual journey continued in Europe, where he was awarded a Marie Curie Fellowship and pursued a joint doctorate between the Department of Engineering at Université de Liège and the Department of Medicine at KU Leuven. His research focused on the intervertebral disc endplate, bridging engineering principles with medical science.

These experiences have shaped Ahmad's interdisciplinary expertise, integrating engineering, medicine, and materials science to address complex challenges at the intersection of technology and healthcare.

Ahmad Alminnawi

Email: ahmad.alminnawi@gmail.com

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Education

- 2021–2026** **Dual PhD in Biomedical Engineering** Leuven and Liège, Belgium
KU Leuven and Université de Liège
Thesis: In Silico and In Vitro Modeling of Cartilage Degeneration Mechanisms. *Supervisor:* Prof. Dr. Liesbet Geris and Prof. Dr. Ioannis Papantoniou.
- 2019–2021** **Research-Based Master's in Bioengineering** Osaka, Japan
Osaka University
Thesis: Shape Memory Alloy Actuated Ankle–Foot Orthosis for Locomotion Force Reduction. *Supervisor:* Prof. Dr. Masao Tanaka and Prof. Dr. Yo Kobayashi.
- 2014–2018** **BEng in Biomechanical Engineering – GPA: 3.5/4.0** Beirut, Lebanon
Lebanese American University (LAU)
Final Year Project: Biomechanics-Based Knee Damper for High Impact Sports. *Supervisor:* Prof. Dr. Barbar Akle.

Abroad Research Stays

- 2025–2026** **PhD Secondment** Barcelona, Spain
Universitat Pompeu Fabra (UPF)
Purpose: Intervertebral Endplate Modeling from Medical Imaging. *Supervisor:* Prof. Dr. Jérôme Noailly.
- 2023** **PhD Secondment** Sheffield, England, UK
University of Sheffield
Purpose: Experimental Modeling of Cytokine-Induced Cartilage Degeneration. *Supervisor:* Prof. Dr. Christine Le Maitre.
- 2021** **PhD Secondment** Bern, Switzerland
University of Bern
Purpose: Bovine Cartilage Harvesting and Cell Extraction. *Supervisor:* Prof. Dr. Benjamin Gantenbein.
- 2019** **Master’s Secondment** Paris, France, and Cardiff, Wales, UK
Arts et Métiers ParisTech & Cardiff University (online due to COVID-19)
Purpose: Developing a Computational Test Bench for Biological Hard–Soft Tissue Interactions. *Supervisor:* Prof. Dr. Pierre-Yves Rohan and Dr. Bethany Keenan.

Technical Certifications

- 2026** **PyTorch: Fundamentals**
DeepLearning.AI
- 2025** **AI and Machine Learning Algorithms and Techniques**
Microsoft
- 2025** **AI Infrastructure and Operations Fundamentals**
NVIDIA
- 2024** **Introduction to AI and Machine Learning for Biomedical Research**
KU Leuven

Professional Certifications

- 2025** **Project Management Professional Certificate**
Google
- 2025** **Exploitation of Research Technology & Knowledge Transfer**
KU Leuven
- 2025** **Early Health Technology Assessment**
EIT Health and Syreon Research Institute

Research and Professional Experience

2021–Present **Doctoral Researcher**, SBE, KU Leuven, Leuven, Belgium, and GIGA Research Center, Université de Liège, Liège, Belgium

- Conducted doctoral research under the supervision of Prof. Liesbet Geris within the Disc4all European consortium, focusing on cartilage tissue degeneration using in silico modeling coupled with in vitro experimental validation.
- Developed a micro-CT-based pipeline for sample-specific structural modeling using Python and 3D Slicer for FEA and CFD.
- Developed a CFD pipeline to test different flow regimes and wall shear stress effects on cells in explants and scaffolds.
- Conducted in vitro cartilage experiments under different environments and biochemical stimulations, coupled with a predictive in silico signal-transduction network model for cartilage degeneration in osteoarthritis and advanced statistical analysis methods.
- Performed CFD analysis on gel carriers to simulate mechanical loading effects on cartilage cells for experiments performed at the University of Bern within the Disc4all consortium.
- Collaborated with the laboratory engineer to design and manufacture a custom perfusion bioreactor to validate CFD simulations.

2024; 2025–2026 **Visiting Researcher**, BCNMedTech Institute, Universitat Pompeu Fabra (UPF), Barcelona, Spain

- Conducted an international research stay under the supervision of Prof. Dr. Jérôme Noailly, focusing on computational modeling and simulation of cartilage endplates.
- Integrated morphological calculations and mechanical property prediction into the micro-CT-based pipeline.
- Trained on motion capture analysis and wearable sensors with a PhD candidate in the group.
- Trained Master's and Bachelor's students in scientific presentations and thesis defense communication under the supervision of a postdoc in the group.

- 2023** **Visiting Researcher**, University of Sheffield, Sheffield, England, UK
- Conducted an international research stay under the supervision of Prof. Christine Le Maitre, focusing on the experimental modeling of the degenerative effects of different cytokine environments on cartilage tissues.
 - Predicted cartilage behavior in different environments using a signal-transduction network model developed for articular cartilage degeneration in osteoarthritis.
 - Performed cartilage tissue harvesting from bovine specimens, tissue culture, and sampling followed by downstream analysis consisting of GAG and DNA quantification, histological and immunohistological staining, CT scanning, and qRT-PCR.
- 2021** **Visiting Researcher**, University of Bern, Bern, Switzerland
- Conducted an international research stay under the supervision of Prof. Benjamin Gantenbein, focusing on the mechanical loading effects on cartilage cells seeded in gel carriers.
 - Modified and optimized an intervertebral disc harvesting and cell isolation protocol to be applied to cartilage tissues.
 - Performed cartilage tissue harvesting and cell isolation from bovine specimens, tissue culture under dynamic compressive load in the bioreactor, and sampling, followed by downstream analysis.
- 2020** **Visiting Researcher (Online due to COVID-19)**, Arts et Métiers ParisTech, Paris, France & Cardiff University, Cardiff, Wales, UK
- Conducted an international research stay under the supervision of Prof. Pierre-Yves Rohan and Dr. Bethany Keenan, focusing on the FEA and modeling of soft tissue interactions with hard tissues.
 - Developed a CAD- and FE-simulation-based test bench to study the hyperelastic biomechanical behavior of soft tissues during daily activities.
 - Remodeled and tested the design and materials of a hospital cushion to minimize internal tissue loads, preventing pressure ulcers for bed-bound patients.

- 2019–2021 Research Fellow (Research-based Master’s degree), Osaka University, Osaka, Japan**
- Conducted master’s-level research under the supervision of Prof. Masao Tanaka and Prof. Yo Kobayashi, focusing on the human gait kinetics and kinematics.
 - Utilized wearable sensors, such as IMUs, to analyze lower limb gait biomechanics and estimate mechanical loading during physiological walking.
 - Designed a passive dynamic ankle-foot orthosis to increase walking efficiency by utilizing shape memory alloys that provided a nonlinear force pattern mimicking the natural movement of human muscles and tendons.
- 2018–2019 University Spin-off Research Assistant, Lebanese American University, Beirut, Lebanon**
- Led and mentored a team of final-year Bachelor’s students in redesigning a patient-specific lower limb exoskeleton to improve mobility for a paraplegic patient, under the supervision of Prof. Barbar Akle.
 - Coordinated communication between the patient, project team, and university stakeholders to facilitate clinical assessments, design reviews, and project meetings.
 - Coordinated and monitored patient testing to evaluate the performance and usability of the redesigned exoskeleton, using patient feedback to guide design refinements.
- 2017–2018 MEP Project Engineer, Caliber Engineering SARL, Beirut, Lebanon**
- Coordinated multidisciplinary teams across design and on-site operations for HVAC and electromechanical systems on a hospital laboratory project in the Gulf region, ensuring technical accuracy, compliance with safety, quality, and GMP standards, and timely project delivery.
 - Prepared and maintained project documentation, including progress reports, technical records, and compliance documentation, to track milestones, identify risks, and support accountability, transparency, and regulatory compliance.

Mentoring, Supervision, and Hands-on Workshops

Mentoring & Supervision

- 2026 Research Secondment Supervision**
Supervised an early-stage PhD candidate from the University of Eastern Piedmont, Vercelli, Italy, on a three-month project in micro-CT-based structural modeling and CFD flow analysis of custom scaffolds to optimize bioreactor flow rates for cell proliferation and differentiation.
- 2024–2026 Academic Mentor and Mock Examiner – Graduate and Undergraduate Level**
Served as a mock thesis defense panel member for Bachelor’s and Master’s students at Pompeu Fabra University, providing constructive feedback, evaluating presentations, and preparing students for final thesis defenses.

Hands-on Workshops

- 2025 Co-Organizer and Instructor, 3D Modeling in Medicine**
EIT Health Innovators Community, PRBB, Barcelona, Spain – Co-organized and delivered a hands-on workshop introducing biomedical, clinical, and life science participants to medical image segmentation, 3D model generation, and visualization from CT/MRI data using 3D Slicer.

Teaching

- 2019–2021 Part-Time English School Teacher**
Taught students at Nuance Inc., Osaka, Japan and Kinran International School, Osaka, Japan, delivering engaging English lessons.
- 2014–2019 Part-Time Private Tutor**
Taught high school Mathematics, Physics, Biology, and Chemistry to students preparing for the Lebanese National Baccalaureate examinations.

Peer-Reviewed Publications

Journal Articles

Under revision

- 2026** Alminnawi, A.; Tejero, P.; Geeroms, C.; Geris, L.*[‡]; Le Maitre, C. L.*[‡] Cytokine-Mediated Responses Across Endplate Subregions in Intervertebral Disc Multi-Tissue Explants. *Under revision*

Accepted and Published

- 2026** Alminnawi, A.*; Nasello, G.; Crump, K. B.; Gantenbein, B.; Noailly, J.; Geris, L.* Semi-Automated μ CT-Based Modeling of Vertebral Bony Endplate Microporosity and Mechanical Properties: Linking Microstructure to Inferred Mechanical Performance. *JOR Spine*, 9(3), e70204. doi: 10.1002/jsp2.70204.
- 2025** Crump, K. B.; Chapallaz, C.; Alminnawi, A.; Bermudez-Lekerika, P.; Geris, L.; Noailly, J.; Gantenbein, B. Exploring Mechanotransduction and Inflammation in Human Cartilaginous Endplate Cells in Blended Collagen–Agarose Hydrogels Under Cyclic Compression. *Gels*, 11(9), 736. doi: 10.3390/gels11090736
- 2025** Crump, K. B.; Kanelis, E.; Segarra-Queralt, M.; Pascuet-Fontanet, A.; Bermudez-Lekerika, P.; Alminnawi, A.; Geris, L.; Alexopoulos, L. G.; Noailly, J.; Gantenbein, B. TNF induces catabolism in human cartilaginous endplate cells in 3D agarose culture under dynamic compression. *Scientific Reports*, 15(1), 15849. doi: 10.1038/s41598-025-00538-w
- 2023** Crump, K. B.*; Alminnawi, A.*; Bermudez-Lekerika, P.; Compte, R.; Gualdi, F.; McSweeney, T.; Munoz-Moya, E.; Nuesch, A.; Geris, L.; Dudley, S.; Karppinen, J.; Noailly, J.; Le Maitre, C.; Gantenbein, B. Cartilaginous endplates: A comprehensive review on a neglected structure in intervertebral disc research. *JOR Spine*, 6(4), e1294. doi: 10.1002/jsp2.1294.
*Shared first authorship
- 2022** Bermudez-Lekerika, P.; Crump, K. B.; Tseranidou, S.; Nuesch, A.; Kanelis, E.; Alminnawi, A.; Baumgartner, L.; Munoz-Moya, E.; Compte, R.; Gualdi, F.; Alexopoulos, L. G.; Geris, L.; Wuertz-Kozak, K.; Le Maitre, C. L.; Noailly, J.; Gantenbein, B. Immuno-Modulatory Effects of Intervertebral Disc Cells. *Frontiers in Cell and Developmental Biology*, 10, 924692. doi: 10.3389/fcell.2022.924692

Conference Papers

- 2021** Alminnawi, A.; Kobayashi, Y.; Otani, T.; Tanaka, M. Shape memory alloy actuated ankle foot orthosis for reduction of locomotion force. *2021 World Automation Congress (WAC)*, 271–276. doi: 10.23919/WAC50355.2021.9559533

Conference Abstracts

- 2025** **Alminnawi, A.** μ CT-Based Calculations of Perfusion-Induced Wall Shear Stresses in Osteochondral Explants of Intervertebral Discs. *Marie Curie Alumni Association (MCAA) Annual Conference & General Assembly 2025*.
- 2025** Crump, K. B.; Chappallaz, C.; **Alminnawi, A.**; Segarra-Queralt, M.; et al. Poster TERMIS25_1015: Dynamically compressed collagen–agarose hydrogels for 3D culture of human cartilaginous endplate cells. *Tissue Engineering*.
- 2024** **Alminnawi, A.** Image-based wall shear stresses calculations in cartilage endplate explants and scaffolds in perfusion culture. *International Society for the Study of the Lumbar Spine (ISSLS)*.
- 2023** Crump, K. B.; Bermudez, P.; **Alminnawi, A.**; Geris, L.; Noailly, J.; Gantenbein, B. In vitro investigation of compression and catabolic cytokines on human cartilage endplate cells in agarose. *Annual Meeting of the Swiss Society of Biomedical Engineering*, 47–47.
- 2023** **Alminnawi, A.** An In Silico Tool to Simulate Fluid Flow and Shear Stresses on CEP Cells in Tissues and Scaffolds. *10th Belgian Symposium of Tissue Engineering (BSTE)*.
- 2023** **Alminnawi, A.**; Crump, K. B.; Bermudez, P.; Croft, A. S.; Geeroms, C.; Le Maitre, C.; Gantenbein, B.; Geris, L. Direction Dependent Mass Transport Through the Cartilage Endplate. *Tissue Engineering and Regenerative Medicine International Society (TERMIS)*.
- 2023** Crump, K. B.; **Alminnawi, A.**; Bermudez, P.; Segarra Queralt, M.; Croft, A. S.; Geris, L.; Noailly, J.; Gantenbein, B. In Vitro and In Silico Investigation of Dynamic Compression on Cartilage Endplate Cells in Agarose. *Tissue Engineering and Regenerative Medicine International Society (TERMIS)*.
- 2022** **Alminnawi, A.** In vitro and in silico modeling of cartilage endplate degeneration mechanisms. *Disc4all Bern Winter School, Bern, Switzerland*.
- 2022** Crump, K. B.; **Alminnawi, A.**; Bermudez, P.; Croft, A. S.; Geris, L.; Noailly, J.; Gantenbein, B. Effects of Dynamic Compression on Cartilage Endplate Cells in Agarose. *eCM20: Cartilage and Disc Repair and Regeneration*, 10–10.
- 2022** **Alminnawi, A.** Cartilage Endplate Permeability: From In Vitro to In Silico. *ORS PSRS 6th International Spine Research Symposium*.
- 2022** **Alminnawi, A.**; Crump, K. B.; Bermudez, P.; Croft, A. S.; Geeroms, C.; Le Maitre, C.; et al. Image-based Analysis of Mass Transport Through Cartilage Endplates. *eCM20: Cartilage and Disc Repair and Regeneration*.

Honors & Awards

Fellowships & Research Funding

- 2025** **FWB Travel Grant**
Fédération Wallonie-Bruxelles, Belgium – Travel grant.
- 2021–2024** **Marie Curie Doctoral Fellowship**
European Commission, Disc4all European Training Network – Three-year doctoral fellowship.
- 2021–2024** **MEXT Doctoral Fellowship**
Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan – Highly competitive three-year Japanese government fellowship; *turned down in favor of the Marie Curie Fellowship*.
- 2019–2021** **MEXT Master’s Fellowship**
Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan – Highly competitive two-year Japanese government fellowship.

Article Awards

- 2025** **Wiley Top Read Article Award**
Wiley – Awarded for high readership of a published article.
- 2025** **Wiley Top Cited Article Award**
Wiley – Awarded for high citation impact of a published article.

Academic Excellence

- 2019–2021** **Master’s Honors**
Osaka University, Osaka, Japan – Academic honors distinction during master’s studies.
- 2014–2018** **LAU Honors**
Lebanese American University (LAU), Beirut, Lebanon – Four-year academic honors distinction.

Student Competitions

- 2013** **MUN Diplomacy Award**
Model United Nations, Beirut, Lebanon – Diplomacy award.
- 2013** **INJAZ Start-Up Company Competition**
INJAZ, Beirut, Lebanon – Top contender in start-up company competition.

Dissemination and Outreach

Oral/Podium Presentations

- 2025 A nano-CT-based approach to intervertebral disc endplate porosity and fluid flow modeling**
20th International Symposium on Computer Methods in Biomechanics and Biomedical Engineering (CMBBE), Barcelona, Spain, 3–5 September 2025.
- 2025 CT-Based Calculations of Perfusion-Induced Wall Shear Stresses in Osteochondral Explants of Intervertebral Discs**
Marie Curie Alumni Association (MCAA) Annual Conference and General Assembly, Krakow, Poland, 21–22 March 2025.
- 2024 Cartilage Endplate Permeability: From In Vitro to In Silico**
4th Disc4All Winter School, Leuven, Belgium, 19–23 February 2024.
- 2023 An In Silico Tool to Simulate Fluid Flow and Shear Stresses on CEP Cells in Tissues and Scaffolds**
10th Belgian Symposium of Tissue Engineering (BSTE), University of Liège, Liège, Belgium, 6–7 December 2023.
- 2023 Cartilage Endplate Permeability: From In Vitro to In Silico**
3rd Disc4All Winter School, Demokritos, Athens, Greece, 20–24 February 2023.
- 2022 Cartilage Endplate Permeability: From In Vitro to In Silico**
ORS PSRS 6th International Spine Research Symposium, Skytop, Pennsylvania, USA, 6–10 November 2022.
- 2022 Image-based Analysis of Mass Transport Through Cartilage Endplates**
eCM20: Cartilage and Disc Repair and Regeneration, Davos, Switzerland, 15–18 June 2022.
- 2022 In vitro and in silico modeling of cartilage endplate degeneration mechanisms**
Disc4All Winter School, University of Bern, Switzerland, 21–25 February 2022.

Poster Presentations and Public Research Exhibitions

- 2025 Cytokine-Mediated Responses in Endplate Subregions of Intervertebral Disc Multitissue Biocomposite Explants**
Engineering and Information and Communication Technologies (EICT) PhD Workshop, Universitat Pompeu Fabra, Barcelona, Spain, 14 May 2025.
- 2024 The Cartilage Endplate**
International Society for the Study of the Lumbar Spine (ISSLS) Annual Meeting, Milan, Italy, 27–31 May 2024.
- 2023 An In Silico Tool to Simulate Fluid Flow and Shear Stresses on CEP Cells in Tissues and Scaffolds**
7th Barcelona VPH Summer School, Barcelona, Spain, 5–9 June 2023.

- 2023 Direction Dependent Mass Transport Through the Cartilage Endplate**
Tissue Engineering and Regenerative Medicine International Society (TERMIS) European Chapter Meeting, Manchester, UK, 28–31 March 2023.
- 2022 Image-based Analysis of Mass Transport Through Cartilage Endplates**
6th Barcelona VPH Summer School, Universitat Pompeu Fabra, Barcelona, Spain, 23–27 March 2022.
- 2021 Shape Memory Alloy Actuated Ankle–Foot Orthosis for Reduction of Locomotion Force**
2021 World Automation Congress (WAC), Taipei, Taiwan, 1–5 August 2021; held online due to COVID-19.

Conference Attendance (Non-Presenting)

- 2025 8th Barcelona VPH Summer School**
Universitat Pompeu Fabra, Barcelona, Spain, 2–6 June 2025.
- 2024 7th Barcelona VPH Summer School**
Universitat Pompeu Fabra, Barcelona, Spain, 3–7 June 2024.
- 2023 Disc4all Advanced Training Event**
Rochester, NY, USA, 11–15 December 2023.
- 2021 5th Barcelona VPH Summer School**
Virtual event organized by Universitat Pompeu Fabra, Barcelona, Spain, 7–11 June 2021.

Professional Service and Academic Activities

Scholarly Reviewing and Evaluation

- 2025** **Project Reviewer**
EPICS in IEEE (Engineering Projects in Community Service), Institute of Electrical and Electronics Engineers – Reviewed four student-led engineering projects focused on community-based technology solutions for real-world problems.
- 2024** **Official Manuscript Reviewer**
Biomolecules and Biomedicine – Reviewed *A repeated strike loading organ culture model for studying compression-associated chronic disc degeneration*. doi: 10.17305/bb.2024.10640; PMID: 39101754.

Conference and Workshop Organization

- 2025** **Co-Organizer and Instructor**
3D Slicer Medical Imaging Workshop for Life Scientists, EIT Health Community, Barcelona, Spain – Contributed to concept design and delivered the hands-on workshop on 3D modeling from medical imaging.
- 2024** **Local Organizing Committee Member**
Belgian Symposium on Tissue Engineering (BSTE), Leuven, Belgium – Coordinated presenter and attendee communications, maintained the event website, and supported social media and marketing activities.
- 2024** **Co-Organizer**
Disc4all Winter School, Leuven, Belgium – Coordinated venue logistics, catering, attendee communications, and keynote speaker correspondence; co-developed the program schedule and delivered opening and closing remarks.

Languages

English	C2 – Fluent (academic proficiency)
Dutch	A2 – Limited (actively improving)
French	B1 – Conversational
Spanish	B1 – Conversational
Arabic	C2 – Native

Professional Memberships

2024– Present	Orthopaedic Research Society (ORS) Research member.
2021– Present	Marie Skłodowska-Curie Alumni Association (MCAA) Member; participant in MCAA Academy activities.
2021– Present	Disc4all European Consortium Early-stage researcher of the European doctoral training consortium.
2023– Present	EIT Health Innovators Community Member of the European health innovation network.
2023– Present	Institute of Electrical and Electronics Engineers (IEEE) Graduate Student Member; Engineering in Medicine and Biology Society (EMBS).
2014–2018	American Society of Mechanical Engineers (ASME) Student Member

References

Supervisor	Prof. Dr. Liesbet Geris Professor and Principal Investigator, SBE, KU Leuven, Leuven, Belgium. PhD supervisor. Email: liesbet.geris@kuleuven.be
Supervisor	Prof. Dr. Jérôme Noailly Professor and Principal Investigator, BCNMedTech, Pompeu Fabra University, Barcelona, Spain. PhD secondment supervisor. Email: jerome.noailly@upf.edu
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