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Bioengineering of a 3D in Vitro Model of Annulus Fibrosus (BIO3D-AF): Dissecting the Impact of Inflammation in Intervertebral Disc Herniation

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BIOENGINEERING OF A 3D *IN VITRO* MODEL OF ANNULUS FIBROSUS (BIO3D-AF): DISSECTING THE IMPACT OF INFLAMMATION IN INTERVERTEBRAL DISC HERNIATION

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*“É este o Meu mandamento:
que vos ameis uns aos outros, como Eu vos amei.
Ninguém tem maior amor do que aquele que dá a vida pelos amigos.
Vós sois Meus amigos, se fizerdes o que Eu vos mando.
Já não vos chamo servos, porque o servo não sabe o que faz o seu senhor;
mas chamo-vos amigos, porque vos dei a conhecer tudo o que ouvi a Meu Pai.
Não fostes vós que Me escolhestes; **fui Eu que vos escolhi e destinei,**
para que vades e **deis fruto e o vosso fruto permaneça.**”*

Jo, 15:12-17

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Abstract

Intervertebral disc (IVD) degeneration is the major contributor to low back pain, a leading cause of global disability. Available clinical treatments primarily address symptoms management, rather than regenerating or restoring native IVD properties. IVD degeneration progression culminates into IVD herniation, characterized by the loss of integrity of Annulus Fibrosus (AF) in containing the Nucleus Pulposus (NP). The pathomechanisms driving herniation are poorly characterized, with research being conducted mainly on NP, while neglecting AF tissue. Most of the studies in IVD herniation have used *in vivo* and *ex vivo* models, with different species that do not resemble human, regarding cell types in the IVD, extracellular matrix (ECM) composition and biomechanical loading.

This thesis aims to fill a gap in the field, by proposing to replicate the pathophysiology of human IVD herniation *in vitro*, with a specific emphasis on AF tissue. By establishing a human-based *in vitro* model, this research intends to replicate and further elucidate the impact of the inflammatory and fibrotic microenvironment on the herniation process.

While some previous studies have implicated fibrosis in IVD degeneration, the correlation between AF fibrosis and progressive IVD herniation in humans had not been thoroughly explored. Therefore, a characterization study in human AF biopsies was first conducted, dissecting the structural, biochemical and cellular alterations observed in different stages of IVD herniation. The results obtained revealed changes in fiber organization, increase of ECM proteins like collagen type I (Col I) and fibronectin (FN) and augmented presence of alpha-smooth muscle actin positive cells with herniation progression. These findings can result in potential disease markers and new cues to replicate *in vitro* and improve disease models.

Subsequent studies focused on developing an AF *in vitro* model (Bio3D-AF) using Col I hydrogels and inflammatory stimulation to mimic disease cues. For that, Human AF cells were cultured in 2D and 3D, and stimulated with IL-1 β , for two or seven days, resembling respectively an acute or chronic inflammatory stimulation. As expected, the results demonstrated that 3D culture and chronic stimulation more accurately replicated AF degeneration, validated by the previously established fibrotic hallmarks in human AF biopsies. Moreover, this model provided new insights into potential inflammatory mediators (e.g. G-CSF and GM-CSF) as diagnostic and therapeutic targets for AF failure.

Finally, to further refine the established model AF model, a collagen-based anisotropic hydrogel was developed, using magneto-responsive polyethylene glycol (PEG) microgels.

This innovative platform allowed for the alignment of collagen fibers and, consequently, as a proof-of-concept of cell alignment, of human fibroblasts, up to 7 days in culture. In the future, this refined model could be used to explore AF cell alignment in the context of native IVD, and thus study the impact on cell alignment on prevention/reversion of IVD herniation.

Overall, this thesis advances the current knowledge on AF tissue and consequently on IVD herniation, providing novel avenues for accurate human-based inflammatory models. By elucidating the underlying pathomechanisms associated with AF failure in herniated human samples, by identifying potential inflammatory therapeutic targets in AF and by bioengineering a new anisotropic *in vitro* model, the work here developed holds promise for improving current diagnosis and treatment of patients suffering from IVD herniation and associated low back pain.

Resumo

A degeneração do disco intervertebral (IVD) é o principal fator que contribui para a dor lombar, uma das principais causas de incapacidade global. Os tratamentos clínicos disponíveis incidem principalmente no controlo dos sintomas, em vez de regenerar ou restaurar as propriedades nativas da degeneração do IVD. A progressão da degeneração do IVD culmina na herniação do IVD, caracterizada pela perda de integridade do anel fibroso (AF) que contém o núcleo pulposo (NP). Os mecanismos patológicos que conduzem à herniação estão mal caracterizados, sendo a investigação conduzida principalmente no NP, negligenciando o tecido do AF. A maioria dos estudos sobre a herniação do IVD tem sido efetuada em modelos *in vivo* e *ex vivo*, utilizando espécies diferentes que não se assemelham ao ser humano, no que diz respeito aos tipos de células do IVD, à composição da matriz extracelular e à carga biomecânica.

Esta tese visa preencher uma falha na área, propondo replicar a fisiopatologia da herniação do IVD humano *in vitro*, com ênfase específica no tecido AF. Ao estabelecer um modelo *in vitro* de base humana, esta investigação pretende replicar e elucidar melhor o impacto do microambiente inflamatório e fibrótico no processo de herniação.

Embora alguns estudos anteriores tenham relacionado a fibrose com a degeneração do IVD, a correlação entre a fibrose do AF e a herniação progressiva do IVD em seres humanos não tinha sido completamente explorada. Assim, foi realizado um estudo de caracterização em biópsias humanas de AF, explorando as alterações estruturais, bioquímicas e celulares observadas em diferentes fases da herniação do IVD. Os resultados obtidos revelaram alterações na organização das fibras, aumento das proteínas da matriz extracelular como o colagénio tipo I (Col I) e a fibronectina (FN) e aumento da presença de células positivas para alfa-actina do músculo liso com a progressão da herniação. Estas descobertas podem resultar em potenciais marcadores de doenças e novas pistas para replicar *in vitro* e melhorar os modelos de doenças.

Os estudos posteriores centraram-se no desenvolvimento de um modelo *in vitro* de AF (Bio3D-AF) utilizando hidrogéis de Col I e estimulação inflamatória para replicar pormenores da doença. Para tal, células de AF humano foram cultivadas em 2D e 3D e estimuladas com IL-1 β , durante dois ou sete dias, assemelhando-se, respetivamente, a uma estimulação inflamatória aguda ou crónica. Como esperado, os resultados demonstraram que a cultura em 3D e a estimulação crónica reproduziram com maior precisão a degeneração do AF, validada pelas características fibróticas previamente estabelecidas em biópsias humanas de AF. Além disso, este modelo forneceu novos

conhecimentos sobre potenciais mediadores inflamatórios (por exemplo, G-CSF e GM-CSF) como alvos diagnósticos e terapêuticos para a falha do AF.

Finalmente, para aperfeiçoar ainda mais o modelo de AF estabelecido, foi desenvolvido um hidrogel anisotrópico à base de colagénio, utilizando microgéis de polietilenoglicol (PEG) magneto-responsivos. Esta plataforma inovadora permitiu o alinhamento das fibras de colagénio e, consequentemente, como prova de conceito do alinhamento celular, de fibroblastos humanos, até 7 dias em cultura. No futuro, este modelo refinado poderá ser utilizado para explorar o alinhamento celular do AF no contexto do IVD nativo e, assim, estudar o impacto do alinhamento celular na prevenção/reversão da herniação do IVD.

De um modo geral, esta tese permite o avanço o conhecimento atual sobre o tecido do AF e, consequentemente, sobre a herniação do IVD, fornecendo novas vias para modelos inflamatórios precisos de base humana. Ao elucidar os mecanismos patológicos subjacentes à falência do AF em amostras humanas herniadas, ao identificar potenciais alvos terapêuticos inflamatórios no AF e ao criar um novo modelo anisotrópico *in vitro*, o trabalho aqui desenvolvido é promissor para melhorar o diagnóstico e o tratamento atuais dos doentes que sofrem de hérnia do IVD e da dor lombar associada.

List of abbreviations

2D	Two-dimensional
3D	Three-dimensional
AB	Alcian Blue
ADAMTS	A Disintegrin and Metalloproteinase with Thrombospondin motifs
AF	Annulus Fibrosus
AIS	Adolescent Idiopathic Scoliosis
BSA	Bovine Serum Albumin
CAM	Chorioallantoic Membrane
CEP	Cartilage Endplate
CMS	Cyclic Mechanical Stretch
Col I	Collagen type I
Col II	Collagen type II
Col III	Collagen type III
CRM	Confocal Reflectance Microscopy
CTS	Cyclic Tensile Strain
CXCL	CXC Chemokine Receptor
DA	Di-acrylate
DMEM	Dulbecco's Modified Eagle Medium
DRG	Dorsal Root Ganglion
ECM	Extracellular Matrix
EDD	Embryonic Development Day
FBS	Fetal Bovine Serum
FFC	Flatfield Correction
FGF	Fibroblast Growth Factor
FN	Fibronectin
FWHM	Full Width at Half Maximum
G-CSF	Granulocyte Colony-stimulating Factor
GM-CSF	Granulocyte-macrophage Colony-stimulating Factor
H&E	Hematoxylin-Eosin
hAF	Human Annulus Fibrosus
HIF	Hypoxia-inducible Factor
IF	Immunofluorescence
IHC	Immunohistochemistry
IL	Interleukin

IL-1RA	Interleukin-1 Receptor Antagonist
IP-10	Interferon-gamma-induced Protein 10
iPSC	Induced Pluripotent Stem Cell
IVD	Intervertebral Disc
LBP	Low Back Pain
LDH	Lumbar Disc Herniation
LPS	Lipopolysaccharide
MCP	Monocyte Chemotactic Protein
M-CSF	Macrophage Colony-stimulating Factor
MF	Myofibroblast
MIG	Monokine Induced by Interferon-gamma
MIP	Macrophage Inflammatory Protein
MMP	Matrix Metalloproteinases
MRI	Magnetic Resonance Imaging
MSC	Mesenchymal Stem Cell
NHDF	Normal Human Dermal Fibroblasts
NP	Nucleus Pulposus
NSAID	Nonsteroidal anti-inflammatory drug
PDMS	Polydimethylsiloxane
PEG	Poly(ethyleneglycol)
PET	Polyethylene Terephthalate
PFA	Paraformaldehyde
PG	Proteoglycans
PLGA	Poly Lactic-co-Glycolic Acid
PLL	Posterior Longitudinal Ligament
PSR	Picro-Sirius Red
RANTES	Regulated on Activation, Normal T-cell Expressed, and Secreted
RFU	Relative Fluorescence Units
RT	Room Temperature
sGAG	Sulphated Glycosaminoglycan
SPION	Superparamagnetic Iron Oxide Nanoparticle
TEM	Transmission Electron Microscopy
TGF	Transforming Growth Factor
TIMP	Tissue Inhibitors of Metalloproteinases
TLBN	Translamellar Bridging Network
TNF	Tumor Necrosis Factor

YAP	Yes-associated Protein
α -SMA	Alpha-Smooth Muscle Actin

Chapter I

General Introduction

1. Intervertebral Disc: from homeostasis to herniation

1.1. The healthy intervertebral disc

The intervertebral disc (IVD) is the largest avascular organ in the human body, responsible for the support, mobility and flexibility of the spine [1]. Situated between the vertebral bodies, the IVD is composed of three distinct tissues: nucleus pulposus (NP), annulus fibrosus (AF) and cartilage endplate (CEP), which differ regarding cellular and extracellular matrix (ECM) composition. The structure of the IVD (depicted in Figure 1) includes the NP in the center, surrounded and restrained by the AF, with both tissues anchored inferiorly and superiorly by two CEPs. NP is characterized by its hydrogel like structure, with an elevated water content, creating a hydrostatic pressure, responsible for the ability of the spine to absorb mechanical loading [2]. NP cells are derived from notochordal cells and its ECM is mainly composed of proteoglycans (PG) and collagen type II (Col II) [3]. AF tissue is distinguished by its organized collagen type I (Col I) lamellae composition, in 15-25 concentric rings [4]. AF cells are differently distributed, with the inner AF cells having an oval/spherical shape, while the outer AF cells are fibroblast-like shaped [5]. CEP is composed by hyaline cartilage, with chondrocyte-like cells, at the highest cellular density within the IVD [6]. As the healthy IVD only has superficial innervation and vascularization, CEP is crucial in maintaining NP and AF homeostasis. CEP acts as a semi-permeable barrier between the vertebrae, being responsible for the regulation of nutrients transport, particularly small solutes [7].

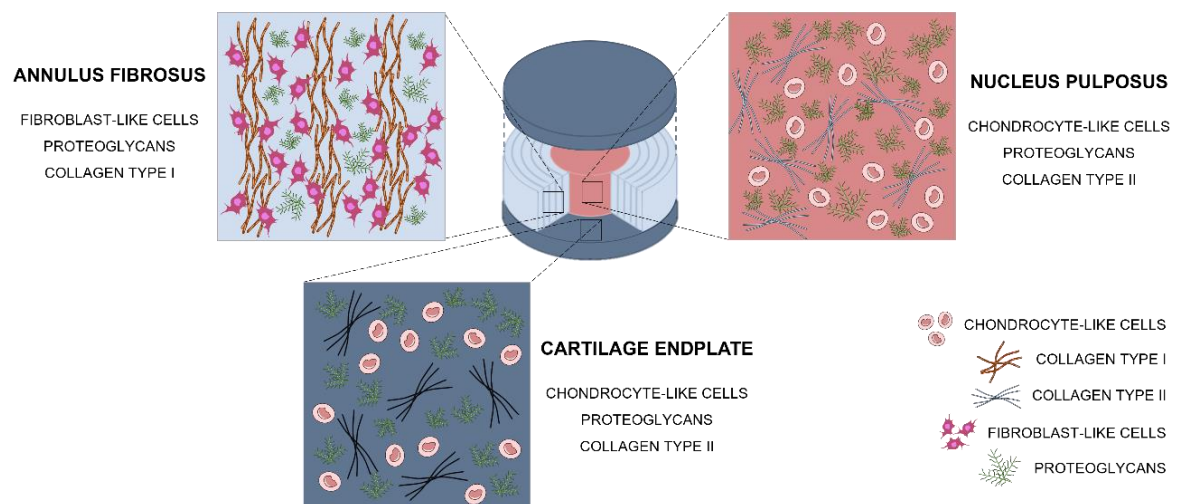


Figure 1 – Schematic representation of the IVD tissue

1.2. IVD Degeneration

Low back pain (LBP) is the leader disorder in number of years lived with disability, in both developed and developing countries [8]. A recent study accounts that by 2020 more than 600 million cases have been reported, estimating an increase up to 800 million by 2050 [9].

LBP is a multifactorial condition but IVD disorders, often associated with degeneration, are considered one of its main causes, affecting around 26-42% of patients suffering with LBP [10, 11].

IVD degeneration is a complex pathophysiology, associated with age, health conditions, trauma, among others. Nevertheless, as age increases, a reduction on the supply of nutrients to IVD cells is observed, leading to a decrease in oxygen supply, pH and ECM alterations, accompanied by a reduction in water content [12]. Moreover, inflammation is associated with IVD degeneration, with pro-inflammatory cytokines and matrix metalloproteinases (MMPs) being linked to tissue homeostasis impairment and ECM weakening [13, 14]. Specifically, a decrease in PGs content is directly related to the hydration loss and osmotic pressure reduction in the IVD [15]. These alterations also include a decrease in collagens content together with an increased expression of fibronectin (FN) within the tissue [16, 17]. ECM alterations are observed both in NP and AF, with fissures occurring in the latter, enabling immune cells infiltration, nociceptive nerve ingrowth and vascularization into the IVD, leading to discogenic back pain [18-20].

Besides degenerative disc disease, IVD disorders also encompass discogenic pain, spinal stenosis, spondylolisthesis and IVD herniation, among others. Specifically, the discogenic LBP is caused by damaged IVDs, from chemical or mechanical sources, due to ageing, trauma, among others [21]. Spinal stenosis consists of the narrowing of the spinal canal, possibly leading to spinal cord compression and in symptoms like pain, numbness, or weakness [22]. Spondylolisthesis involves vertebra slipping (forward or backward) and results from degenerative changes in IVDs and facet joints, leading to instability and compression [23]. IVD herniation occurs upon loss of IVD height and AF failure, with the displacement of inner tissue through a tear, often causing nerve compression and radiating pain [15, 24].

1.3. The herniated IVD

Lumbar disc herniation (LDH) is a major contributor to LBP, affecting 1-3% of world population per year, most commonly between 30 and 50 years of age [25]. Although many herniated IVD are asymptomatic, LDH is the main cause of spine surgery [26, 27]. Commonly, this condition is idiopathic, although it can be a secondary consequence to trauma or natural aging process within the IVD [25]. Risk factors such as diabetes, obesity, smoking and genetic predisposition can cause individuals to develop LDH [28]. Patients with symptomatic LDH often experience back pain and associated leg discomfort and, in severe cases, motor and sensory issues, due to compression of nerve roots [25]. Most of the individuals diagnosed with LDH can be treated with conservative treatments, after

correlating symptoms with imaging techniques, using magnetic resonance imaging (MRI), the gold standard to evaluate herniated IVDs [29]. Nonoperative treatment is effective in symptom relief in up to 85% of patients, after 8-12 weeks, with nonsteroidal anti-inflammatory drugs (NSAIDs) being the most common prescribed oral medications [30]. Physical therapy is also strongly recommended as soon as the first symptoms arise, to complement the medication prescribed [25]. When conservative approaches are not successful and symptoms are persistent, LDH patients are referred to operative treatments. Microdiscectomy is the most common surgical approach, where the herniated IVD material is excised, ensuring that the nerve roots are free of pressure upon surgery, attenuating back pain and leg discomforts [25, 31]. However, growing evidence has showed that discogenic pain persists even upon surgical interventions [32].

According to the North American Spine Society, the American Society of Spine Radiology and the American Society of Neuroradiology and upon the update on Lumbar disc nomenclature, version 2.0, “herniated disc” refers to the displacement of IVD material, with localized displacement of nucleus, cartilage, fragmented apophyseal bone or fragmented annular tissue beyond the IVD space [33]. Upon compressive loading in the spine, the pressure in the NP increases, which pushes against the AF fibers, placing them under tension [2]. In homeostasis, this tension is dispersed by the AF, which maintains the NP in the center of the IVD. However, when the AF integrity is compromised, as observed in IVD degeneration, the NP tissue can be pushed through the AF fibers, resulting in herniation and consequent compression of nerve roots, causing radicular pain [32]. To be considered a herniated disc, a tissue displacement from its normal location must occur, while an excessive growth beyond the tissue’s edges is not sufficient to be considered LDH [2]. Therefore, the disruption of AF tissue is intrinsically involved in IVD herniation [33].

Although several biological modifications are believed to contribute to IVD herniation, the causing event that ultimately leads to the herniated disc remains unknown. As mentioned in section 1.2., degenerative alterations, as ECM alterations and inflammation, lead to alterations in AF integrity, facilitating IVD herniation. Association between degeneration and genetic factors has been recently addressed, showing that genes encoding structural and support proteins, together with cytokines, MMPs, apoptotic and growth factors contribute to IVD degeneration and herniation [34]. Nevertheless, not only aging and degeneration cause herniation, with spinal overloading also being suggested to induce fissuring of the AF and consequently contribute to the lack of containment on the NP [35].

Regenerative medicine research has addressed LDH, aiming to resolve discogenic pain, by reversing the degenerative cues and restoring the IVD tissue (partial or completely) [36].

Current attempts include tissue engineering approaches, gene and cell-based therapy and growth factors delivery, aiming to modulate the metabolic activity of disc cells and increase ECM production, essentially regarding the PG content. Nevertheless, clinical translation falls short, with several side effects, cost-effective approaches and integration with native tissue. Also, partial or complete spontaneous hernia regression has been observed without any surgical treatment in 70-80% of the cases, in a mechanism still to be characterized [37]. Overall, more and better studies are required, as the main challenge when tackling LDH resides on the lack of understanding the pathway of herniation, essentially regarding the impact of IVD degeneration on AF tissue, concerning composition and architecture [38].

2. IVD herniation study models

As the intricacies of pathomechanisms underlying IVD herniation remain unclear, the demand for study models rises. These models are indispensable tools to better understand the complexities of IVD herniation, offering valuable insights regarding the disease process and its implications. By resembling anatomic structures, biomechanical features and biochemical composition, herniation study models have the potential to unravel novel insights into the pathophysiology of LDH. By possibly exploring the underlying mechanisms driving IVD herniation, these models can act as an important step towards the development of more effective diagnostic and therapeutic strategies, acting at the same time as a testing platform for innovative treatment approaches, ultimately improving LDH patient outcomes.

To disclose the different herniation study models currently used in IVD research, a literature search was conducted, using keywords in combination as “IVD” “herniation” “study model”, “IVD” “herniation model”, “IVD herniation” “model”, “NP” “herniation model”, “AF” “herniation model”, “IVD herniation” “*in vitro* model”, “IVD herniation” “*in vivo* model” and “IVD herniation” “*ex vivo* model”. The search was limited to studies in English language only with full-text accessibility. Retracted studies were also screened. PRISMA checklist was used to validate the research process, as described in flow diagram in Figure 2 [39]. Articles were eligible for full-text screening if the title and/or abstract mentioned herniation model, study model, *in vivo* model, *ex vivo* model or *in vitro* model. Articles were reviewed if they reported studies where IVD herniation was induced and further evaluated *in vivo*, *ex vivo* and *in vitro* contexts. *In silico* studies were retrieved upon the literature search, however as they were not the focus of the present review, they were not further considered.

A total of 127 studies were retrieved from the literature search in this study. After the exclusion of duplicates, 104 studies were included. 90 articles remained after title and abstract analysis and considering paper language, retraction of the study and inaccessibility to the full-text. Ultimately, 57 studies were included in this literature review, upon full-text analysis regarding the goals of the current study.

2.1. *In vivo* models

The use of animal models to study IVD diseases has been extensively reviewed [40, 41]. In specific for IVD herniation, animal models are widely used due to the feasibility and reproducibility of *in vivo* experiments. Nevertheless, there is not a consensus on the optimal animal model to replicate IVD herniation, with different *in vivo* models being currently employed, involving diverse animals and herniation induction methods.

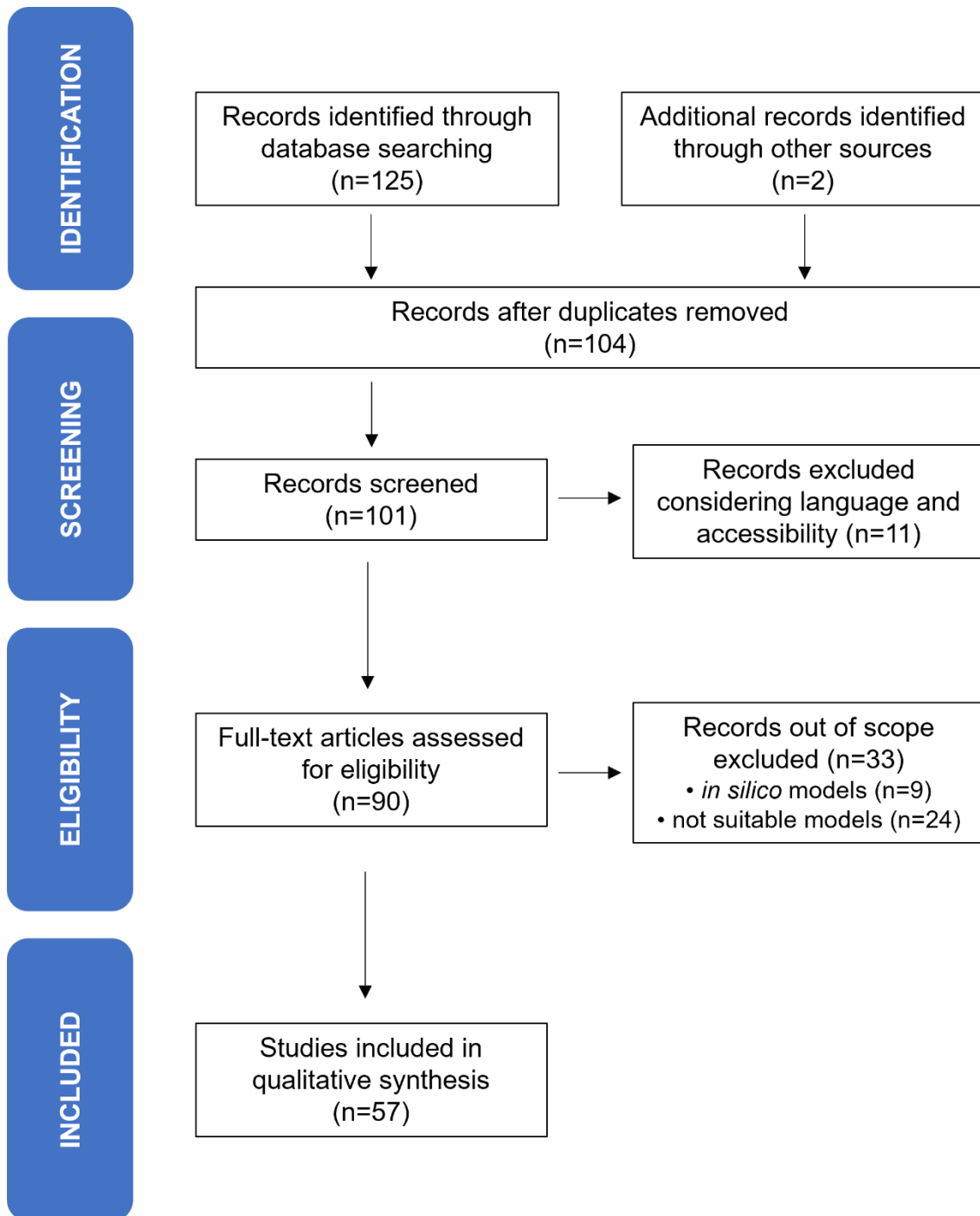


Figure 2 – PRISMA flow diagram for herniation study models literature review

2.1.1. Species

Different animals have been explored to study IVD herniation, as pictured in Figure 3. The majority of the studies found and analyzed here were performed in rats, more specifically in Sprague Dawley Rats. Studies have also been performed in dogs, sheep, rabbits, guinea pigs, goats and mice. Key aspects to take into consideration when pursuing animal experiments rely on the resemblance to human features. For example, the absence of

notochordal cells observed in human adult IVD is also verified in sheep, chondrodystrophoid dog and goat, but not in pig, rat, mouse and rabbit, which retain these cellular types throughout adult life [41]. Achieving the biomechanical features of the human spine is also challenging, as most of the models use quadrupedal animals. Nevertheless, as it happens with sheep, some cases have biomechanical human resemblance regarding the stress that the IVD is under [42]. Last, disc size and geometry varies between species, which have been previously studied and compared, concluding that, among the animals evaluated, mouse and rat lumbar IVDs are the ones that closely resemble the human lumbar IVD [43].

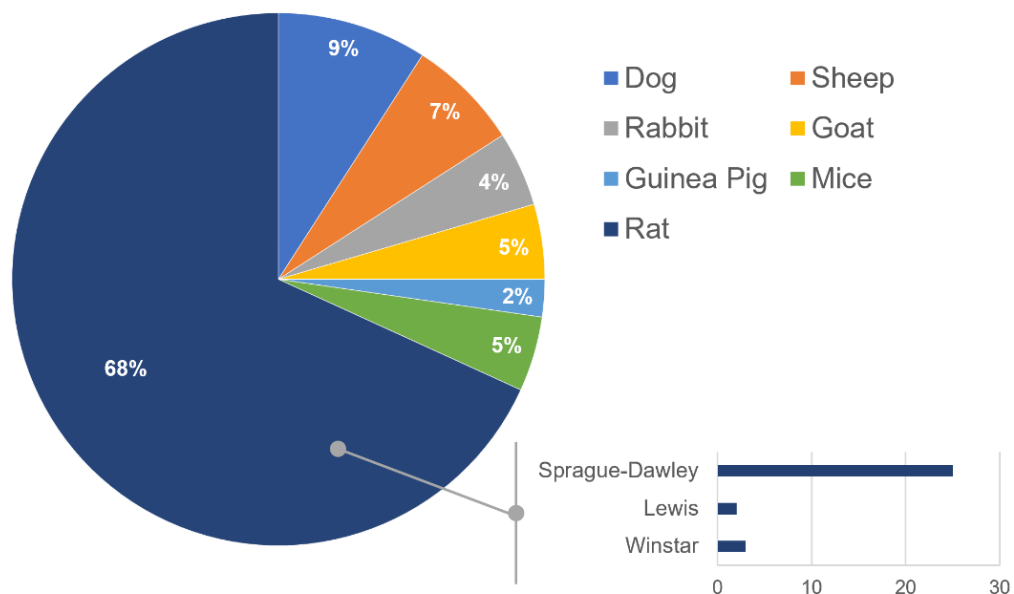


Figure 3 – Animal distribution in *in vivo* study models of IVD herniation

2.1.2. Herniation induction and evaluation

All methods to induce and later evaluate herniation are summarized in Table 1, per animal category. As for the dog models, spontaneous herniation is studied, with most cases selecting an inclusion criterion, based on clinical examination and considering secondary conditions upon herniation (as myelopathy and paraplegia) [44, 45]. For the other animals used in *in vivo* studies, induced herniation is required. Most of the herniation research conducted in animals (in the 24 out of 44 studies evaluated) focuses exclusively only on the application of autologous NP applied to a lumbar dorsal root ganglion (DRG) in the IVD spine. This method resembles a non-compressive disc herniation, inducing neural alterations in the associated DRG [46]. Other studies have performed annulotomy or nucleotomy, which involves the surgical removal of the tissue to simulate the absence/defect observed upon extrusion in IVD herniation. Throughout most of the animal

models, herniation has also been induced by injuring the AF, either with scalpel defects or puncture procedures, to stimulate the failure observed in AF tissue.

To evaluate the biochemical and biomechanical alterations upon IVD herniation, different methods have been used, depending on the animal model chosen, the specific aim of the work and whether it involved therapeutics tests. For example, the majority of the studies conducted in rat and mouse have addressed behavioral tests and evaluation of motor function. Often, these methods were complemented with protein detection by histology, immunohistochemistry (IHC), western blot and RNA analysis. In general, histological and IHC analysis were the most common evaluation performed independently of the animal model. Analysis was occasionally complemented by evaluation of biomechanical properties and detection of pro-inflammatory mediators, among others, as indicated in Table 1.

2.1.3. Limitations

As these models are currently being used as a gold standard to study herniation, caution should be taken regarding the limitations of each animal model. Researchers have highlighted the absence of control healthy groups when using dogs as *in vivo* models for IVD herniation. Moreover, when using these animals, researchers are dependent on the clinical information from patient records at the time of hospitalization, which might lack detail regarding pain symptoms and duration and neurological alterations in the animals [44, 47]. The ovine studies here discussed have not been performed in full resemblance of human IVD herniation, as, for example, the left lateral IVD aspect was used, when human herniation occurs more frequently near the posterolateral aspect [49]. Also in ovine models, researchers have mentioned the limitations of the use of drill based IVD injury methods that only induce acute defects and might not fully reflect the severity of IVD herniation [51]. As abovementioned, the use of animals with high notochordal cell number during adulthood, like rabbits, rat and mice, has to be done with precaution, as these animals have an increased regenerative capacity than humans, which do not have these types of cells in adult life [41]. In general rat studies dependent on the autologous NP application, prudence is advised as this method does not reflect fully the pathology of LDH, focusing specifically on the neurological alterations observed at the DRG [76]. Mice studies were conducted with a short observation window instead of long-term analysis and one study revealed alterations in body weight, which might induce analysis errors [87, 88]. Regardless of the model, the low sample size and IVD biochemical/biomechanical differences are highlighted several times as an important limitation of *in vivo* models to study IVD herniation [49, 50, 89].

Table 1: *In vivo* Herniation Models

ANIMAL	SPECIES	HERNIATION INDUCTION	EVALUATION	REFERENCE
DOG	Dachshund	Natural (inclusion criteria: myelopathy secondary to IVD herniation)	Cross sectional area and fat infiltration of the back muscles	[44]
	ND	Natural	Magnitude and localization of fatty infiltration	[47]
	ND	Natural (inclusion criteria: paraplegia after IVD herniation)	Neurological evaluation	[45]
	ND	Natural (based on clinical examination)	Histological and IHC analysis	[48]
SHEEP	ND	Annulotomy	Biomechanical evaluation, disc height assessment, micro-CT, histological/histomorphomeric analysis	[49]
	Swiss White Alpine	Annular defect	Biomechanical and histological evaluation	[50]
	ND	Drill bit IVD injury	ND	[51]
RABBIT	Japanese white	Autologous IVD application in L4	Histological analysis	[52]
		Needle puncture with manual compression to induce NP extrusion	Hernia size assessment, histological and IHC analysis	[53]
GUINEA PIG	ND	Annular defect	Disc height assessment, water, collagen and PG content evaluation	[54]
GOAT	Dutch Milk	NP excision	Histological analysis	[55]
	ND	Annular injury	Histological and biomechanical evaluation, MRI and micro-CT	[56]
RAT	Sprague Dawley	Autologous NP applied to L5 DRG without compression	Behavioral tests, cytokine evaluation (real-time PCR and ELISA) and mechanistic analysis	[57]
		Autologous NP applied to L5 DRG	Behavioral tests, needle electromyography and histological analysis	[58]
		Autologous NP and AF applied to L5 DRG	Behavioral tests and motor function assessment	[59]
		Autologous NP applied to L5 DRG	Cytokine evaluation	[60]

Autologous NP applied to L5 DRG without compression	Western blot, ELISA and IF analysis	[61]
Autologous NP applied to L5 DRG	Behavioral tests and neurological evaluation	[62]
Nucleotomy	Disc height assessment, histological and IF analysis	[63]
Autologous NP applied to L5 DRG without compression	Behavioral tests, Western blot, ELISA and IHC analysis	[64]
Autologous NP applied to DRG without compression	Motor function and mechanical sensitivity evaluation	[65]
Autologous NP applied to DRG	Behavioral tests and IHC analysis	[66]
Autologous NP applied to L5 DRG	Behavioral tests and IHC analysis	[67]
Autologous NP applied to DRG without compression	Behavioral tests, Western blot, ELISA and IHC analysis	[68]
Needle puncture and autologous NP applied to L5 DRG	Neurological evaluation	[69]
Needle puncture with IL-1 β	Behavioral tests, MRI imaging, PCR and histological analysis	[70]
Laminectomy hole and autologous NP applied to L5 DRG	Behavioral tests	[71]
Autologous NP applied to L5 DRG	Behavioral and stress tests, IHC analysis	[72]
Autologous NP and AF applied to L5 DRG	Behavioral tests, IHC and mRNA expression analysis	[73]
Autologous NP applied to L5 DRG	Behavioral tests, Western Blot and PCR evaluation.	[74]
Autologous NP applied to L5 DRG	Behavioral tests, Western Blot and chromatography analysis	[75]
Autologous NP applied to L5 DRG	Behavioral tests, Immunoblot and IHC analysis	[76]
Autologous NP applied to L5 DRG	Behavioral tests and IHC analysis	[77]
Autologous NP applied to DRG	Electrophysiological analysis	[78]
Autologous NP applied to L5 DRG	Behavioral tests and IHC analysis	[79]
Drill with L-shaped stainless-steel rod	Behavioral tests, siRNA and IHC analysis	[80]
Autologous NP applied to L5 DRG	IF analysis	[81]

	Lewis	Autologous NP applied in the spinal dorsal nerve roots	Electrophysiological analysis, mRNA expression evaluation	[82]
		Autologous NP applied to L5 DRG	Behavioral tests and neurological evaluation	[83]
		Needle puncture	Histological, IHC, flow cytometry, cytokine production and proteomic analysis	[84]
	Wistar	Autologous NP applied to L5 DRG	Behavioral tests, depressive evaluation, cytokine production analysis	[85]
		Needle puncture	Disc height assessment, hernia volume evaluation, histological, IF and IHC analysis	[86]
	MICE	MAFIA transgenic	Needle puncture	Histological, IF, IHC and flow cytometry analysis
	Balb/c	Needle puncture	Behavioral evaluation, Histological and IHC analysis, inflammatory response evaluation	[88]

IL - Interleukin, IF - Immunofluorescence; IHC - Immunohistochemistry

2.2. *Ex vivo* models

Ex vivo models allow the induction of herniation while maintaining a biochemical and mechanically controlled environment [89]. These models can be used to explore different experimental conditions, reducing the number of animals needed for *in vivo* trials [90]. Moreover, in these approaches, a thorough analysis regarding ECM alterations, mechanistic cellular studies and biomechanical properties is possible. As for *in vivo* models, different tissue sources and induction/evaluation methods have been applied in herniation *ex vivo* research.

2.2.1. Tissue source

Current herniation research using *ex vivo* models use a variety of tissue sources, ranging from human to rat, as detailed in Table 2. Although human material is difficult to obtain, several studies have been reported to use human samples to study herniation processes *ex vivo*. Nevertheless, as animal tissue is easily obtained and facilitates the reproducibility of the experiences, with high sample size, most of the *ex vivo* studies of herniation found in literature (10 out of 14) are from animal origin [41]. Porcine tissue, more specifically the cervical spine, has shown close resemblance to the human lumbar spine, regarding biochemical and anatomical features [91]. Similarly, ovine lumbar spine has shown to resemble biomechanical, anatomical and biochemically the features of human spine, as although quadrupeds, the ovine spine is under axial loading (as in humans) due to muscular forces [92]. Bovine tissue has also been explored in herniation studies, as bovine IVD have similar cell density and morphology and biomechanical properties [93]. As above mentioned, rat lumbar IVD has been reported to be the closest to resemble lumbar human IVD regarding disc geometry and size [43].

2.2.2. Herniation induction and evaluation

Apart from a specific human study reporting the use of herniated samples, the herniation induction methods performed in *ex vivo* models rely either on mechanical loading or annular defects, as depicted in Table 2. The mechanical loading applied, either in human, pig, sheep and bovine, varies between compressive loading, flexion and extension, anterior-posterior and lateral shear, axial rotation, and lateral bending. Annular defects were performed by removing AF tissue, exposing NP or also removing part of NP tissue.

The majority of evaluation methods in the *ex vivo* models reviewed relies firstly in confirming that herniation was achieved. Moreover, researchers focus on biomechanical analysis (regarding stiffness alterations, failure load and strength and tissue tension). Disc height and microstructural alterations have also been evaluated upon herniation induction across

Table 2: *Ex vivo* Herniation Models

SPECIES	HERNIATION INDUCTION	EVALUATION	REFERENCE
HUMAN	Mechanical loading (flexion, compression and axial rotation)	Stiffness alteration with time	[97]
	Herniated samples	Angiogenesis	[96]
	Mechanical loading (flexion, extension, load bearing and axial rotation)	Failure load, disc height and evidence for herniation	[98]
	Compressive loading	Failure load, disc height and evidence for herniation/fracture	[99]
PIG	Mechanical loading	Intradiscal pressure, flexion-extension moments and disc height	
	Mechanical loading (flexion, extension and compression)	Evidence for herniation	[101]
	Mechanical loading (flexion and extension) with and without vibration	Evidence for herniation, disc height, annular tissue tension and biomechanical properties	[102]
SHEEP	Low mechanical loading (flexion, lateral bending, axial rotation, anterior–posterior shear and lateral shear)	Failure load and microstructural alterations	[103]
	High mechanical loading (flexion, lateral bending, axial rotation, anterior–posterior shear and lateral shear)	Failure load and microstructural alterations	[104]
	Annular defect	Biomechanical analysis	[49]
	Mechanical loading (compression)	Evidence for herniation, histological analysis	[94]
BOVINE	Compressive loading	Failure load, disc height and evidence for herniation/fracture	[99]
	Annular defect	Biomechanical properties, disc height, failure strength, morphology and histology.	[95]
RAT	Annular defect	Biomechanical analysis	[105]

the majority of the species used for *ex vivo* models. Histological analysis was also conducted in ovine and bovine models [94, 95]. Specifically, the interaction and the angiogenic potential of herniated human samples with different cell types has also been addressed [96].

2.2.3. Limitations

Both the species used for *ex vivo* herniation studies and herniation induction methods have their limitations and caution should be taken when translating the results towards human. Herniation methods based on mechanical loading application do not fully resemble the physiological loading of the human lumbar IVD, which is subjected to different loading rates and moves in six degrees of freedom [95, 101]. Moreover, for logistic reasons as the implementation of the pressure sensor system, the CEP had to be damaged which can further de-pressurized the NP and ultimately contribute to AF buckling [100]. Moreover, as exemplified for *in vivo* models, different species have their limitations regarding the IVD biochemical composition and the presence of notochordal cells throughout adulthood. Lastly, caution should be taken as *ex vivo* culture lack native complexity regarding vascularization, innervation and immune cell infiltration, all characteristics of IVD disorders [106].

2.3. Final remarks

All discussed, IVD herniation study models provide new opportunities to better understand the pathophysiology of this disorder. Nevertheless, they all also present important limitations regarding the resemblance to the human disease, essentially considering biochemical resemblance and higher regenerative capacity. Notably, no *in vitro* study model was found in the current literature review. *In vitro* models are important tools as they allow for precise experimental control and reproducibility. Moreover, from an ethic point of view, they provide an animal-free alternative to study diseases, while being cost-effective and providing high throughput platforms for therapeutic screening. The development of *in vitro* models mimicking IVD herniation, with the possibility to naturally or artificially resemble AF failure, facilitating NP extrusion, is of paramount importance to overcome the disadvantages of current *in vivo* and *ex vivo* models and further advance the treatment of LDH.

3. *In vitro* AF models

In vitro models resembling LDH are needed, ideally providing a platform that allows for AF failure and NP extrusion. As current research has focused widely on NP tissue rather than AF, novel *in vitro* AF models are required to further develop a complete herniation model. In this chapter, the current research on AF *in vitro* models will be reviewed together with the identification of the several components to consider when developing the optimal AF study model.

3.1. AF development and maturation

The genesis of AF remotes to embryonic development, during the morphogenesis of the vertebral column, as it has been extensively reviewed elsewhere [107, 108]. Briefly, AF tissue rises upon the formation of NP from the notochord, when the surrounding paraxial mesoderm is segmented into somites and patterned, forming the sclerotome [107]. Sclerotomal cells originate the inner hyaline-cartilaginous and outer AF of the embryonic IVD, migrating and condensing around the notochord [109]. Cells elongate during the development of AF to organize into concentric circles around the developing NP, orchestrating the formation of the distinct lamellar structure characteristic of the mature AF. This orientation is caused by the intracellular organization of actin stress fibers and is proposed to precede the deposition of aligned collagen matrix, responsible for the lamellar structure of the AF [110-112]. During this developmental phase, intricate signaling pathways regulate the expression of key transcription factors and ECM components towards AF fate [113]. Mfh1 and transforming growth factor (TGF) β signaling are important players towards AF fate determination and their disruption has shown to lead to abnormal AF formation [114-116]. Moreover, mechanical loading is also described as an important feature towards AF development, as an *in vivo* study as shown that fetal movements affect pineal curvature and segmentation [117].

AF mature tissue is characterized by Col I lamellae (50-70% dry weight), which are organized in an angle-ply structure, oriented between 30°-65°, depending on tissue localization [4, 5]. Individual lamellae are separated by a non-organized interlamellar tissue, rich in PG (10-20% dry weight), elastic fibers and cells, which are connected by the translamellar bridging network, mainly composed of elastin, fibrillin, PGs and Col IV [5, 118, 119]. Previous studies have highlighted the importance of this complex structure on the mechanical properties of AF, as it has been proposed to allow the sliding between lamellae, preventing damage under extreme mechanical loadings [119-121].

Mature tissue displays high cellular diversity in homeostasis, with cell density varying between 3000 to 9000 cells/mm³ [122]. Despite this, current research has only identified

few markers to allow the distinction between cell populations [118]. Previous work exclusively focused on human AF cells gene expression analysis has highlighted over 1000 genes with higher expression in AF (compared to NP tissue), with 125 genes being specific to membrane-associated markers [123]. Torre et al. have proposed that, among the different cell types, the native population primarily derived from the sclerotome should be labeled as “annulocyte” [118]. Additionally, an ECM-related transcriptional signature has also been proposed to distinguish AF cells from other tissues, including expression of COL1A1, COL5A1, COL12A1 and keratan sulfate, all commonly found in annulocytes of human samples [124, 125]. Moreover, typical markers from other fibrous connective tissues, namely *Tnmd* and *Mkx*, have also been reported to be present in AF cells from the outer tissue layer [126, 127]. In this same layer, Nakai et al. have identified a cell population expressing CD146 and SM22 α [128]. In short, although several markers are proposed, a definitive set of markers that unequivocally identify AF cells and distinguish them from the other IVD cell populations is still lacking.

3.2. AF Tissue degeneration

During degeneration, AF integrity is compromised, in a combination of different factors, as mechanical forces, inflammation and biochemical imbalance [129, 130]. For example, alterations in mechanical loading in AF tissue, characteristic from degenerative processes, have been described to influence ECM and cellular processes. Static compressive stimulation led to reduction in the expression of collagens and aggrecan, while dynamic compressive loading resulted in an increased anabolism of regarding Col I, Col II and aggrecan [131]. Osmolarity alterations in diseased environments also play an important role in AF cells behavior, as observed by the inhibition of Col I expression at high levels of osmolarity [132]. These alterations affect AF ability to restrain NP tissue, leading to IVD herniation. The impact of biomechanical alterations extends over inflammatory responses within AF tissue, affecting ECM catabolism and contributing to the degenerative process [133, 134]. In general, interleukin (IL)-1 β and tumor necrosis factor (TNF)- α are the main cytokines whose expression is increased in IVD degeneration, together with IL-2, IL-4, IL-10, IL-12 and IL-17 [135]. Analysis of human herniated discs has revealed increased levels of IL-8 and monocyte chemotactic protein-1 (MCP-1) [136]. Moreover, an up-regulation of IL-1 has showed to induce alterations in MMPs and a Disintegrin and Metalloproteinase with Thrombospondin motifs (ADAMTS) homeostasis [137]. Altogether, these alterations in inflammatory mediators weaken the AF tissue and can also impact the sensory nerves and increase vascularization, contributing to discogenic pain and AF failure, ultimately leading to IVD herniation [21, 138].

3.3. Current treatment strategies for AF repair

Different approaches combining tissue engineering, therapeutics development and biological interventions have focused on mitigate the degenerative alterations observed in AF failure, promoting tissue regeneration, as reviewed elsewhere [139]. Stem cell-based therapies and growth factor delivery systems, among others, attempt to promote tissue repair, regenerating ECM components, and restoring IVD homeostasis [139]. Also, biomechanical interventions, such as biomaterials-based annular repair strategies, are performed to attempt to restore structural integrity and biomechanical stability to the degenerated disc [140]. Nevertheless, none of the current treatments repairs or regenerates the herniated IVD, highlighting the importance of developing novel therapies, to improve clinical outcomes and enhance patient quality of life [38]. Research on disease biomarkers such as MMPs, inflammatory cytokines, and degradation products of ECM components act as potential indicators of AF failure and might serve as important tools to monitor AF health and disease progression. For that reason, understanding the complexities of AF biology and pathology is crucial. Study models rise as important tools to better dissect the pathomechanisms behind AF failure and IVD herniation and hopefully develop targeted therapies and alleviating the burden of spinal disorders on global health.

3.4. AF tissue *in vitro* study models

As research has focused predominantly on the study of NP disorders and somehow neglected AF tissue, only a few *in vitro* three-dimensional (3D) studies are currently described in literature focusing on understanding AF pathomechanisms, as depicted in Table 3.

Part of these studies aim to replicate the interaction between AF tissue and the other tissues in IVD: NP and CEP. Chong et al. have developed an *in vitro* model to replicate the interaction between AF (more specifically the outer AF) and cartilage tissue [141]. For that, the researchers have used polycarbonate urethane scaffolds organized in multi-lamellae and oriented at 30°, to resemble AF tissue anisotropy. The construct was cultivated with bovine AF cells and placed in direct culture with cartilage constructs, using Col II coated membranes and articular chondrocytes. The team was successful in recreating the interface between AF and CEP, through the analysis of ECM production and biomechanical evaluation. Moreover, Srivastava et al. have studied the interface between NP and AF under inflammatory stimulation, more specific to evaluate the crosstalk between inflamed NP and healthy AF tissue [142]. To achieve this, researchers have used collagen based hydrogels to resemble both tissues. For AF specifically, Col I hydrogels with poly(ethyleneglycol) (PEG)-cross linker were produced. Evaluation of ECM components, pro-inflammatory

Table 3: *in vitro* AF study models

FOCUS OF THE STUDY	SPECIES	CELLS	PLATFORM	REFERENCE
Mimic the interaction between AF tissue (specifically outer AF) and cartilage	Bovine	AF	Polycarbonate urethane scaffolds, with 30° orientation (for AF)	[141]
Reproduce the interaction between NP and AF tissue and evaluate the effect of inflammation exposure (using IL-1 β)	Bovine	NP and AF	Collagen type I hydrogel with PEG-cross linker (for AF)	[142]
Evaluate the effect of inflammation exposure (using LPS) in AF cells	Rat	AF	Collagen type I hydrogel	[143]
Dissect the effect of inflammation exposure (using IL-1 β) and contact with endothelial cells in NP and AF cells	Human	NP and AF + endothelial	Microfluidic platform	[144]
Assess the effects of concentration of proinflammatory mediators on AF cells	Human	AF + THP-1	Microfluidic platform	[145]
Assess the effect in AF cells of a co-culture with THP-1 cells	Human	AF + THP-1	Alginate	[146]
Compare the response to LPS stimulation between IVD and cartilage cells	Bovine	NP, AF and articular cartilage	Alginate	[147]
Characterize the production of PGs and lactate in the presence of macrophage proinflammatory mediators	Human	NP and AF + THP-1	Alginate	[148]
Evaluate the immunomodulatory effect of notochordal cells on AF cells	Rabbit	AF + notochordal	Self-assembled pellet aggregates	[149]
Decode the interaction between AF cells and neuron cells interactions upon inflammation exposure (using IL-1 β and TNF- α)	Human	AF + neuroblastoma	Self-assembled pellet aggregates	[150]
Understand the interaction of rabbit notochordal cells and AF cells under inflammation, through THP-1 cells	Human and rabbit	AF + notochordal and THP-1	Self-assembled pellet aggregates	[151]
Evaluate the interaction between mast cells and IVD cells	Bovine	NP and AF + mast	Agarose	[152]

mediators and glycan analysis highlighted the resemblance of the proposed study model to decipher alterations during IVD pathology. Molladavoodi et al. have proposed an exclusively AF tissue model, using Col I hydrogels to culture rat AF cells under inflammatory stimulation (using lipopolysaccharide (LPS)) [143]. The proposed model induced alterations in cell viability and morphology together with a reduction in biomechanical properties, mimicking the observations reported in AF pathomechanisms. Moreover, researchers have also developed *in vitro* models to characterize the effect of other cell types on human AF cells. A microfluidic platform was established by Hwang et al. to study the effect of exposing human NP and AF cells under inflammation to endothelial cells [144]. This *in vitro* platform allowed for the study of inflammatory mediators' expression and cell migration towards the endothelial cells, therefore enhancing the understanding of the vascularization process upon IVD disorders. Also, Hwang et al. have developed a microfluidic-based spine-on-a-chip that was used to thoroughly characterize the response of human AF cells to different IL-1 β concentrations and to macrophage conditioned media, using THP-1 cells [145]. Results showed modulation of important disease players, as pain-related and angiogenic factors, with increased concentrations of pro-inflammatory stimulus. Different studies have also used alginate as a biomaterial to establish 3D *in vitro* AF models, through cell encapsulation [146-148]. Byvaltsev et al. encapsulated human AF cells in alginate to study the effect of inflammation exposure and THP-1 cells presence [146]. A thorough analysis on cytokine production was performed, revealing increases on IL-1 β and IL-8 upon co-culture with THP-1 cells, similar to the alterations in IVD disorders. Also, Byvaltsev's team has used this alginate encapsulation to characterize the production of PGs and lactate under proinflammatory stimulation, mediated by the presence of THP-1 cells, showing that glycolysis is promoted accompanied by reduced PGs synthesis [148]. Aota et al. have used alginate encapsulation as well, to perform a comparison study between IVD cells and cartilage cells, both from bovine origin, under LPS stimulation [147]. The study was focused on PGs production and revealed suppressed PGs synthesis, although with less impact on AF cells. A 3D model approach using self-assembled AF cell pellet aggregates has also been explored in different studies [149-151]. Initially, Moon et al. used this approach to disclose the immunomodulatory effect of notochordal cells on AF cells, from rabbit origin, by testing different concentrations of IL-1 β and TNF- α [149]. Results showed that the conditioned media from notochordal cells was able to attenuate the gene expression of different inflammatory mediators, as iNOS and COX-2 in AF cells. Moreover, Moon et al. used the AF cell pellet strategy to understand the interaction between human AF cells and neurons [150]. The team was able to study which growth factors and cytokines are associated with the neural contact upon IVD degeneration. Last, this approach has also been used to dissect the interaction between human AF cells, rabbit notochordal cells and

THP-1 cells, by Kim et al. [151]. Cytokine analysis showed that when AF cells are cultured in contact with both notochordal and THP-1 cells, levels of inflammatory factors as IL-6, IL-8 and iNOS are reduced, when compared to AF cells only in culture with THP-1 cells. Agarose gels have also been used for AF studies, with Wiet et al. using this approach to study the interaction between human IVD cells and mast-cells in a 3D microenvironment [152]. However, on the analysis conducted in proinflammatory mediators, no alterations were observed in AF cells.

Nevertheless, limitations have been exposed through the different study models. First, the scarcity of healthy human cells is a general drawback across different IVD studies [144]. Researchers have also highlighted the need to enhance the study conditions regarding the resemblance to the IVD homeostatic microenvironment, with privation of oxygen and glucose [142, 146]. Moreover, the importance of model refinement is mentioned, essentially regarding the architecture of the proposed constructs, as it often lacks the organization of collagen fibers observed in native tissue [143]. Last, it is also imperative to address the need for biomechanical stimulation during *in vitro* studies to better resemble the mechanical loading of human spine [142]. Altogether, a question arises: “What is crucial to have into consideration when designing an ideal AF study model?”

3.5. Key requirements for an (optimal) AF study model

When planning the development of an effective study model for understanding the complexities of AF biology and pathology, researchers are tasked with a multifaceted challenge: to replicate, as faithfully as possible, the native tissue and microenvironment. Long et al. have previously reviewed the requirements considering material properties when approaching AF repair [153]. Nevertheless, the process of designing a 3D AF study model demands thorough consideration at every stage of model development, beginning with the choice of biomaterials, extending to the mechanical properties and architecture features, the microenvironment cues and the inclusion of specific cell types. Ultimately, researchers should attempt to minimize discrepancies with native AF, maximizing, therefore, the translation potential of the model.

3.5.1 Biomaterial composition

Native AF tissue is mainly composed of Col I, which accounts for 50-70% of its dry weight, in combination with PGs and other ECM proteins [7]. Among PGs, aggrecan is the most abundant within AF ECM, binding to the collagen fibrils [111]. Different sulphated glycosaminoglycans (sGAG) as chondroitin sulfate and keratan sulfate, present in the side chains of PGs, are important molecules on AF ECM, contributing to water retention and the compressive resistance of the tissue [154]. Moreover, elastin also plays an important role

on AF homeostasis, providing elasticity to the tissue and allowing it to maintain its shape upon mechanical loadings [155]. Therefore, when choosing the biomaterial to serve as scaffold for an AF study model, the choice towards natural biomaterials is almost immediate, to resemble *in vivo* tissue. A few AF study models have been developed using Col I hydrogels, as described in the previous section of this chapter, while the others rely on other choices, both natural and synthetic. Nevertheless, the characteristics of the tissue are not exclusively dependent on Col I, as abovementioned. Biomaterials involving a mixture of AF ECM components have not been described to be used for AF study models. However, they have been reported for other tissues. For example, collagen/elastin hydrogels have been developed for lung applications (exploring different ratios of soluble elastin) and for heart valve studies [156, 157]. Hybrid hydrogels using collagen and chondroitin sulfate have also been developed for dermal applications and for cartilage defects (in combination with hyaluronic acid) [158, 159]. Thus, there is an opportunity to translate these biomaterial combinations towards the study of AF tissue, enhancing its resemblance.

When addressing the issue of mimicking the ECM components of native tissue in an *in vitro* model, decellularization rises as an interesting tool. IVD tissues, essentially the NP, have been decellularized, as reviewed elsewhere [160]. AF tissue has been successfully decellularized, maintaining native mechanical properties and ECM components (Col I and sGAG) [161, 162]. Decellularized scaffolds enabled cell seeding and proliferation and were tested for different applications as AF patches and hydrogels [162, 163]. Therefore, decellularized scaffolds are attractive approaches when considering the development of AF study models resembling native tissue composition.

3.5.2 Architecture

As described in section 3.1., AF tissue is characterized by a distinct anisotropic architecture. Characterized by concentrically arranged collagen fibers, these fibers run predominantly parallel to one another within the AF's lamellae [4]. Interspersed among this collagen framework are PGs and elastin, pivotal components responsible for regulating tissue hydration and osmotic pressure, forming the inter-lamellar matrix [120, 164]. Although without aligned organization, this inter-lamellar structure, connected by the translamellar bridging network (TLBN), is important for the replication of AF tissue homeostasis and pathomechanisms, as it contributes significantly to the AF's ability to tolerate tensile stresses and maintain structural integrity [119]. Consequently, faithfully reproducing this sophisticated architecture *in vitro* becomes paramount for accurately mimicking the AF tissue.

Among the current AF study models, Chong et al. have used electrospinning techniques to develop an anisotropic polycarbonate urethane construct to serve as AF tissue, showing that bovine cells aligned with the scaffold orientation [141]. In a previous study, this team also addressed the impact of coating these scaffolds to facilitate cell alignment and oriented collagen deposition [165]. Other studies have explored anisotropic techniques, mainly regarding the production of AF patches for *in vivo* applications. Using electrospinning techniques, Dewle et al. have developed an aligned polycaprolactone-supported electro compacted Col I patch, with promising results in proliferation and alignment of goat AF cells, stimulating Col I and GAGs production [166]. Extrusion techniques were also explored, by 3D fiber deposition, to develop an angle-ply oriented polycaprolactone scaffold for AF applications [49, 167]. Similar to what was described in the previous subsection 3.5.1., decellularization methods also show advantages concerning the replication of AF tissue architecture. By using decellularized pericardium tissue, McGuire et al. proposed a try-layer scaffolds, with induced angle-ply orientation, where bovine AF cells were able to elongate with the construct orientation, followed by collagen deposition, that were used in several studies [168-170]. This construct was improved in a posterior study by incorporating hyaluronic acid between the lamellae, to resemble the inter-lamellar GAG layer [171].

Nevertheless, the current anisotropic strategies here described always compromise the composition of the construct, when compared to the native AF tissue. Regarding the recreation of the AF inter-lamellar structure, only Borem et al. have addressed it, by improving the decellularized scaffold through hyaluronic acid incorporation [171].

All the other described studies have used synthetic materials (sometimes in combination with Col I) to produce anisotropic approaches. A shift towards collagen-based anisotropic approaches applied to AF studies is required, ultimately to closely recreate native ECM, regarding both composition and architecture. The current techniques employed to develop collagen anisotropic scaffolds have been reviewed elsewhere [172]. In brief, methods as microfluidics, magnetic alignment and freeze-drying have shown promising outcomes regarding the production of aligned collagen scaffolds. Throughout the different approaches, preserving collagen fibrillogenesis profile and avoid its denaturation, while enabling cell viability and proliferation are the main concerns when producing these constructs [172]. These strategies have been applied to distinct tissue and organs, as neural system, bone and cornea, opening opportunities to translate them towards AF tissue [173-175].

Finally, it is important to reinforce that the anisotropy of the scaffold does not straightforwardly imply cell orientation, as improvements on the constructs have been

performed to achieve better results. For example, coating application and scaffold modification have shown improved AF cell alignment compared to constructs controls [165, 166].

3.5.3 Biomechanical properties

The AF is a crucial component in providing structural support and facilitating various biomechanical functions in the human spine [139]. Taking the mechanical properties of AF tissue into consideration is essential for designing study models that accurately mimic its behavior and response to physiological and pathological conditions.

AF tissue experiences tensile forces primarily along its circumferential direction due to spinal motion and loading [118]. Its collagen-rich structure provides high tensile strength and stiffness, allowing it to resist deformation and maintain disc integrity [171]. Moreover, AF tissue is under compressive loading, enduring the weight-bearing demands of the spine while preserving disc height and integrity [139]. As mentioned earlier, PGs are key players on attracting and retaining water within AF ECM, granting compressive resistance to mechanical loads [164]. Last, the AF also exhibits viscoelastic behavior, characterized by time-dependent deformation under applied loads, that allow shock absorption during spinal movements [176]. Altogether, these properties result in a unique biomechanical profile in AF tissue, characterized by different parameters as tensile strength, stiffness, compressive modulus, and viscoelastic properties, as reviewed elsewhere [153]. For example, the tensile strains of AF tissue have been reported to range from approximately 10 to 90 MPa, depending on factors such as age, anatomical location within the disc, and testing methodology [153, 177]. The compressive modulus, reflecting the tissue resistance to compression, can vary between 0.4 and 3 MPa [153].

While developing study models, it is crucial to incorporate these numerical values to accurately replicate the mechanical behavior of AF tissue. Considering the AF study models discussed in section 3.4., the majority of them did not address the mechanical properties of the construct. Nevertheless, Chong et al., when developing the AF-CEP interface study model, addressed the mechanical strength of the interface through uniaxial tensile testing, comparing it to native bovine AF-CEP interface. Results showed a mechanical strength approximately 57 times weaker in the engineered constructs (21kPa vs 1.2-1.4 MPa in native bovine tissue) [141]. Also uniaxial tensile tests were conducted in the Col I based study model proposed by Molladavoodi et al.. Although the purpose was to compare the mechanical properties of acellular versus cellular constructs, with and without LPS stimulation, results showed values ranging from 20 to 30 kPa, on average, far away from the native tissue values [143]. Both models report mechanical properties that do not

resemble AF tissue, highlighting the importance to include novel approaches when proposing new research models.

Similar to what was described for 3.5.2., other AF biomaterial-based research has considered mechanical properties, mainly for patch applications. Uniaxial tensile testing was conducted in polycaprolactone-supported electro compacted Col I patches and revealed a tensile strength of approximately 4 MPa, closer to what is observed in the native tissue [166]. The biaxial tensile mechanical properties of decellularized pericardium scaffolds proposed by Borem et al. for AF applications revealed similar biaxial responses (regarding elastic modulus in circumferential and axial), when compared to native bovine AF tissue [168]. Moreover, this team has also incorporated hyaluronic acid in the construct, which, besides the advantages previously mentioned considering biochemical composition resemblance, also enhanced the mechanical properties of the scaffold [169]. Clearly, AF patch research has come closer to mimic the native mechanical properties, rather than the current options for AF study models, highlighting the importance to integrate the different research being conducted in AF tissue.

Additionally, integrating techniques such as finite element analysis, taking advantage of numerical data on AF biomechanical properties, can aid developing more realistic and predictive models [178]. This analysis will possibly allow for the prediction of stress and strain distributions within the AF under different loading scenarios, supporting the validation and refinement of study models.

3.5.4 Cell sources

To study AF biology and pathology, researchers have different cell sources available. AF cells can be obtained from different origins, including human and animal tissue specimens, cadaveric donations, and even through reprogramming techniques such as induced pluripotent stem cells (iPSCs). Until now most of AF cells are obtained from AF tissue macroscopic dissection. However, there is a need to develop reliable markers to specifically isolate AF cells and guarantee the purity of this cell population within the other IVD cells. Each cell source offers distinct advantages and considerations, contributing to the versatility and comprehensiveness of studies. Human AF cells are isolated directly from surgically obtained AF tissue specimens, typically from patients undergoing spinal surgery for disc-related disorders. These primary AF cells are harvested through enzymatic digestion or explant culture techniques, wherein the tissue is minced, digested with enzymes such as collagenase, and then cultured to allow cell outgrowth [179, 180]. Human AF tissue provides a physiologically relevant cell source for studying tissue-specific responses to mechanical loading, matrix remodeling, and degenerative processes [179, 181]. Primary human AF cells

exhibit phenotypic characteristics resembling native AF cells, including expression of Col I and inflammatory mediators [179]. Considering clinical relevance and translational potential, human AF cells closely resemble those found *in vivo*, providing physiologically relevant insights into human disc biology and pathology, that can lately be easily applicable to clinical practice [182]. Also, human AF cells allow the possibility to conduct patient-specific studies, tailoring interventions through personalized medicine approaches. However, access to human IVD tissue is scarce and limited to surgical specimens from patients undergoing spinal surgery, limiting sample size and raising ethical consideration [89]. Also, donor variability, regarding age, other comorbidities, among others, may play a role during study interpretation.

Human AF cells can also be obtained from human cadaveric tissue, obtained postmortem from organ donors or anatomical donations, represents another human source of AF cells. The isolation of these cells involves similar techniques as those used for live tissue, although considerations regarding tissue preservation and quality may apply [181, 183]. In this approach, AF tissue integrity is preserved, providing a standardized and consistent cell source. These cells are more readily available and cost-effective source, and allow for controlled studies considering age, gender and other factors that might influence AF homeostasis and disease. Nevertheless, similar to human AF tissue, there is a limited availability of tissue, with ethical considerations involved [184]. Also, postmortem alterations must be taken into considerations, as they may occur regarding tissue physiology [185].

AF cells from different animals, including rodents and larger animals (porcine, bovine, canine, ovine), serve as valuable sources for AF and IVD research purposes [186]. Isolation methods for animal AF cells are similar to those for human, involving enzymatic digestion or explant culture of AF tissue specimens [141, 166]. Animal AF cells provide an accessible and reproducible cell source for investigating AF biology and pathology, complementing studies performed in human cells [187]. Also, this source provides a higher experimental control regarding external variables, while being easily accessible throughout time, hence allowing for mechanistic and longer studies [187]. However, differences between species cannot be neglected, as AF tissue varies in structure and composition [43]. Moreover, ethical considerations and constraints are superior, considering animal welfare and regulatory processes, approved by ethical boards and animal committees [187].

Mesenchymal Stem Cells (MSCs) can also be used, differentiating into AF-like cells under appropriate culture conditions [188, 189]. The robust proliferative capacity of these cells enables their expansion *in vitro*, providing the scalability of AF cell generation [190]. As MSCs can be isolate from various tissues, these cells are easily accessible, facilitating the

development of standardized protocols [191]. However, MSCs populations have high heterogeneity and a limited lineage-specificity, which may impact the reproducibility of AF-like cells experiments [192]. Standardized protocols for directing MSC differentiation into AF-like cells are lacking, leading to possible variability in differentiation efficiency and cell phenotype.

In alternative, iPSCs have also been used to obtain AF-like cells by differentiation with developmental cues and microenvironmental factors [113]. iPSCs have the capacity to differentiate into multiple cell lineages, including AF-like cells, offering a potentially unlimited cell source for regenerative medicine applications and disease modeling [193]. Also, iPSCs can be patient-specific, allowing for personalized medicine studies and treatments [194]. Nevertheless, iPSC-derived cell populations may retain residual pluripotency and tumorigenic potential, while being highly heterogeneous, which may affect the interpretation of experimental outcomes [195, 196]. Achieving efficient and reproducible differentiation of iPSCs remains challenging, as current differentiation protocols often yield low yields and variable cell phenotypes [197]. Last, the use of iPSCs also involves ethical considerations and significant costs and resource requirements, which have to be considered when planning AF experiments [198].

In the current AF study models described in section 3.5., as depicted in Table 3, 50% of the work has been conducted already using human AF cells. Nevertheless, the other studies were performed using animal cells, highlighting the importance to translate the studies towards human approaches. Moreover, the inclusion of MSCs or iPSC in the design of AF study models has not been addressed yet, enabling opportunities for future approaches when planning novel models.

3.5.5 Physicochemical microenvironment cues

The unique microenvironment within the IVD impacts cellular behavior, ECM synthesis and tissue homeostasis. The IVD is characterized by its acidic, avascular, hyperosmotic and hypoxic hostile environment, together with low nutrient supply [199]. It is important to replicate these physiological cues accurately when developing AF study models to ensure the clinical relevance and reliability of experimental outcomes.

Lactic acid is highly accumulated in IVD tissues, due to cellular anaerobic glycolysis, with concentrations varying between 2 to 6 mM, and resulting in a slightly acidic pH (7.0-7.2) in homeostasis [200, 201]. Recently, researchers have linked the overproduced lactic acid, more specific lactate, with an increase on AF cells capacity on ECM production [202].

IVD tissue is characterized by a high osmolarity, due to its increased aggrecan composition, characterized by negatively charged GAG chains [203]. The hyperosmotic profile is responsible for the swelling properties of the IVD in homeostasis. The IVD osmolarity, more specifically in NP, suffers cyclic alteration throughout the day, considering the application of mechanical loading. At rest, osmolarity values range from 430 to 500 mOsm/L, while during the diurnal activities a reduction up to 25% can be observed [204, 205]. IVD studies have used NaCl / KCl solutions to recreate *in vitro* the native osmolarity [119, 179, 206].

Hypoxia is associated with cellular and ECM metabolism, with hypoxia-inducible factor (HIF) being one of the main factors involved [207, 208]. Studies comparing 1% and 6% O₂ have been conducted in bovine NP cells, showing minor differences on aggrecan expression [209]. Nevertheless, studies have addressed the importance of simulating hypoxic conditions (ranging from 2 to 20% O₂) when culturing human NP and AF cells and results showed different responses between tissues, with smaller effects on AF cells [210].

Currently, the AF study models proposed and described in Table 3 have not included any of the mentioned physicochemical cues in their experimental setups.

3.5.6 Considerations for AF disease models

As discussed in section 3.1., during degeneration and herniation, AF tissue is compromised. The alterations in AF integrity are related with mechanical traumas and inflammation progression, which can lead to discogenic pain, through angiogenesis and innervation. When designing a study model, disease induction must be carefully planned, to resemble at best the *in vivo* pathology. In the case of AF studies, and general IVD studies, a combination of mechanical loading, inflammation and external cell types should be planned to better resemble degeneration and herniation.

The current AF study models, described in section 3.4., do not include mechanical stimulation while inducing disease. However, studies have used mechanical loading to simulate herniation in *ex vivo* models, as discussed in Chapter 2. Different loadings have been applied, comprising compression, flexion and extension, bending and other, in samples from different origins, as human, pig and bovine. The translation between the techniques established *ex vivo* towards *in vitro* studies is of paramount importance, to better induce pathology in AF models. Nevertheless, considering other applications of mechanical loading *in vitro*, essentially in two-dimensional (2D) approaches, a cyclic tensile strain (CTS) device has been used to stimulate human AF cells, leading to increased inflammation [211, 212] and production of Col I [179]. Another study using cyclic mechanical stretch (CMS) in rat AF cells has successfully shown the induction of cell apoptosis, promoting degenerative disease induction [213]. Yet, one of the studies using CTS, also addressed the impact of

mechanical loading, inflammatory stimulation or the combination of both in human AF cells, with results showing that inflammation induces better AF pathology [179]. Also, moderate mechanical stimulation has shown to revert inflammation in rat AF cells, through pathways related with caveolin-1 and Yes-associated protein (YAP), important in mechanotransduction [214, 215]. Altogether, the current research on the impact of mechanical loading in AF cells emphasizes the importance of including mechanical stimulation when designing *in vitro* 3D study models, to understand its impact on the pathophysiological processes.

Regarding the induction of the inflammatory milieu observed in native disorders, the current AF study models, described in section 3.4., perform stimulation with IL-1 β , LPS and TNF- α to induce disease [142-145, 147, 149-151]. Notably, exogenous administration of IL-1 β and TNF- α stand out as the predominant choice for many studies conducted on IVD tissues due to its central role in driving inflammatory pathways and tissue degeneration [216]. Human AF cells were stimulated with IL-1 β in different studies, evaluating the efficacy of therapies [179, 215, 217-220] and the production of related chemokines [221]. Other animal studies, using porcine, rabbit and rat cells, have also used this pro-inflammatory cytokine to better understand the pathomechanisms of the disease [222-225] and to test therapeutic agents [226-229]. Considering stimulation with TNF- α , similar to IL-1 β , human studies addressed the interplay with other inflammatory mediators [221], the efficacy of therapeutic agents [218] and the deeper characterization of the AF pathomechanisms [230]. The latter was also conducted in bovine and porcine studies using TNF- α [228, 231]. Another important aspect regarding the *in vitro* replication of the inflammatory processes is the duration of stimulation. Inflammation in IVD disorders can become chronic. Nevertheless, the current *in vitro* research performed in general IVD tissues relies on stimulation up to 72h [142, 145, 218, 232, 233]. By example, examining the current *in vitro* AF study models described in section 3.4., only one has a higher duration of inflammatory stimulus (6 days) [143]. It is important that besides selecting the adequate cytokine to replicate inflammation the duration of stimulation is considered and evaluated for longer periods.

Other cell types, besides IVD cells, are also involved in the diseased mechanisms within AF, namely regarding the immune system, neovascularization and nerve system. This factor should also be contemplated upon the establishment of a study model to dissect AF pathology. There are studies reporting the existence of immune resident cells within IVD tissue [234, 235]. Nevertheless, the mechanisms of immune cell recruitment are still unclear and might be related with chemoattractive cytokines and ECM fragments, upon increased inflammation [14]. Human studies have reported increased presence of macrophages in AF tissue upon degeneration, in cervical IVD, with alterations in macrophage polarization [236]

and lumbar IVD [235, 237]. To resemble this *in vitro*, the co-culture of AF cells with immune cells can be addressed, as it is reported in the study models mentioned in section 3.4, that used macrophage-like THP-1 cells and their conditioned media to successfully induce the AF inflammatory microenvironment [145, 146, 148, 151]. The interaction between mast-cells and AF cells has also been addressed in one of the current AF study models, described in Table 3 [152]. Also, 2D co-culture studies with human AF cells and THP-1 cells were conducted, to better understand the disease mechanism and possible therapy targets [238, 239]. Similar studies have been conducted using animal cells and macrophages / macrophage conditioned media [240-244]. In general, most studies addressed monocytes/macrophages, but other immune cells should not be neglected, as, for example, T-cells and dendritic cells have been studied in IVD context [245]. These cells should be included when planning study models, to disclose their participation during AF failure.

Innervation is linked with IVD tissue, with studies addressing its presence within AF tissue [246-249], its importance on maintaining homeostasis, through ECM interactions [250] and its connection with IVD degeneration [251, 252]. Moon et al., while purposing their 3D *in vitro* AF study model, performed a co-culture with human neuroblastoma to evaluate the AF/neuron interactions [150]. Other studies were performed aiming to understand the interplay between the nervous system and AF tissue. Neurite growth was evaluated when in contact with human AF cells or the corresponding conditioned media [253]. The interaction of DRG and AF explants in bovine tissue has also been addressed, under inflammatory stimulation [254]. The impact of *Cutibacterium acnes* infection on nerve growth in AF tissue has also been evaluated in rat cells [255]. Also, the interplay between a degenerative microenvironment and sensory neurons was investigated using rat DRG and bovine explants [256]. It is important to translate the current findings related with innervation in AF tissue, mainly produced in *ex vivo* approaches, towards *in vitro* 3D study models, to systematically characterize the interaction of neurons and AF cells.

Although an avascular tissue, IVD has increased angiogenesis under pathological environmental conditions. A study on degenerated IVD samples showed a significant up-regulation of VEGF compared to control samples [257]. Also, through the study of an anti-angiogenic factor (tenomodulin), researchers have shown that by inhibiting it, IVD degeneration was amplified, with increased migration of human umbilical vein endothelial cells [258]. Therefore, replicating the interactions between AF tissue and endothelial cells is also relevant to better study IVD disorders. As described in section 3.4., Hwang et al. have studied the impact on human NP and AF cells to endothelial cells exposure, upon inflammation, although showing that NP cells exhibit an increased response to these cells, considering migration and invasion [144]. Conditioned media from AF culture under

degenerative microenvironments has been used to culture human endothelial cells, promoting higher expression of invasive biomarkers [259]. Contrarily, endothelial cells conditioned media has also been used to stimulate AF cells, allowing for the study of possible therapeutic agents on mitigating the conditioned media impact on inflammatory outcomes [260]. Human AF cells in 2D studies were cultured with extracellular endothelial microparticles, inducing matrix catabolism through an up-regulation on MMPs [261]. In brief, investing on the inclusion of angiogenic approaches in 3D AF study models is crucial to better understand the vascularization processes within IVD herniation.

3.6. Final remarks

This chapter reviews the current research on *in vitro* 3D models to study AF tissue, while providing a through discussion on the key parameters to take into consideration when proposing a new model (Figure 4). Researchers are still far from the ideal model, as current studies have addressed few of the factors here described and crucial to faithfully replicate AF tissue. In one hand, a biomaterial-based option that meets the requirements for biochemical composition, architecture and mechanical properties is still lacking, as the current options imply a compromise of at least one of these characteristics in order to attempt to fully resemble the other two. In another hand, from the more biological side, the use of human cells together with appropriate microenvironment cues (both in homeostasis and disease) is still far from the native processes. Also, although not the main focus of this

Considerations to design AF *in vitro* Study Models

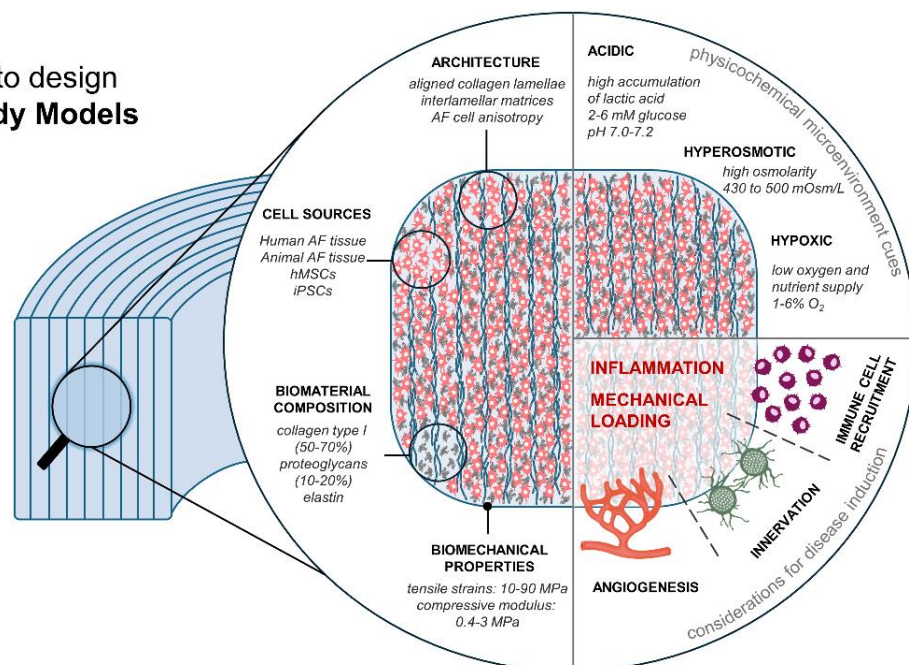


Figure 4 - Key considerations to include when proposing an AF study model

discussion, it should also be noted that upon achieving a reliable *in vitro* AF model, it is imperative to conjugate it with NP and CEP models and address their interaction and their relevance to the study of IVD herniation. Moreover, AF research can benefit intensively from the current advances on biofabrication techniques towards more complexed models, using organ-on-a-chip approaches, for example, to, at the same time, establish different but relevant conditions and understand the impact of distinct players here discussed.

Hence, it is clear that novel models are needed to enhance the current knowledge on AF tissue and provide researchers with better insights on AF failure and IVD herniation, ultimately improving patients' life after diagnosis.

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Chapter II

Aim of the thesis

The main aim of this thesis was to comprehensively enhance the knowledge of the process of IVD herniation with a particular emphasis on AF tissue, through the development of a novel bioengineered 3D study model designed to explore the impact of inflammation on AF pathomechanisms.

For such, this work was strategically divided into three distinct yet interrelated segments:

1. Investigating the fibrotic alterations within AF tissue during the progression of human IVD herniation. For that purpose, a systematic characterization of human tissue samples covering various stages of herniation was performed, employing a multi-faceted approach including structural, biochemical and cellular analysis. The primary goal was to investigate possible connections between these observed fibrotic alterations within AF tissue and the hernia type, thereby enhancing our comprehension of the dynamic changes occurring within this crucial IVD component during IVD herniation.
2. Translating the identified fibrotic cues into an *in vitro* 3D AF study model (Bio3D-AF), using Col I hydrogels and human AF cells. With that intent, disease induction was conducted using acute (48h) vs chronic (7 days) inflammatory stimulation, followed by a comprehensive evaluation of ECM dynamics, pro-inflammatory cytokine and MMPs/TIMPs profile characterization.
3. Continual refinement of the Bio3D-AF previously established, focusing on accurately replicate the complex architecture of human AF tissue. For that end, anisotropic strategies to develop collagen-based aligned hydrogels were explored, aiming to mimic the properties of native AF tissue, facilitating a deeper understanding of herniation pathomechanisms within a more physiologically relevant *in vitro* setting.

Overall, the work here presented not only unravels new perspectives on the diseased mechanism of IVD herniation within AF tissue, while also proposes a novel study model that can recreate the diseased cues *in vitro*. Moreover, this work proposes a step forward on the accurately *in vitro* replication of AF tissue and IVD disorders, namely by the development of an anisotropic collagen-based platform and the induction of chronic inflammation environment. Altogether, the work here presented allows for the enriched understanding of IVD herniation, while holding promise for the design of innovative therapeutic interventions and prognosis targets, supported by the availability of a novel testing *in vitro* study model.

Chapter III

Fibrotic alterations in human annulus fibrosus correlate with progression of intervertebral disc herniation

Fibrotic alterations in human annulus fibrosus correlate with progression of intervertebral disc herniation

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Abstract

Background

Intervertebral disc (IVD) herniation is characterized by annulus fibrosus failure (AF) in containing the nucleus pulposus (NP). IVD herniation involves cellular and extracellular matrix (ECM) alterations that have been associated with tissue fibrosis, although still poorly investigated.

Methods

Here, fibrotic alterations in human AF were evaluated, by characterizing the herniated ECM. Human AF samples (herniated lumbar IVD (n = 39, age 24–83) and scoliosis controls (n = 6, age 15–21)) were processed for transmission electron microscopy and histological/immunohistochemical analysis of fibrotic markers. Correlations between the fibrotic markers in AF ECM and the degree of NP containment (protruded, contained and uncontained) and patients' age were conducted.

Results

Our results demonstrate that with herniation progression, i.e. loss of NP containment, human AF presents less stained area of sulphated glycosaminoglycans and collagen I, being collagen I fibers thinner and disorganized. On the other hand, fibronectin stained area and percentage of α -smooth muscle actin+ cells increase in human AF, while matrix metalloproteinase-12 (MMP12) production and percentage of macrophages (CD68+ cells) remain constant. These structural and biochemical fibrotic alterations observed in human AF with herniation progression occur independently of the age.

Conclusions

The characterization of human AF here conducted evidence the presence of fibrosis in degenerated IVD, while highlighting the importance of considering the herniation progression stage, despite the patients' age, for a better understanding of the mechanisms behind AF failure and IVD herniation.

1. Introduction

Intervertebral disc (IVD) disorders are a major cause of low back pain (LBP) [1], often associated with ageing and degeneration [2,3]. During IVD degeneration, the annulus fibrosus (AF) integrity is compromised and its extracellular matrix (ECM) becomes disorganized, impairing the nucleus pulposus (NP) integrity [1]. IVD bulging may occur, without rupture of the AF, resulting in the so-called contained disc herniation, when NP extrusion is prevented by either the AF or the posterior longitudinal ligament (PLL) (hereby designated by protruded and contained hernias, respectively, following the Lumbar Disc Nomenclature: version 2.0) [4]. When AF and PLL rupture occurs, the NP may be extruded to the epidural space [5,6] (hereby designated by uncontained hernias). IVD degeneration is currently best evaluated through magnetic resonance imaging (MRI) that allows the analysis of both disc morphology and hydration [7], which is clinically assessed by one of the five Pfirrmann grades that classify disc degeneration regarding disc height, structure and signal intensity [7,8]. Nevertheless, the Pfirrmann grade does not relate to pain symptoms and hernia containment.

IVD degeneration is characterized by biochemical and mechanical alterations, with disorganization and degradation of ECM, and chronic inflammation, with increased expression of pro-inflammatory cytokines [9]. Recently, the dysregulation of the IVD ECM during degeneration has been associated to tissue fibrosis [10,11,12]. Fibrosis is a pathological event occurring in several organs, characterized by a dysregulation of conventional tissue repair, in response to chronic inflammation, resulting in ECM remodeling and excessive accumulation of specific ECM components, which may alter the biomechanical properties of the tissue [13]. Particularly, ECM alterations are characterized by an increase in collagen I (Col I) content and fibronectin (FN) deposition, accompanied by matrix disorganization, leading to scar tissue formation and eventually resulting in organ dysfunction [10,14]. Alterations in the concentration and morphology of Col I, collagen II (Col II) and FN have been associated with the progression of human IVD degeneration [11,15]. In human AF (hAF) tissue, a reduction in Col I has been associated with the degeneration level and ageing [1,11,16,17], suggesting a dual effect of both factors in Col I reduction. Col II has also been shown to be reduced in degenerated IVD, compared to healthy IVD [11,18,19] and to increase from low to high degeneration grades (evaluated by Thompson's scale) [15]. The same trend was observed in herniated IVD increasing from protrusion (bulging condition where the AF remains intact) to extrusion conditions [18]. Nevertheless, to the best of our knowledge, fibrotic alterations have not been systematically investigated in human IVD herniation. Myofibroblasts (MFs) and macrophages are the major contributing cells for the progression of fibrosis [13,20]. MFs express alpha-smooth muscle

actin (α -SMA) [21] and are responsible for extensive ECM deposition, namely collagen, within fibrotic tissue, altering tissue architecture and function [21,22,23]. In the IVD, upon injury, MFs have been suggested to be involved in the AF repair process [24, 25]. The presence of α -SMA+ cells in IVD herniation has also been studied, being increased in degenerated and extruded IVDs, both in human and rat [12]. Macrophages regulate fibrosis since they are high producers of transglutaminases and matrix metalloproteinases (MMPs) [21,26]. Their presence in herniated IVDs has been extensively reported [27,28], as well as MMPs, but only MMP12 has been suggested as fibrotic marker for IVD degeneration [12,29].

Here, we hypothesize that AF fibrosis is associated with hernia progression. Therefore, a systematic analysis of structural and biochemical fibrotic markers in hAF from herniated IVDs was performed and correlated the obtained results with the clinical information on hernia containment.

2. Methods

2.1. Study population

Human herniated IVD samples were obtained from patients undergoing microdiscectomy, after informed consent and ethics committee approval, in collaboration with Centro Hospitalar Universitário São João and Hospital CUF (Porto, Portugal). A total of 39 samples (median age 44 (IQR: 35–53 years old)) were dissected to isolate AF tissue (Figure 1A). Samples were characterized considering the medical information provided regarding hernia type: protruded, contained uncontained (Figure 1B). Control discs without visible morphological alterations were collected from adolescent idiopathic scoliosis (AIS) patients undergoing surgery [$n = 6$, median age 16 (15–19.5 years old)]. Age, gender and Pfirrmann distribution of the donors/samples collected are shown in Figure 1C. The individual information for all the samples received is presented in Supplemental Table S1.

2.2. Sample collection, processing and analysis

Human IVD samples were collected and incubated with IVD Medium [Dulbecco's modified Eagle medium (DMEM, Invitrogen) supplemented with 5% fetal bovine serum (FBS, Biowest), 0.5% fungizone (Invitrogen), 1% penicillin/streptomycin (Invitrogen) and 1.5% of 5 M NaCl/0.4 M KCl solution, for osmolarity adjustment to physiological conditions, as previously described [30, 31] and kept at 4 °C overnight after the surgery procedure until further analysis on the following day (total maximum of 18 h). Samples were macroscopically separated into NP and AF tissue. Fixed hAF tissue was processed for transmission electron microscopy (TEM) analysis, using 3 representative samples from

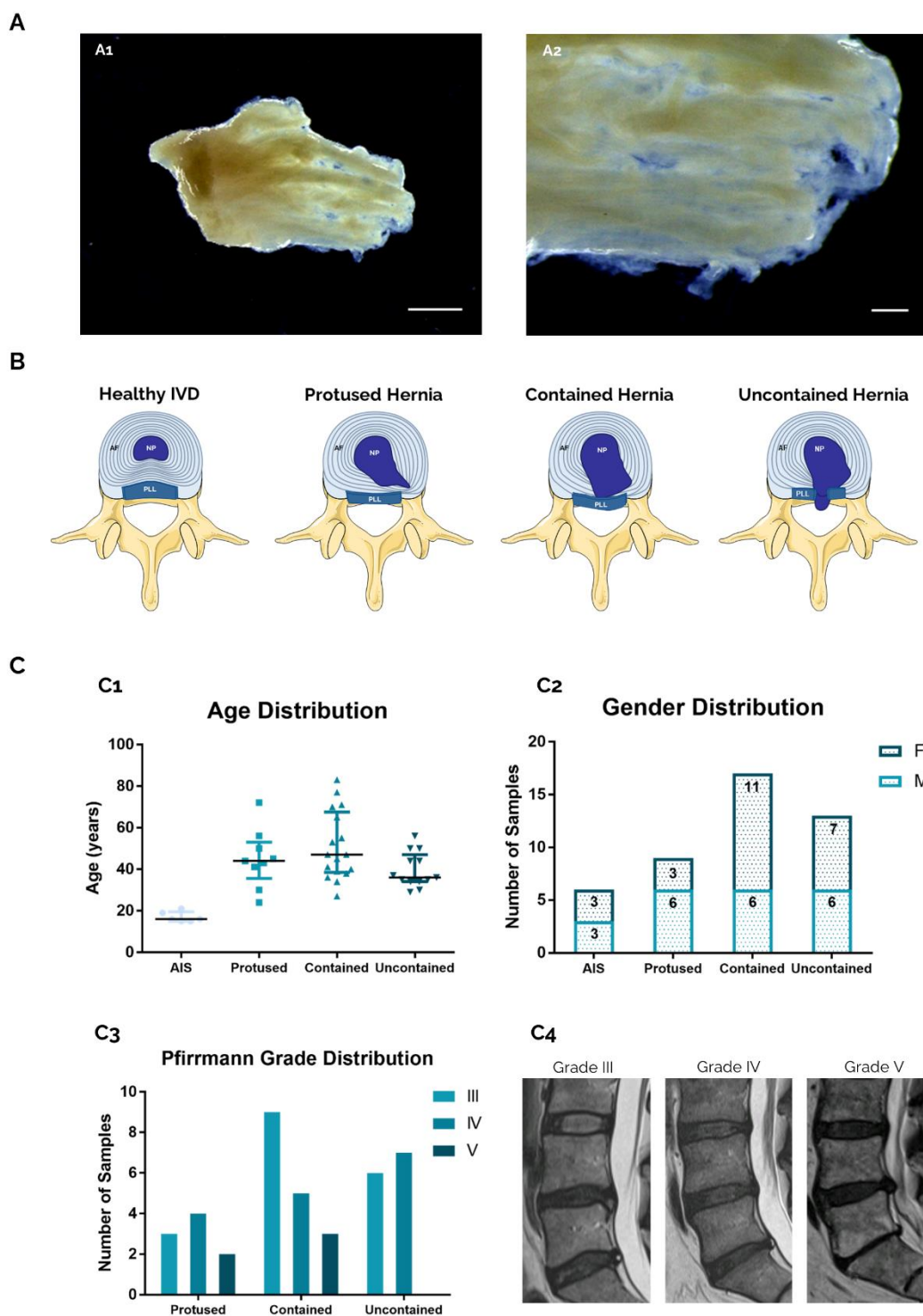


Figure 1 - Human AF tissue obtained from LBP patients. **A**: Native hAF tissue after macroscopic separation of IVD samples. Scale bar: A1—5 mm; A2—1 mm. **B**: Schematic representation of human IVD degeneration/herniation. **C**: Clinical data from hAF donors, from scoliosis and LBP patients distributed by hernia containment type (protused, contained, uncontained). C1: age, C2: gender and C3: Pfirrmann grade distribution; C4: representative MRI images of Pfirrmann grade III, IV and V, all in L5-S1 level

each hernia type condition. Dissected AF samples were fixed (10% neutral buffered formalin), embedded in paraffin and sectioned (3 μ m). Sections were stained for Alcian Blue (AB) and Picro-Sirius Red (PSR) to analyze sulphated glycosaminoglycans (sGAG) (blue)/collagens (red) stained area, respectively. Col I, FN, α -SMA, MMP12, Col II and CD68 expression in AF sections was assessed by immunohistochemistry (IHC), using Novolink™ Polymer Detection Kit (Leica Biosystems), according to manufacturer's instructions. Both optimized antigen retrieval and antibody dilutions are described in Table 1. The protocols used for the different techniques are extensively described in Supplementary Methods.

2.3. Bioimage analysis

Sections were imaged using a Motic 3.0 camera coupled to a Zeiss Axioskop2 microscope (Carl Zeiss International), except for PSR polarization and CD68 images, which were imaged using an inverted fluorescence microscope (Zeiss Axiovert 200 M).

Bioimage analysis was performed using FIJI free software (v2.0.0). For the AB/PSR staining, area quantification was carried on using 5 images (10x magnification) per sample, and a color threshold was applied to separate areas stained in blue/red. For the polarized light analysis, a mosaic was performed and red/yellow/green collagen fibers were quantified as described above. Regarding IHC quantification, for matrix antibodies (Col I, FN and Col II), 5 images per sample (10x magnification) were used for the quantification of the stained area, through threshold application. For cell-specific antibodies (α -SMA, MMP12), 7 images per sample (20x magnification) were used, and positive cells were counted using the Cell Counter plugin of FIJI. To better enlighten this process, a schematic workflow can be found in Figure S1. Finally, for CD68+ area quantification, a mosaic was performed (20x magnification), and color threshold was applied.

2.4. Statistical analysis

Statistical analysis was performed using GraphPad Prism software (v5). Results are presented using median percentage and interquartile ranges. Data normality was assessed using D'Agostino-Pearson normality test. Non-parametric Mann-Whitney tests were performed for comparison between different hernia containment levels. Uni- and multivariate regression analysis was conducted to identify any association between fibrotic markers expression and type of hernia containment. The effect of interaction between age and IVD herniation level on fibrotic markers expression was evaluated in the statistical regression model using statistical software R version 4.0.2 (Project for Statistical Computing). Statistical significance was considered at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

Table 1: Antibodies select for IHC, antigen retrieval procedure, optimized dilution and incubation period

Antibody	Antigen Retrieval	Incubation	Reference / Brand
Collagen type I	10 mM citrate buffer, pH=6.0, 1' MW 1,5 U/mL hyaluronidase (Sigma-Aldrich), 45' 37°C	Dilution: 1:100 Incubation overnight	600-401-103-0.1 / Rockland Immunochemicals, Inc. Limerick, PA
Fibronectin	10 mM citrate buffer (pH=6.0), 1' MW 1,5 U/mL hyaluronidase (Sigma-Aldrich), 45' 37°C	Dilution: 1:50 Incubation overnight	SC-8422 / Santa Cruz Biotechnology
Alpha Smooth Muscle Actin	10 mM citrate buffer (pH=6.0), 1' MW 1,5 U/mL hyaluronidase (Sigma-Aldrich), 45' 37°C	Dilution: 1:400 Incubation overnight	SC-53015 / Santa Cruz Biotechnology
Matrix Metalloproteinase 12	10 mM citrate buffer (pH=6.0), 1' MW 1,5 U/mL hyaluronidase (Sigma-Aldrich), 45' 37°C	Dilution: 1:400 Incubation overnight	SC-390863 / Santa Cruz Biotechnology
Collagen type II	10 mM citrate buffer (pH=6.0), 1' MW 20 µg/mL proteinase K (Sigma-Aldrich), 15' 37°C	Dilution: 1:100 Incubation overnight	SC-52658 / Santa Cruz Biotechnology
CD68	Tris-EDTA buffer (pH=9.0), 1' MW	Dilution: 1:100 Incubation 30'	PG-M1 / Dako

MW – microwave

3. Results

3.1. hAF matrix structural analysis with herniation progression

The hAF was obtained from herniated IVD biopsies collected from patients undergoing microdiscectomy. A macroscopic dissection of IVD samples to isolate the AF tissue was first performed (Figure 1A). hAF from herniated samples was separated into three categories, according to the information provided by the neurosurgeon: protruded, contained and uncontained (Figure 1B). As controls, hAF was obtained from non-herniated IVD of AIS patients [n = 6, median age 16 (15–19.5 years old), 3 males/3 females]. Age, gender and Pfirrmann grade distribution per hernia type can be observed in Figure 1 C1, 1C2 and 1C3, respectively. Gender distribution is similar in the scoliosis and uncontained groups. In contained hernias, almost two thirds of the samples were from females, and in protruded samples, the reverse was observed. Pfirrmann grade distribution among herniated samples shows a low presence of samples with grade V in all categories. In addition, the samples

with Pfirrmann grade III and IV are evenly distributed, except in contained hernias, in which samples with Pfirrmann grade III are more numerous (Figure 1 C3).

ECM ultra-structural alterations in hAF were first analyzed, regarding matrix density, collagen fibers maturation and distribution/orientation. For that, TEM analysis was performed in representative samples from hernias with distinct containment levels (Figure 2A). Highly organized collagen fiber bundles (white arrows), although some organization (white arrows) was still present (Figure 2A, c, g, k). On the other hand, uncontained hernias exhibit loss of lamellar organization of the fibers, being these randomly dispersed within the tissue (red arrows) (Figure 2A, d, h, l). In addition, the analysis of the thickness of collagen fibers was performed by the analysis of PSR staining under polarized light, as previously described [32]. This evaluation allows the identification of collagen fibers thickness, depending on their birefringence under polarized light. The visualization of red and yellow collagen fibers corresponds to thicker fibers, while green correspond to thinner fibers, related with more youthful collagens [33]. This analysis is usually associated with Col I and collagen III (Col III) fibers, although, in this study, the fibers are most probably associated with Col I, since Col III is not abundant in AF and is described only as a pericellular protein within the tissue [34,35]. Nevertheless, this analysis should be performed using second harmonic microscopy [36]. Figure 2B shows representative images of hAF samples stained with PSR and imaged under polarized light, while Figure 2C shows the proportion of the collagen fibers (in red, yellow and green) for the different conditions analyzed. Results clearly show a lower frequency ($< 7\%$) of the red birefringence (thicker fibers) for all the samples, in comparison with the more mature fibers (yellow/green birefringence). Moreover, fibers with red birefringence were reduced in the herniated samples, compared with AIS. On the other hand, the thinner fibers (green) were present in higher proportion (38–86%) in all the conditions analyzed, while the frequency of collagen fibers with intermediate thickness (yellow) range from 12 to 60%. Together with thicker fibers, the intermediate size collagen fibers are also reduced in all herniated samples compared with AIS control, more specifically in protruded hernias (12%, $**p < 0.01$). These fibers slightly increase in contained hernias (30%, $p = 0.0718$) compared with protruded hernias.

In what concerns thinner collagen fibers, an opposite trend is observed. In this case, the AF from all herniated discs present a higher proportion of green fibers, compared with AIS discs, being this significantly higher in the case of protruded hernias (87.85%, $**p < 0.01$). With herniation progression, the presence of these fibers is slightly reduced (69.35%, $p = 0.0656$ contained hernias). These results suggest an increased synthesis of new collagen fibers in the AF from herniated discs, compared with AIS controls, particularly in protruded hernias.

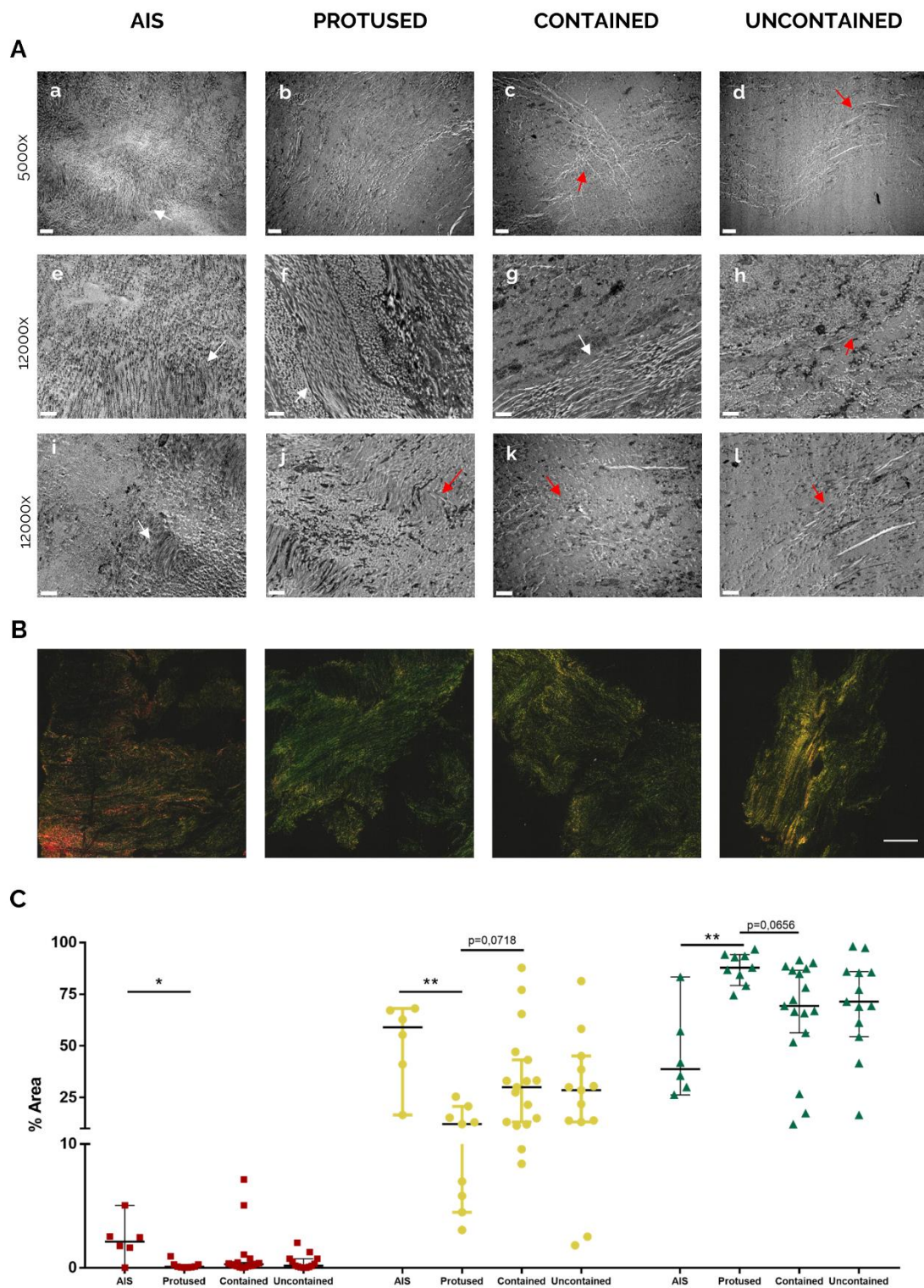


Figure 2 - Human AF matrix ultra-structural characterization with herniation progression (n = 3).

A: a–d: 5000x magnification, scale bar: 2 μ m; e–l: 12,000x magnification, scale bar: 1 μ m. White arrows point to well organized collagen fibers, while red arrows point to disorganized fibers. **B:** Picro-Sirius Red staining under polarized light, scale bar: 500 μ m. **C:** Quantification of collagen fibers per birefringence color. Kruskal-Wallis test followed by corrected Dunn's were performed.

*p < 0.05; **p < 0.01

3.2. hAF matrix biochemical analysis with herniation progression

A biochemical analysis of the ECM of hAF was performed by histological/IHC analysis of Col I, Col II, FN and sGAG, generally lost during fibrosis (Figure 3A) (low magnification images are presented in Supplementary Figure S2). From the images, the percentage of staining area was quantified and the results are presented as the median percentage of area or positive cells and respective interquartile range for each marker (Figure 3B). Figure 3A (a–d) shows representative images for sGAG staining (blue) in the hAF of different samples. By AB/PSR staining, it can be observed a clear higher stained area of sGAG (blue) in hAF from most AIS samples [77.1% (43.8–90.6)] compared to all herniated samples, particularly in contained hernias [7.1% (2.1–21.3), $p = 0.0515$] and uncontained hernias [1.78% (0.2–81.9), $p = 0.0555$].

Regarding the expression of Col I (Figure 3A (e–h) and 3B), it is highly present in AF tissue from AIS samples (Figure 3A, e) [84.0% (65.4–98.1)] and significantly decreased in the AF of all herniated samples [8.0% (3.1–16.0), $**p < 0.01$, in protruded hernias; 11.9% (2.2–49.7), $**p < 0.01$, in contained hernias; and 13.5% (1.4–47.4) $*p < 0.05$, in uncontained hernias] (Figure 3A, f–h). Col II stained area was also assessed (Figure 3A (i–l) and 3B), showing an opposite trend of Col I. The stained area of Col II is lower in hAF from AIS samples [20.5% (3.5–35.8)] (Figure 3A, i) increasing in hAF from herniated samples (Figure 3A, j–l). This increase is close to significant in hAF of contained hernias [65.4% (32.8–82.5), $p = 0.0717$] and uncontained hernias [68.9% (26.8–88.8), $p = 0.0976$].

Relatively to FN expression (Figure 3A (m–p) and 3B), the results show low and heterogeneous FN expression in protruded hernias [0.5% (0.04–15.8)], with 2 (out of 9) samples showing higher FN stained area (about 25.8 and 30.3%). Nevertheless, FN expression tends to increase in hAF from herniated IVD, being significantly higher in contained hernias samples [23.5% (3.2–59.0), $*p < 0.05$], and in uncontained samples [14.8% (6.6–42.8), $p = 0.0516$].

In addition, to discard that the differences observed might be due to age differences, the interaction between the variables “age” and “hernia containment” for all the markers analyzed was addressed using a multivariate analysis. The p values obtained (Figure 3C) demonstrate an absence of interaction between the variables “age” and “herniation progression” ($p > 0.05$) for sGAG, Col I, Col II and FN presence in hAF, reinforcing that the differences in the biochemical composition of hAF ECM with herniation progression are not due to age differences between the different donors.

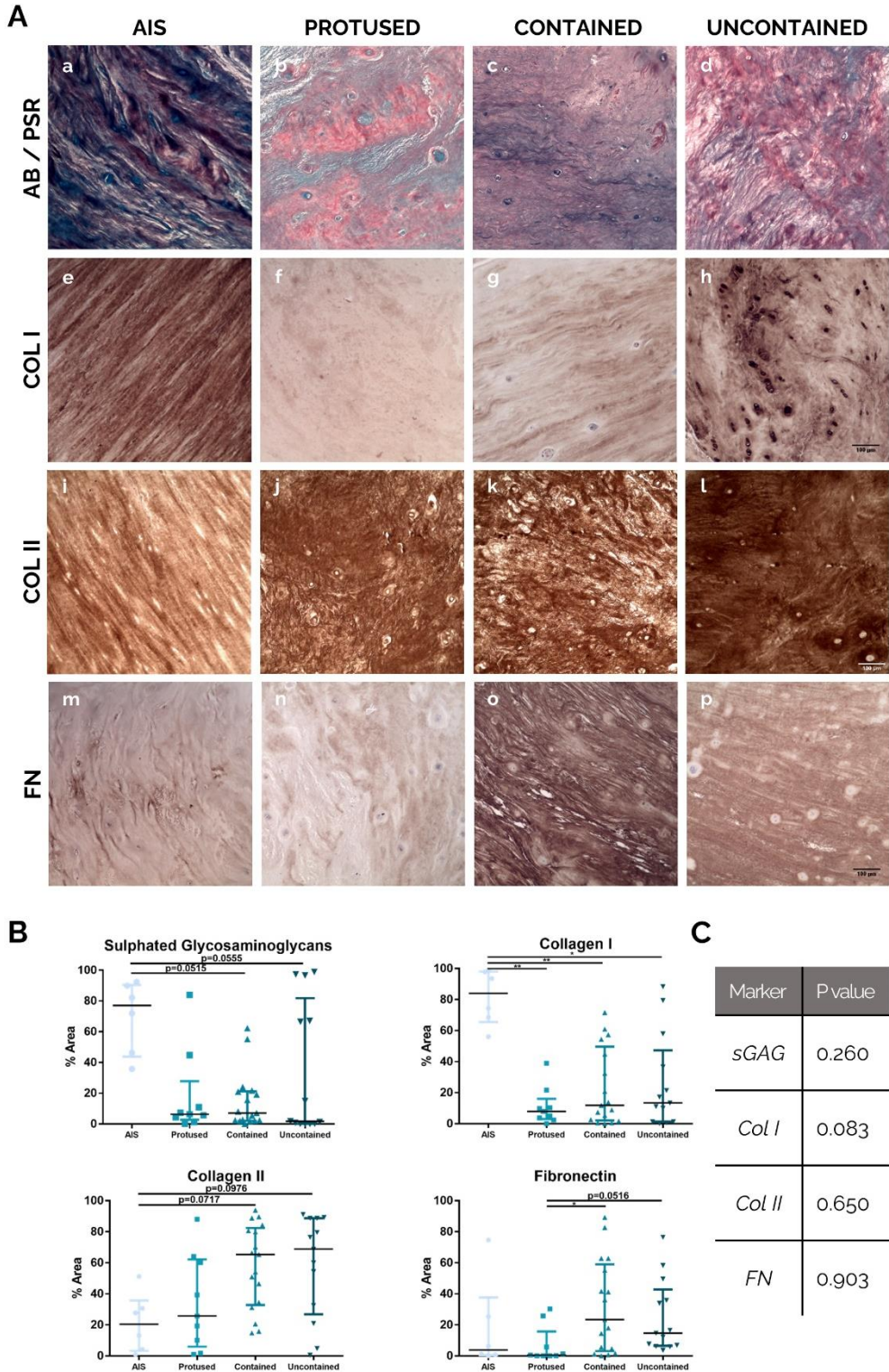


Figure 3 - Human AF matrix biochemical characterization with herniation progression. **A:** Histological/IHC staining for a–d: Alcian Blue/Picro-Sirius Red, scale bar: 100 μ m; e–h: Collagen I, scale bar: 100 μ m; i–l: Collagen II, scale bar: 100 μ m; m–p: Fibronectin, scale bar: 100 μ m. **B:** Quantification of each staining per herniation type. Data presented using dot plots, with median and interquartile range. Kruskal-Wallis test followed by corrected Dunn’s were performed. * $p < 0.05$; ** $p < 0.01$. **C:** Multivariate analysis of interaction of hernia containment level with age for each staining

Moreover, for each herniation stage (protruded, contained and uncontained), a linear regression between hAF ECM stained area and donor age was performed (Figure 4). With this analysis, it is possible to verify a positive correlation for sGAG and Col I with ageing only in the group of hernias contained by PLL ($r^2 = 0.2346$, $p = 0.0488$ for sGAGs and $r^2 = 0.259$, $p = 0.037$ for Col I, respectively), suggesting that, within this herniation containment level, Col I and sGAG increase with ageing.

3.3. hAF fibrotic analysis with herniation progression at the cell level

Additionally, relevant cellular markers to tissue/IVD fibrosis (α -SMA+ cells, MMP12+ cells and CD68) were also evaluated in hAF samples by IHC analysis.

The α -SMA+ cells were absent from hAF of AIS samples (Figure 5A (a–d) and 5B), as well as in a high percentage of herniated samples (in 33.3% of the protruded hernias, 47% of the contained hernias and 38.5% of the uncontained hernias), suggesting a high heterogeneity of the α -SMA expression in hAF. Nevertheless, in the samples presenting α -

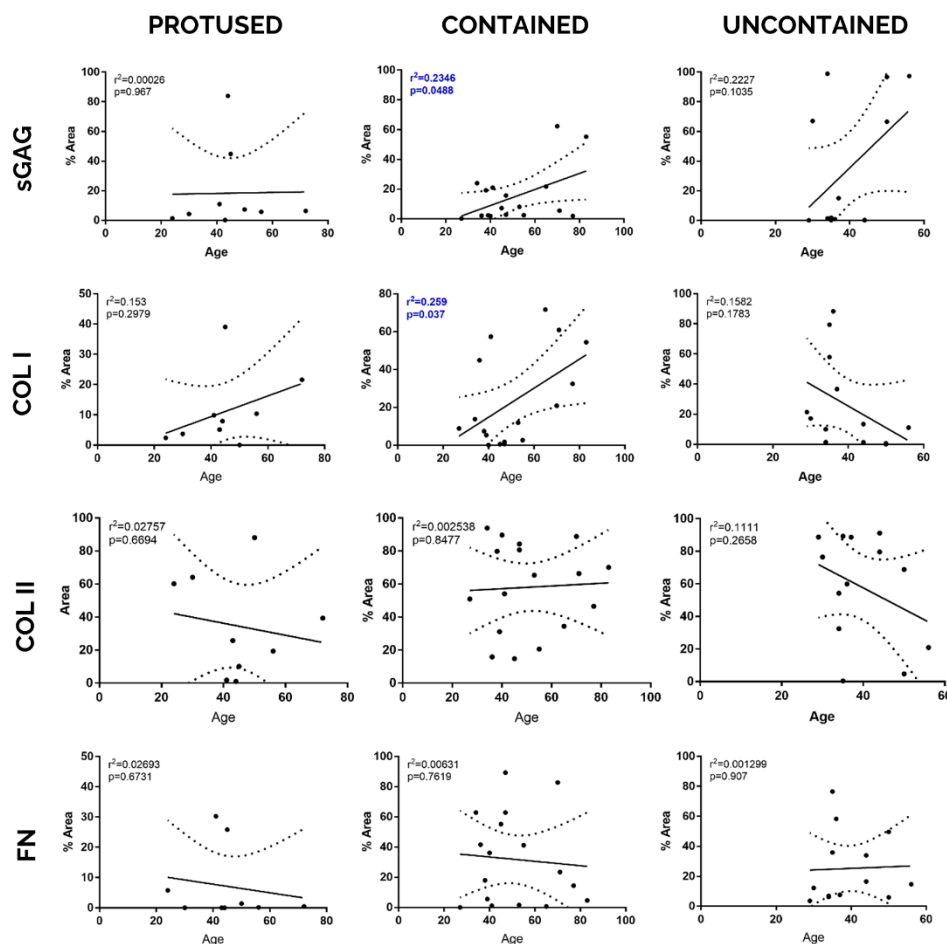


Figure 4 - Correlation of expression levels of hAF matrix components with age, within each herniation type. Data presented using dot plots, with 95% confidence intervals and indication of r^2 and p value for each linear regression

SMA+ cells, an increased expression of α -SMA in the AF from herniated tissues was observed, ranging from 2.27 to 94.71%, when compared to AIS samples.

In what concerns MMP12 (Figure 5A (e–h) and 5B), another marker associated with IVD fibrosis, heterogeneity was also observed, since MMP12+ cells were absent in 33.3% of AIS samples, in 11.1% protruded hernias, in 5.9% of contained hernias and in 9.1% of uncontained samples. No differences were observed regarding the presence of MMP12 in the different herniated conditions, with most samples exhibiting MMP12+ cells below 50%.

Furthermore, macrophage presence in hAF samples was also assessed by the expression of CD68+ cells (a common marker used for macrophages) (Figure 5A, i–l, and Figure 5B). CD68+ cells were observed in 33% of AIS samples, in 24% of contained hernias and in 11.7% of uncontained samples, but not in protruded hernias (Figure 5A, i–l). Macrophages were present in AF tissue both, dispersed (Figure 5A, k) and agglomerated (Figure 5A, l), the last predominantly in hAF tissue borders (Figure 5A, l). Due to this observation, macrophage infiltration area was quantified (Figure 5B), instead of percentage of positive cells. Nevertheless, the quantification of CD68+ area revealed no significant differences between hernia conditions.

As previously described for ECM markers, the interaction between the variables “hernia containment” and “age” was addressed for α -SMA expression using a multivariate analysis (Figure 5C), but no interaction was found between the two variables (p value = 0.291). Moreover, no linear correlations were found between the increase of α -SMA expression with ageing for each hernia containment group.

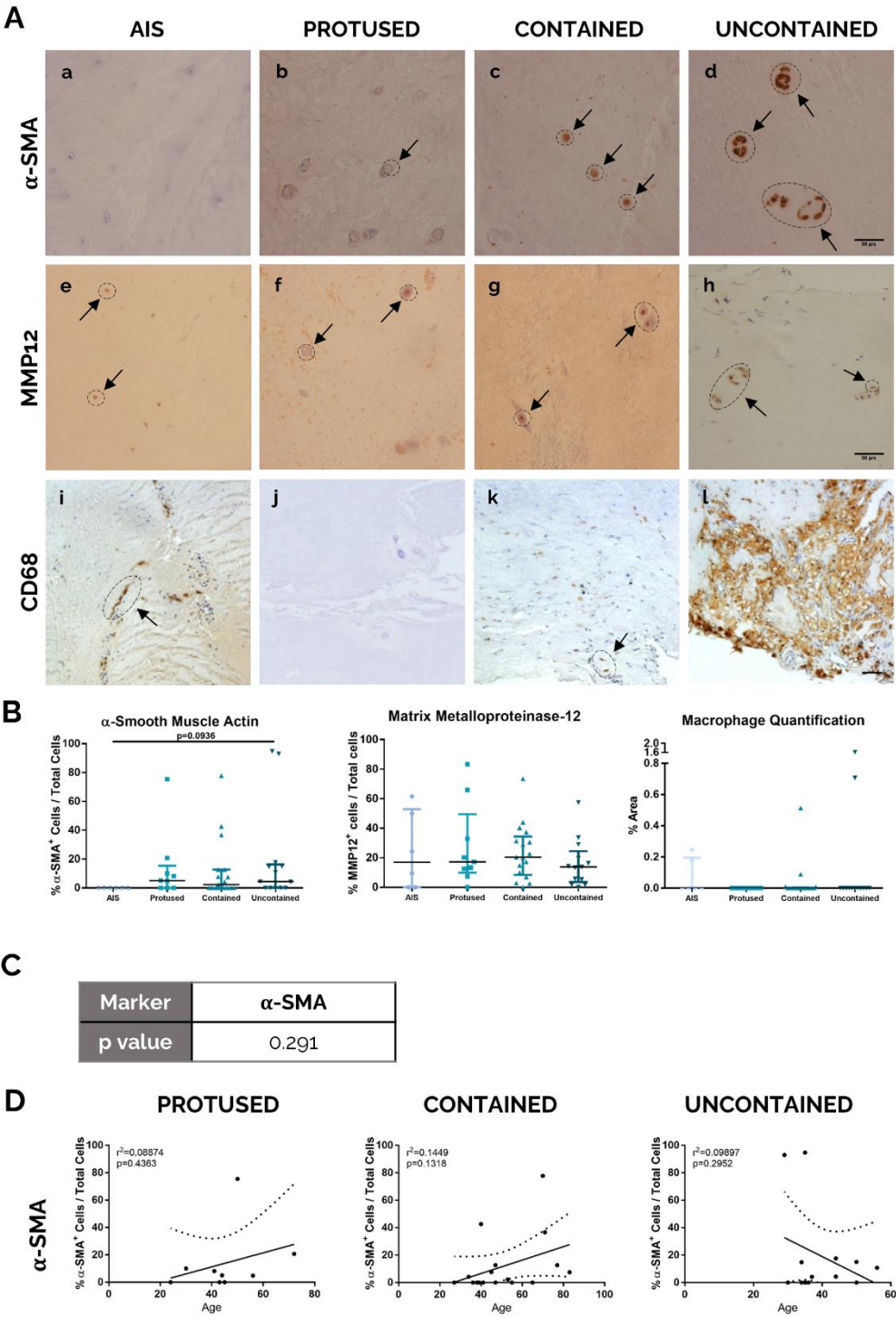


Figure 5 - Human AF fibrotic analysis at the cell level with herniation progression. **A:** IHC staining for a-d: α -SMA, scale bar: 50 μ m; e-h: MMP12, scale bar: 50 μ m; i-l: macrophages (CD68) scale bar: 50 μ m. **B:** Quantification of each staining per herniation type. Data presented using dot plots, with median and interquartile range. Kruskal-Wallis test followed by corrected Dunn's were performed. * $p < 0.05$; ** $p < 0.01$. **C:** Multivariate analysis of interaction of hernia containment level with age for α -SMA. **D:** Correlation of α -SMA staining quantification with age for each herniation type. Data presented using dot plots with 95% confidence intervals and indication of r^2 and p value for each linear regression

4. Discussion

The AF plays a functional and crucial role in IVD homeostasis since it is responsible for the containment of the NP, preventing its extrusion. A pro-inflammatory environment and extensive mechanical stresses can weaken AF mechanical properties [37], ultimately leading to IVD herniation. But the mechanisms behind IVD herniation and AF disruption remain unclear. The IVD, and particularly the AF ECM, undergoes structural, biochemical and biomechanical alterations that have been studied in different models, as canine, rabbit, mice or bovine [38,39,40,41]. IVD degeneration has been associated with tissue fibrosis [10,11,12], but this has been poorly addressed. It is crucial to understand tissue fibrosis in the IVD, so we could disclose novel cellular and molecular cell targets to stop/revert IVD herniation. Taking this into account, this study aimed to evaluate the fibrotic alterations of AF with hernia progression (here referred to the medical evaluation of hernia containment levels).

Currently used in IVD clinical evaluation, Pfirrmann's MRI-based classification lacks suitability to assess herniation progression, among other problems [7, 8]. Although considering the structure, brightness and height of the disc [8], Pfirrmann grading system does not distinguish disc herniation progression. For example, in this study, only 5 samples were described as Pfirrmann grade V, characterized by the collapse of IVD to the epidural space [8], but 13 samples were identified as extruded to the epidural space by the surgery team. It is important to combine different classification systems that embrace both image analysis and clinical evaluation, to a more accurate definition of each herniation condition. In the future, systemic markers would be important to improve the accurate diagnosis of IVD herniation progression stages.

The ECM of hAF was first analyzed regarding its ultra-structure and biochemical composition. Matrix disorganization and impairment in the AF tissue are characteristics of IVD degeneration and herniation. As expected, in the most severe herniation stage, a more disorganized matrix was found. A low percentage of thicker (more mature) collagen fibers (red) was revealed in all samples. A higher amount of thinner collagen fibers (green birefringence) present within the herniated tissue was observed, suggesting recent ECM remodeling.

Regarding ECM biochemical alterations of hAF, this work used a panel of fibrotic ECM-related markers (Col I, Col II, FN, sGAG) and compared AF from herniated IVD with AF from AIS patients, as control group, due to the impossibility of obtaining human adult healthy IVD. A tendency for lower sGAG stained area in AF tissue from contained hernias and uncontained compared to AIS samples was observed. These changes are in line with the

loss of sGAGs previously reported for IVD degeneration in human, as well as in canine and rabbit models [16, 41,42,43]. Regarding Col I, a decreased expression in herniated IVD was observed compared with AIS samples but increasing with herniation progression. The reduction of Col I stained area in AF from herniated tissue, together with increased disorganization of the fibers may contribute to an increase in AF susceptibility to rupture, impairing NP confinement and allowing herniation [1]. A reduction in Col I in AF tissue has been associated with ageing [1, 11, 16], and with the degeneration level in both goat [44] and human (*in vitro* study) [17].

Col II stained area in the AF showed a trend to increase in the most herniated tissues, in opposite to Col I, suggesting ECM turnover regarding the collagen composition of the AF. It is possible that this ECM turnover is also accompanied by alterations in the mechanical properties of the tissue and, therefore, it may become more susceptible to herniation, since Col II has a smaller elastic modulus than Col I [45]. Further biomechanical tests should be conducted to evaluate this possibility. Nevertheless, the literature describes a reduction of Col II from non-degenerated to degenerated or herniated IVD, both in AF tissue [11] or whole IVD [18, 19]. In fact, there is a lack of knowledge of the herniation containment level in most of the literature. Additionally, FN stained area was shown to increase from hernias protruded to more severe herniation stages. This is in accordance with the literature, in which FN showed to increase from low to high degeneration grades (evaluated by Thompson's scale) [15], as well as between protrusion and extrusion [18], both in human IVD.

Concurrently, hAF fibrotic markers were also evaluated at the cellular level. In the current work, α -SMA+ cells were absent in all AIS samples, suggesting MF activity in AF tissue only during IVD herniation. α -SMA+ cells have been previously reported to increase from non-degenerated to degenerated IVD, both in human and rat [12]. Hastreiter et al. reported an increase percentage of α -SMA+ cells in extruded tissues, when compared to scoliosis samples [46]. In our study, most of the samples present low levels of α -SMA+ cells (< 20%), in agreement with previous reports (4–15% of α -SMA expression) [46]. However, some heterogeneity between the samples was observed, suggesting that, although a reliable marker for fibrosis in several tissues, α -SMA might not be optimal for IVD herniation.

We have also investigated the expression of MMP12, a pro-fibrotic marker upregulated under inflammatory conditions [12]. MMP12 has been associated with IVD degeneration in a rat model [29] and in human AF [12]. However, although Lv et al. suggested MMP12 as a degeneration marker [12], our study did not show any correlation between MMP12 and the progression of IVD herniation, since MMP12+ cells were observed in most of control

samples and absent in some of the herniated samples. Moreover, although MMP12 has been associated with α -SMA, herein our results indicate higher expression of MMP12 than α -SMA [12]. Lastly, we also evaluated the presence of macrophages, important players in tissue fibrosis. We observed macrophage infiltration in a small number of samples and in a low percentage ($< 2.0\%$), distributed within the outer layers of the AF. Moreover, although macrophages were found in some AIS samples, these cells were not present in protruded hernias. Previous studies detected CD68+ macrophages in the AF of pig, mice and human [27, 28, 47]. Gronblad et al. highlighted their abundant presence in AF of human herniated tissue, but without analyzing differences between hernia types [48]. Our findings are in agreement with previous reports where macrophages were detected in herniated IVD and are believed to be key players in hernia resorption through phagocytosis [3, 49]. Additionally, an evaluation of a possible interaction of age within the comparisons regarding hernia containment was conducted, revealing no influence of this factor in the observed differences. However, no differences were observed between different herniated conditions suggesting that the ECM fibrotic alterations do not correlate directly with the frequency of macrophages present on hAF, indicating that other players might be involved.

This study presents some limitations. The use of thoracic/lumbar discs collected during anterior release surgeries for AIS may not be the ideal control for lumbar discs collected from patients operated for herniated lumbar discs. As in practice IVD samples from healthy donors are almost inaccessible, AIS samples were the most suitable option for control group. However, it has to be considered that although MRI analysis suggests that the NP from scoliosis patients retains in normal conditions of water, the IVD of these conditions might be under abnormal spine load and effects on AF tissue are still not well defined [12, 50]. AIS discs have been described to present sparse elastic fibers with some disorganization in collagen and elastic fiber networks, loss of lamellar structure in the AF and presence of cell clusters [51]. In addition, AIS AF presents a decrease in elastin and Col I and decrease of Col III and Collagen IV [52]. Therefore, caution should be taken when analyzing the results since some differences can either be covered or less attenuated [12]. Moreover, a reduced sample size of different hernia types might influence the statistical power of comparisons, but still it was possible to observe significant differences between parameters analyzed in the hAF tissues. Although our conclusions have shown to be independent of the age of the patients, we cannot exclude the influence that other external factors (gender, body mass index, smoking habits or other co-morbidities) might have in the analysis. In the future, it would be relevant to address other fibrosis markers for a better understanding of AF failure in IVD herniation.

5. Conclusions

To the best of our knowledge, this is the first study that characterizes fibrotic events in the AF tissue of herniated IVD in a systematic way, at distinct stages of hernia containment. This study suggests important alterations in the hAF tissue during herniation that have been associated with tissue fibrosis. Structural changes were observed in the distribution of collagen fibers in herniated AF, in particular their lack of organization in uncontained samples. The stained area of sGAG and Col I were decreased in all the containment levels of herniated AF when compared with AIS AF, while FN and Col II were increased in AF of more advanced stages of IVD herniation. Moreover, α -SMA tends to increase in the AF of uncontained hernias. Hence, our work indicates herniation progression stage as an important parameter to understand the process of tissue fibrosis in hAF and consequently, of AF failure and IVD herniation.

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Ethics Declaration

Ethics approval and consent to participate

This study was conducted after patients' informed consent and approval from local ethics committee approval (2018/05 Ulm University, Germany and Centro Hospitalar Universitário S. João, Porto, Portugal, 29-11-2011).

Consent for publication

All authors have read and reviewed the final draft of the article and approve its submission for publication.

Competing interests

The authors declare that they have no competing interests.

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Supplementary Data

Methods

Transmission electron microscopy analysis

Fixed hAF tissue was processed for TEM analysis. Samples were washed in PBS and fixed in 2.5% glutaraldehyde (2h) and 2% paraformaldehyde in 0.1 M sodium cacodylate (pH 7.4). After a 30 min wash in 0.1 M sodium cacodylate buffer, the tissue was fixed in 2% (v/v) osmium tetroxide in 0.1 M sodium cacodylate overnight followed by fixation (1% uranyl acetate) overnight. Samples were dehydrated in a gradient series of ethanol solutions: 50% ethanol (10 min), followed by 70%, 80%, 90%, 96%, 100% and propylene oxide (v/v). Further, samples were included in EPON resin in a silicon mold, using gradually increasing series of propylene oxide to EPON as follows: 2:1, 1:1, 1:2 and 0:1 (60 min each). EPON polymerization was performed at 60 °C (48h). Samples were cut (50nm sections) using a diamond knife (Diatome, Hatfield, PA, USA) and recovered to 200 mesh Formvar Ni-grids. Prior to observation, sections were stained with 2 wt% uranyl acetate and saturated lead citrate solution (7 min each). Visualization was performed at 80 kV in a JEOL JEM 1400 microscope (Japan) (5000x and 12000x magnifications). 15-20 images for each sample were analyzed (n = 3).

Histological analysis

Dissected AF samples were fixed (10% neutral buffered formalin), embedded in paraffin and sectioned (3 µm). Sections were stained AB/PSR to analyze sGAG (blue)/collagens (red) content, respectively. Briefly, samples were dewaxed, rehydrated and incubated in AB solution (pH=1.0, 30 min), washed in tap water, and stained in PSR solution (1 h). Afterwards, a final wash in acidified water (0,5% acetic acid) (30 s) was performed and sections were cleared and mounted with Entellan (Merck).

Immunohistochemistry

Col I, FN, α -SMA, MMP12, Col II and CD68 expression in AF sections was assessed by IHC, using Novolink™ Polymer Detection Kit (Leica Biosystems, Newcastle, UK), according to manufacturer's instructions. Both optimized antigen retrieval and antibody dilutions are described in Table 1. Briefly, for each staining, slides were dewaxed and rehydrated, followed by antigen retrieval and an endogenous peroxidase and protein block (5 minutes each). Next, sections were incubated with the primary antibody, under the conditions described in Table1. Slides were then incubated in post-primary solution and Novolink™ Polymer (30 min each). A final incubation with peroxidase-substrate DAB

solution (5 minutes) in the dark allowed the revelation of bound antibodies, followed by a hematoxylin staining. Slides were mounted with Entellan.

Tables**Table S1:** Donor information regarding gender, age, Pfirrmann scale and condition

ID	Gender	Age	Pfirrmann	Disc Level	Condition
1	F	45	4	L5-S1	Protruded
2	F	30	4	L5-S1	Protruded
3	F	44	4	L5-S1	Protruded
4	M	50	3	L4-L5	Protruded
5	M	43	3	L4-L5	Protruded
6	M	41	3	L4-L5	Protruded
7	M	24	4	L4-L5	Protruded
8	M	72	5	L4-L5	Protruded
9	M	56	5	L5-S1	Protruded
10	F	50	3	L4-L5	Uncontained
11	F	34	4	L5-S1	Uncontained
12	F	50	3	L5-S1	Uncontained
13	F	29	4	L5-S1	Uncontained
14	F	44	3		Uncontained
15	F	37	4	L5-S1	Uncontained
16	F	44	4	L5-S1	Uncontained
17	M	30	3	L5-S1	Uncontained
18	M	56	3	L2-L3	Uncontained
19	M	34	3	L4-L5	Uncontained
20	M	35	4	L5-S1	Uncontained
21	M	35	4	L5-S1	Uncontained
22	M	36	4	L5-S1	Uncontained
23	F	40	4	L5-S1	Contained
24	F	70	3	L4-L5	Contained
25	F	47	5	L4-L5	Contained
26	F	45	3	L4-L5	Contained
27	F	55	3		Contained
28	F	71	3	L4-L5	Contained
29	F	36	3	L4-L5	Contained
30	F	41	4	L4-L5	Contained
31	F	39	5		Contained
32	F	27	3	L5-S1	Contained
33	F	65	4	L3-L4	Contained
34	M	38	3	L4-L5	Contained
35	M	83	5	L4-L5	Contained
36	M	34	3	L5-S1	Contained
37	M	47	3	L5-S1	Contained
38	M	77	4	L5-S1	Contained
39	M	53	4	L4-L5	Contained

40	F	16	-	AIS
41	F	21	-	AIS
42	F	16	-	AIS
43	M	15	-	AIS
44	M	15	-	AIS
45	M	19	-	AIS

F- female; M- male;

Figures

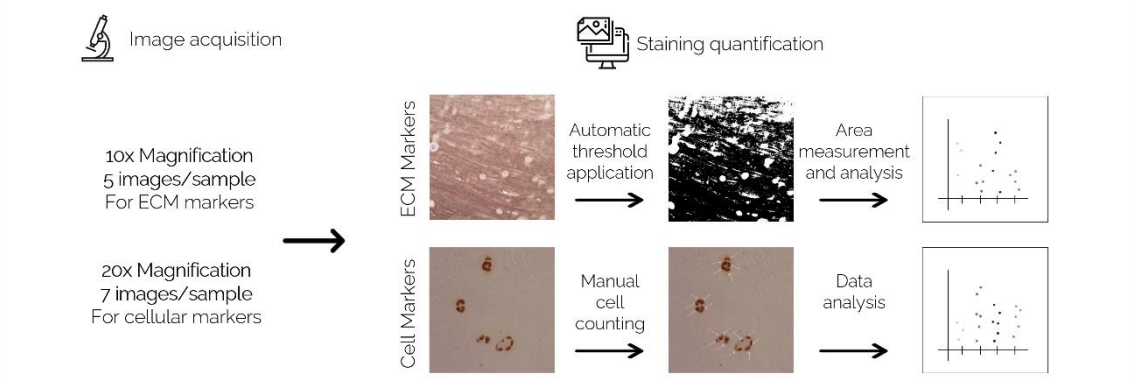


Figure S1 - Image analysis workflow. Schematization of the image analysis process, for both ECM and cellular markers

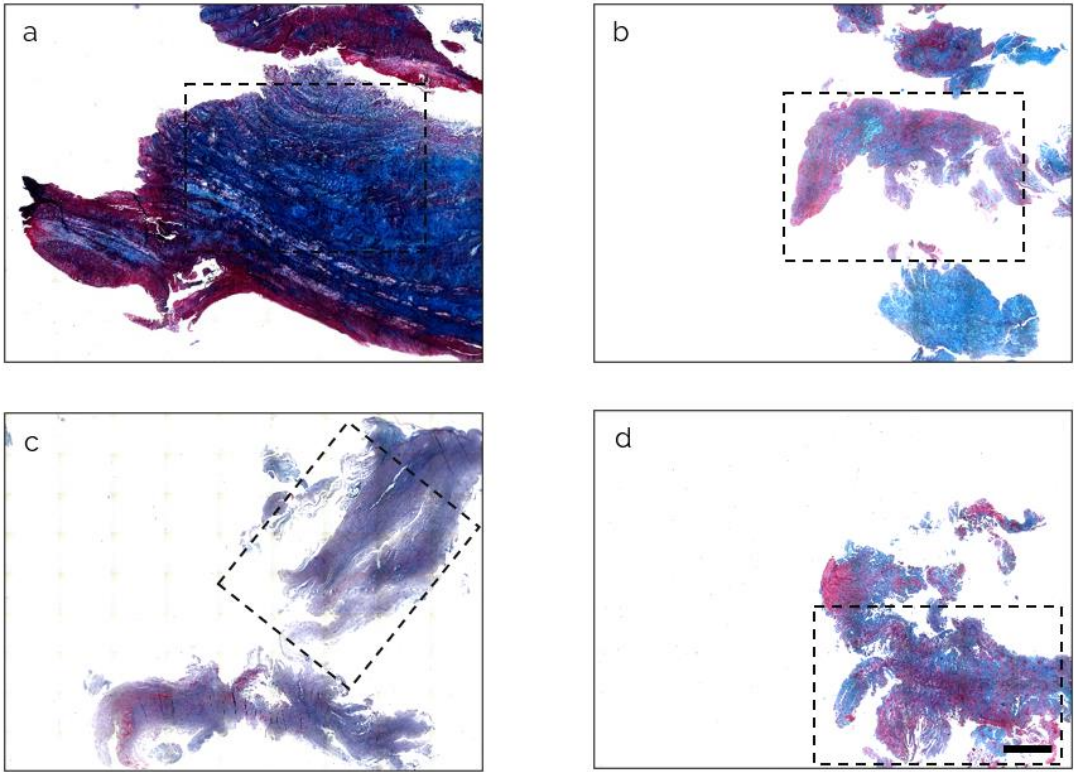


Figure S2 - Human AF mosaic representation with histological staining Alcian Blue / Picro-Sirius Red. Distinct hernia containment levels are presented, as follows (a) AIS, (b) Protruded, (c) Contained and (d) Uncontained. Rectangles indicate the area where staining analysis was further conducted. Scale bar: 500 μ m

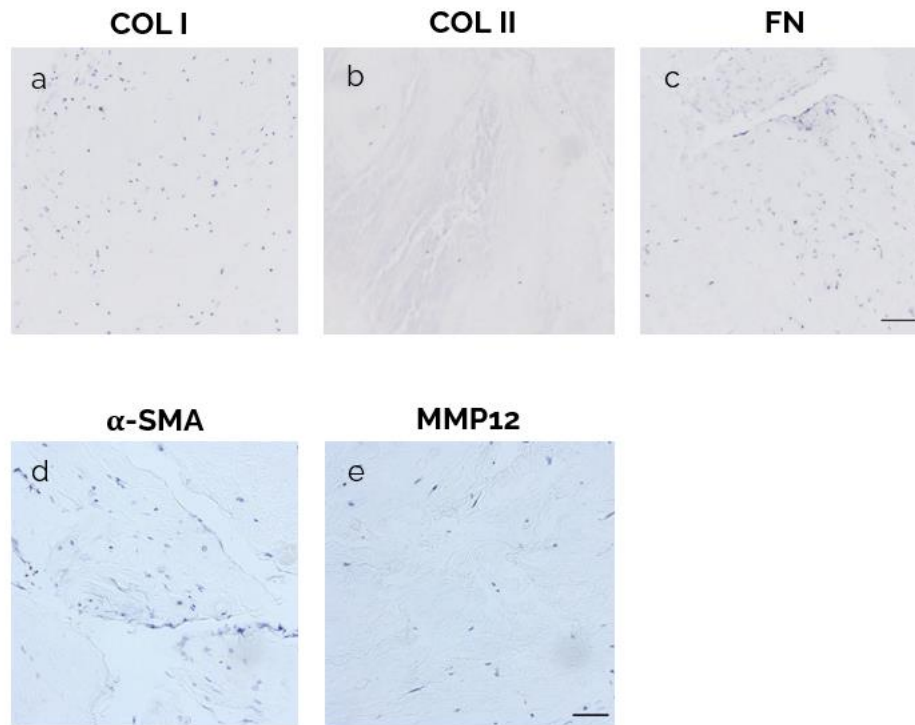


Figure S3 - Example representation of negative IHC controls for each staining. (a) Collagen I; (b) Collagen II; (c) Fibronectin, scale bar: 100 μ m; (d) α -SMA; (e) MMP12, scale bar: 50 μ m.

Chapter IV

Unveiling Inflammation Dynamics in Human Annulus Fibrosus Degeneration/Herniation through 2D and 3D models

Unveiling Inflammation Dynamics in Human Annulus Fibrosus Degeneration/Herniation through 2D and 3D models

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In preparation

Abstract

Intervertebral disc (IVD) herniation involves failure of the annulus fibrosus (AF) under chronic inflammation, related with AF extracellular matrix (ECM) disorganization and tissue fibrosis, among other alterations, in a process still under investigation. More specifically, human AF (hAF) degeneration and fibrosis, under inflammatory conditions, has been poorly addressed and mostly under acute inflammatory stimulus.

Here, we explored the role of inflammation progression in human AF cells and also compared two-dimensional (2D) and three-dimensional (3D) microenvironments, to improve the knowledge on AF failure in IVD herniation. For that, hAF cells from herniated IVD of patients with low back pain (LBP) were cultured in 2D and 3D, embedded in collagen type I (Col I) hydrogels for 7 days, in: basal and inflammatory (IL-1 β 10 ng/mL) conditions, the latter divided in acute (2 days) and chronic (continuous) conditions. hAF cell morphology analysis revealed alterations in actin filaments only in 3D, under both acute and chronic inflammation. hAF ECM deposition, namely Col I and Fibronectin (FN), was reduced with inflammation progression only in 3D, with chronic inflammation promoting a recovery in FN production as observed in human herniated IVD samples. hAF cell secretion profile showed that chronic inflammation promoted the expression of matrix-degrading enzymes, both in 2D and 3D, without significant differences, while for the production of pro-inflammatory cytokines/chemokines, 3D cultures evidenced that granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-1 receptor antagonist (IL-1RA), IL-10 and macrophage colony-stimulating factor (M-CSF), were significantly increased under chronic inflammation. *In vivo*, *in ovo* experiments demonstrated that hAF cells under chronic inflammation in 3D, promoted increased immune cell infiltration.

Altogether, this work highlighted the importance of 3D architecture to better mimic hAF and its degeneration, namely ECM alterations. Moreover, this study demonstrated key differences in hAF with a chronic inflammatory stimulus, compared with acute, namely in immune cell recruitment and specific inflammatory mediators. This represents a step further in the comprehension of the pathomechanism of AF failure and proposes new disease targets and prognosis markers for IVD herniation.

1. Introduction

Lumbar disc herniation is the main cause of spine surgery worldwide and one of the major contributors for low back pain (LBP), the leader disorder in years lived with disability, affecting 1-3% world population per year. LBP is a multifactorial condition, which the main cause associated has been intervertebral disc (IVD) degeneration, and includes different pathologies, including IVD herniation [1]. In 2020 LBP accounted more than 600 million cases estimating an increase up to 800 million by 2050 [2].

The IVD degenerative process, that culminates with IVD herniation, is characterized by alterations at biochemical and mechanical levels, including extracellular matrix (ECM) disorganization and degradation, and persistent inflammation, with increased levels of pro-inflammatory mediators [3,4], that culminate into disruption of annulus fibrosus (AF) integrity, consequently allowing the extrusion of the nucleus pulposus (NP) [5]. This process involves alterations in the AF ECM, both at structural and biochemical levels more specifically a reduction in sulphated glycosaminoglycans (sGAG) and collagen type I (Col I) content, accompanied by an increase in fibronectin (FN) and collagen type II (Col II), as previously evaluated in human herniated biopsies [3]. However, the pathomechanism of AF failure remains to be fully understood, particularly at the early stages of degeneration.

Current treatments for IVD herniation include both conservative (physical therapy, anti-inflammatory treatments) and invasive (usually microdiscectomy) approaches to remove the herniated IVD, which is not always successful in resolving pain symptoms, extending patients' limitations with this disorder [6]. In particular, being lumbar disc herniation the main cause of spine surgery, involves a high economic burden for healthcare systems worldwide and ultimately, it is not specific to resolve this IVD pathology [7]. Presently, the study of IVD degeneration/herniation involves diverse models aiming to mimic the pathological process of human IVD degeneration. These models range from: 1) 2D cell cultures, which fall short of accurately resembling the *in vivo* IVD cell microenvironment [8, 9]; 2) *ex vivo* organotypic cultures, lacking the capability for mechanistic studies of specific IVD cell types [10,11]; 3) *in vivo* models, unable to mimic the biomechanics and loading of human spine [12,13]; and 4) humanized IVD models, constrained by limited access to both healthy or early degenerated IVD samples [14,15]. Therefore, the development of more advanced spine study models that replicate more accurately the IVD degenerative microenvironment is imperative for a comprehensive understanding of IVD degeneration/herniation, unravel novel and more specific therapeutic targets, and ultimately to facilitate the design of innovative therapeutic solutions to prevent AF failure.

Inflammation has been strongly linked to IVD degeneration/herniation, by increasing the catabolism of AF and NP cells, contributing to the ECM metabolic imbalance in IVD, that leads to tissue weakening [16,17]. Hence, understanding and replicating *in vitro* the inflammatory cascade in IVD tissue is essential to better treat and repair lumbar disc herniation. Most of the *in vitro* studies performed in the field stimulate IVD cells or IVD organotypic cultures with an acute inflammatory stimulus (10 ng/mL IL-1 β , up to 48h of stimulation) [18-27]. These studies were conducted either to reveal novel biomarkers of IVD degeneration [20], to evaluate the efficacy of drug treatments [18,19,22,25,27], to understand the interplay of IVD cells and other cellular populations [21,24,26] or to design diseased IVD *in vitro* models [23]. However, the inflammation associated with IVD degeneration/herniation is sustained for longer periods of time, becoming chronic [28]. This chronic inflammatory response is known to lead to ECM alterations, including tissue fibrosis, recruitment of immune cells, namely monocyte/macrophages, which then contributes to AF weakening [4,29,30]. Recent studies have established a chronic inflammatory environment in AF cells up to 6 days, using IL-1 β or lipopolysaccharide (LPS) stimulation, evaluating its impact on the mechanical properties of the constructs (bovine tissue and collagen type I hydrogels, respectively) [31,32]. Incorporation of a chronic pro-inflammatory microenvironment into the development of *in vitro* study models for IVD disorders, particularly within AF tissue, represents an important step towards an enhanced knowledge on the interplay between inflammation and ECM deregulation.

A 3D microenvironment has been described as essential to culture IVD cells for many years in order to replicate the native cell morphology, proliferation kinetics and collagen type I and type II production [33]. This work aims to contribute to disclose the pathomechanism of AF failure in human IVD herniation, namely regarding the role of chronic inflammation in a 3D microenvironment. For that we propose an *in vitro* study model comparing 2D and 3D culture (using Col I hydrogels) of human AF cells in basal, acute (2 days) and chronic (7 days) inflammation (IL-1 β supplementation), as described in Figure 1A. This study aimed to unveil alterations in AF cell morphology, ECM and pro-inflammatory mediators' production in a systematic way (Figure 1B). Inflammatory response was further corroborated using a chick chorioallantoic membrane (CAM) *in vivo* assay (Figure 1C). Our hypothesis proposes that the implementation of a chronic pro-inflammatory humanized 3D model will more accurately replicate the early stages of AF failure and consequent IVD herniation.

2. Methods

2.1. Human AF cell isolation and culture

Human AF cells were isolated from patients undergoing surgery in the lumbar region. Detailed patient information is summarized in Supplementary Table S1, following the Lumbar Disc Nomenclature: version 2.0 [34]. AF tissue was dissected from NP tissue macroscopically through visual identification of fibrous tissue with lamellar structure, as previously described [35, 36]. Cells were obtained upon overnight enzymatic digestion of the tissue in 1.1 mg/ml collagenase type II (Sigma-Aldrich) in IVD medium without fetal bovine serum (FBS): low-glucose Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 1% penicillin/streptomycin, 0.5% amphotericin B, and 1.5% 5M NaCl/0.4 M KCl solution (to adjust osmolarity to 400 mOsm), under agitation at 37°C. Cells were then expanded under sterile conditions in IVD medium containing 10% FBS (Biochrom), up to passage 4, as previously described [35] (Figure 1A).

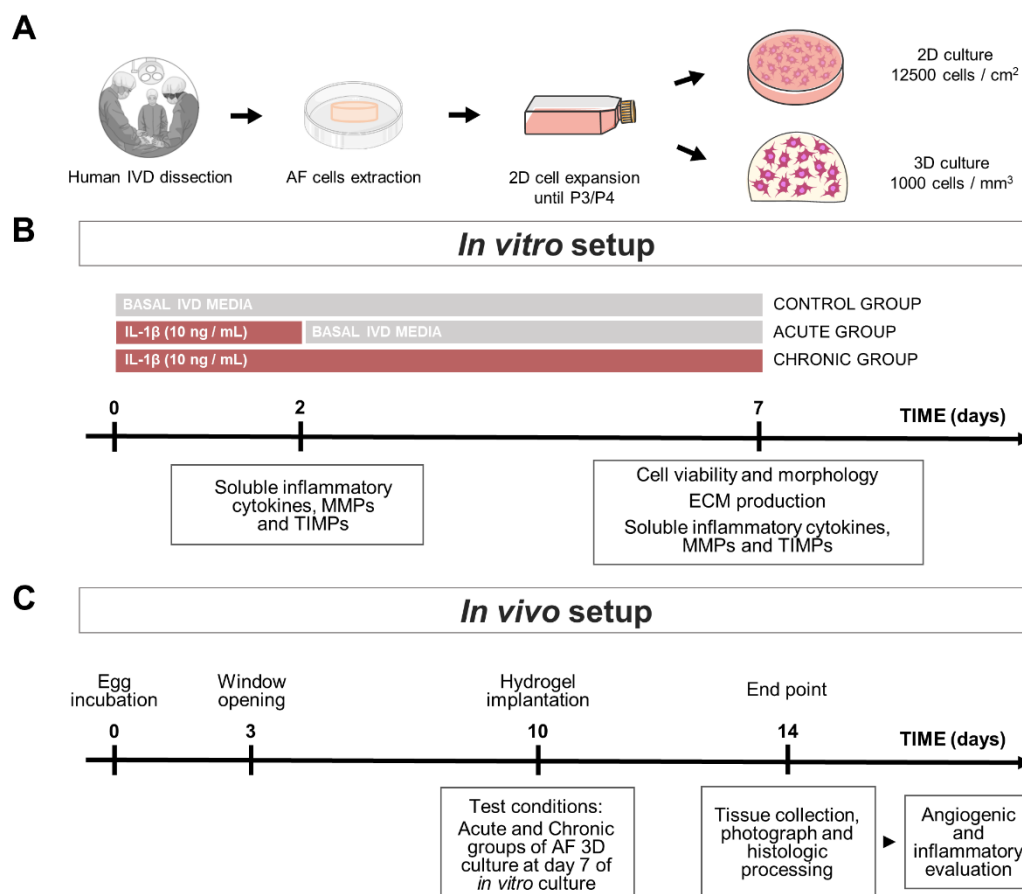


Figure 1 - Schematic representation of the experimental setup. **A**: human AF cells isolation and culture; **B**: *In vitro* experimental groups and timeline; **C**: *In vivo* experimental groups and timeline

2.2. 2D and 3D cell culture and IL-1 β stimulation

For 2D experiments, AF cells (passages 3 to 4) were seeded in 13 mm plastic coverslips (12500 cells / cm²). After 24h for cell stabilization, experimental conditions were supplemented with 10 ng/mL IL-1 β (Peprotech) (day 0 in Figure 1B). Media change was performed at day 2, 5 and 7 of stimulation and conditioned medium were collected and frozen at -20°C for posterior analysis. Two inflammatory experimental conditions were established: Acute, with basal IVD medium after day 2; and Chronic, with continuous renewal of IL-1 β in all media changes (Figure 1C).

Col I hydrogels were prepared as previously described for 3D experiments and casted on μ -Slide 15 Well 3D (Ibidi, Gräfelfing, Germany). Cell concentration conditions (500 or 1000 cells / mm³) were optimized at day 2 of stimulation. Similar to 2D experiments, 10 ng/mL IL-1 β stimulation was performed, with two conditions established (Acute and Chronic). Media change was performed at day 2, 4, 6 and 7 of stimulation and conditioned medium were collected and frozen at -20°C for posterior analysis. Brightfield images were captured for each collagen gel at the end of experiment. Hydrogel contraction was evaluated by area measurements using Fiji software.

2.3. Measurement of lactate dehydrogenase release

To determine cytotoxicity upon IL-1 β stimulation, conditioned medium from day 2 of stimulation in both 2D and 3D experiment was collected and subjected to a quantitative cytotoxicity assay (CytoTox 96 Non-Radioactive Cytotoxicity Assay, Promega). The presence of lactate dehydrogenase was determined following the manufacturer's instructions and absorbance was recorded in a microplate reader Synergy MX (BioTek, Winnoski, VT, USA) at 490 nm. Lactate dehydrogenase positive control provided by the assay was used to calculate cell cytotoxicity (percentage of lactate dehydrogenase activity per sample, relative to lactate dehydrogenase activity of the positive control).

2.4. Resazurin reduction and Live/Dead assays

AF cells metabolic activity in 3D experiments was determined through resazurin reduction assay. Briefly, at day 2 of stimulation, cells were incubated with resazurin redox dye (0.1 mg/mL) (Sigma-Aldrich Co., St Louis, MO, USA) for 4 h. Samples were transferred to a black 96-well microplate and fluorescence intensity (530 nm Excitation/590 nm Emission) was measured using the microplate reader Synergy MX (BioTek, Winnoski, VT, USA). Data was presented in relative fluorescence units (RFU).

To evaluate AF cells viability in 3D experiments, Live/Dead staining was performed at day 2 of stimulation. Briefly, collagen hydrogels were incubated with a solution of calcein (dilution

1:500; Thermo Fisher Scientific) and Hoestch (dilution 1:1000), prepared in PBS, for 20 min, at 37 °C. Then, a solution of propidium iodide (dilution 1:500, Sigma- Aldrich) was added and imaging was immediately performed.

2.5. Cellular and extracellular matrix evaluation by immunofluorescence staining

For both 2D and 3D experiments, AF cells were fixed with 4% paraformaldehyde (PFA) at room temperature (RT) (15 min for coverslips and 40 min for collagen hydrogels), and then washed three times with PBS. Immunofluorescence staining for Col I and FN was performed for all tested conditions. Both optimized protocols and antibody dilutions are described in Table S2. Briefly, cell membrane permeabilization was performed with 0.1% Triton X-100 (Sigma-Aldrich Co., St Louis, MO, USA) in PBS. Bovine serum albumin (BSA, Sigma-Aldrich Co., St Louis, MO, USA) in PBS was used for blocking nonspecific protein binding. Then, samples were incubated overnight at 4°C with the primary antibodies, washed three times in PBS and incubated with the respective secondary antibodies, together with Phalloidin-iFluor 488 Reagent (ab176753, Abcam, 1:2000) and DAPI (Sigma-Aldrich, 1:1000). Samples were imaged with an Operetta high-content imaging system (PerkinElmer) at 20x magnification. For 2D approaches, a minimum of 20 fields per sample was analyzed, while for 3D experiments a minimum of 5 fields per sample and 30 µm of z-stack range were evaluated.

2.6. Bioimage analysis sequence

Images were analyzed using Harmony software (PerkinElmer) and the following analysis sequence. Building blocks are defined with [] and adjustable parameters in *italic*.

1. [Input Image] – Flatfield correction (FFC): Basic; Stack Processing: Maximum projection
2. [Filter Image] – Channel: DAPI; Method: Smoothing (Filter: Gaussian). Output image: Gaussian Smoothed
3. [Find Nuclei] – Channel: Gaussian Smoothed; Method: B; Output Population: Nuclei
4. [Modify Population] – Population: Nuclei; Method: Cluster by Distance (4 µm); Output Population: Modified Nuclei; Output Region: Region
5. [Find Surrounding Region] – Channel: Alexa 488; Population: Modified Nuclei; Region: Region; Method: D; Output Region: Surrounding Region
6. [Select Population] – Population: Modified Nuclei; Method: Common Filters (Remove Border Objects); Output Population: Final Cells

7. [Calculate Morphology Properties] – Population: Final Cells; Region: Surrounding Region; Method: Standard (Area; Ratio Width to Length)
8. [Calculate Texture Properties] – Channel: Alexa 488; Population: Final Cells; Region: Surrounding Region; Method: SER Features
9. [Find Surrounding Region] – Channel: Alexa 594; Population: Final Cells; Region: Region; Method: A; Output Region: Region 594
10. [Find Surrounding Region] – Channel: Alexa 633; Population: Final Cells; Region: Region; Method: A; Output Region: Region 633
11. [Calculate Morphology Properties] – Population: Final Cells; Region: Region 594; Method: Standard (Area)
12. [Calculate Morphology Properties] – Population: Final Cells; Region: Region 633; Method: Standard (Area)
13. [Calculate Intensity Properties] – Channel: Alexa 594; Population: Final Cells; Region: Region 594; Method: Standard
14. [Calculate Morphology Properties] – Channel: Alexa 633; Population: Final Cells; Region: Region 633; Method: Standard
15. [Define Results]

2.7. AF cells pro-inflammatory cytokine and MMPs/TIMPs quantification

Cell culture supernatants were collected at day 2 and 7 of stimulation under sterile conditions, centrifuged at 13,000 rpm, 5 min, 4 °C (Eppendorf Centrifuge 5810R, VWR International LLC, Radnor, PA, USA), to remove cell debris, and stored at -20 °C until further analysis.

Cytokines were quantified using the Human Cytokine/Chemokine Panel A 48-Plex Discovery Assay (Eve Technologies Corp., Calgary, AB, Canada). Matrix Metalloproteinases (MMPs) and Tissue Inhibitors of Metalloproteinases (TIMPs) were quantified using the Human MMP and TIMP Discovery Assay® Array for Cell Culture and non-blood samples (Eve Technologies Corp., Calgary, AB, Canada).

2.8. Inflammatory response in CAM model

The chicken embryo CAM model was used to evaluate the angiogenic response as well as *in vivo* the inflammatory response of AF cells under inflammation, a CAM assay was performed using AF 3D constructs previously under acute and chronic inflammatory

conditions for 7 days. According to the European Directive 2010/63/EU, ethical approval is not required for experiments using embryonic chickens. Correspondingly, the Portuguese law on animal welfare does not restrict the use of chicken eggs for experimental purposes. At Embryonic development day 0 (EDD0), fertilized chicken eggs were horizontally incubated at 37.8 °C in a humidified environment. On EDD3, a square window was opened in the shell, after the removal of 1.5–2 mL of albumen, to allow detachment of the developing CAM. Then, the opening was sealed using transparent adhesive tape, and the eggs returned to the incubator. At EDD10, 3D AF constructs, previously in culture for 7 days as described in subsection 2.2., were positioned onto the expanding CAM within a silicon ring and hydrated with the respective conditioned media. The egg was sealed, returned to the incubator and at EDD14, embryos were euthanized by adding 10% v/v formalin (2 mL) and the CAM inoculation area were excised and imaged *ex ovo* under a stereomicroscope at 20x magnification (Olympus, SZX 16, with a DP 71 camera). Newly formed vessels (<20 µm in diameter) growing radially towards the inoculation site were counted in order to quantify hydrogel induced angiogenesis. Excised CAMs were then embedded in paraffin, sectioned (3 µm) and stained with hematoxylin-eosin (H&E) for histological examination. Imaging was conducted using a brightfield microscope (Leica DM2000 LED) at 10x and 20x magnification.

2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism software (v8). A Shapiro Wilk normality test was used to assess data normality. When a normal distribution was verified, one-way ANOVA analysis followed by Tukey's multiple comparison test was performed, and results were presented using mean and standard deviation. For non-normal distribution, a Kruskal-Wallis test followed by corrected Dunn's were performed, and results were presented using median and interquartile range. For evaluations regarding paired experiments, upon normality assessment, Friedman test followed by Dunn's comparison were conducted. Statistical significance was considered for * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

3. Results

3.1. AF cell culture under inflammatory stimuli

In this study, the impact of chronic inflammatory stimuli on AF cell culture was assessed, employing both 2D and 3D experimental setups (Figure 1). Parameters as IL-1 β production, cytotoxicity, cell viability and metabolic activity were at first studied to characterize AF cell culture under inflammation.

The efficacy of the establishment of the pro-inflammatory conditions described in Figure 1B was initially assessed in 2D experiments, through analysis of IL-1 β production at day 7. Results showed a significant increase on IL-1 β from acute to chronic conditions [2.88 pg/mL (1.48-2.97) vs 3646 pg/mL (2876-4436), *p value < 0.05, Figure 2A1]. To evaluate the impact of the chronic inflammatory stimulation on cell cytotoxicity, Lactate dehydrogenase release was measured at day 2 in all conditions, showing low cytotoxicity (below 12%) with no statistical difference between the experimental groups (Figure 2A2). After 7 days in

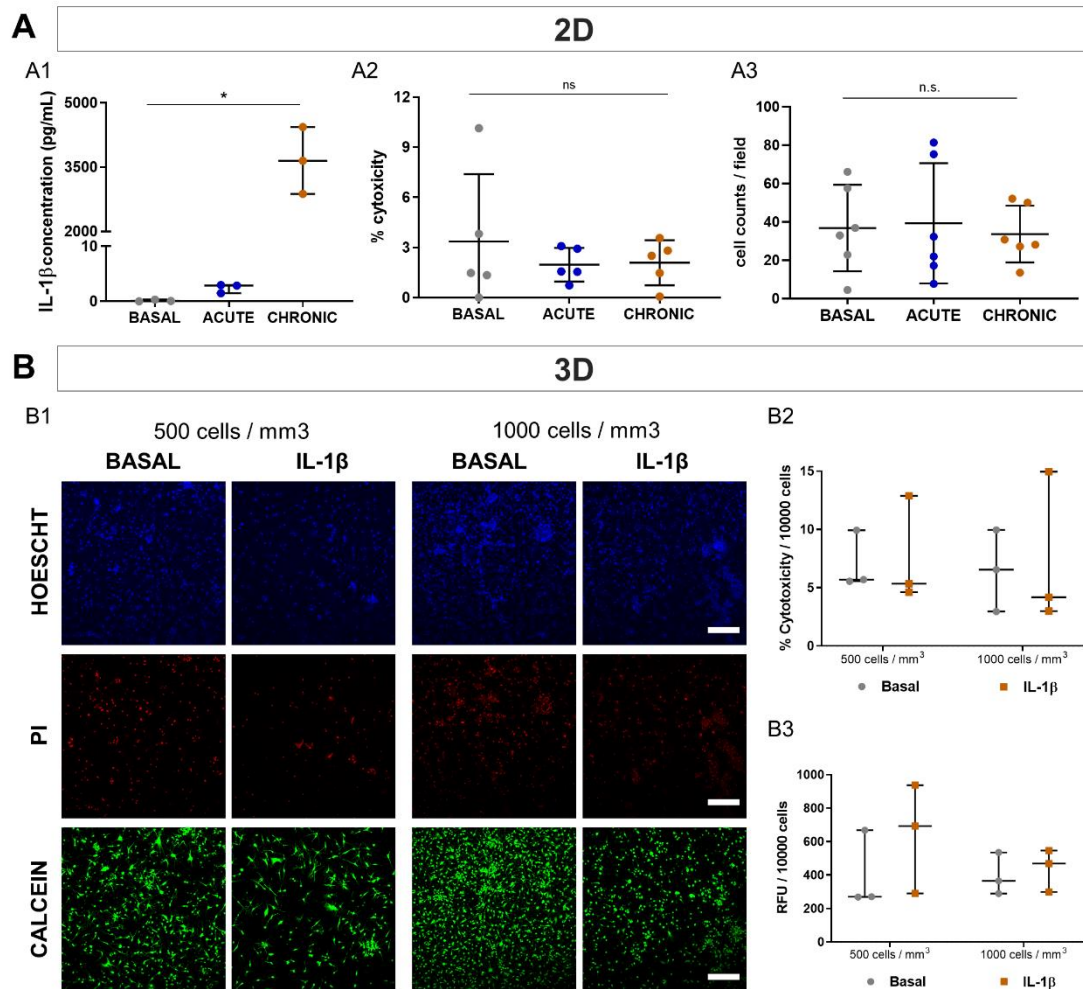


Figure 2 – A: AF cells evaluation in 2D culture. A1) IL-1 β concentration analysis. Data presented as dot plot, with median and interquartile range. N= 3 biological replicas. Friedman test followed by Dunn's comparison was conducted, * p<0.05; A2) Cell cytotoxicity evaluation. Data presented as dot plot, with median and interquartile range. N= 5 biological replicas A3) Cell counts analysis. Data presented as dot plot, with mean and standard deviation. N= 3 biological replicas. **B:** AF cells concentration optimization in Col I hydrogels. B1 – LIVE/DEAD staining. Scale bar = 300 μ m. B2 – Cytotoxicity evaluation. Data presented as dot plot, with median and interquartile range. N= 3 biological replicas. B3 – Metabolic activity assessment. Data presented as dot plot, with median and interquartile range. N= 3 biological replicas.

culture, cell counts indicate no significant alterations between different experimental groups, thus suggesting that cell growth was not affected by chronic inflammatory stimulus (Figure 2A3).

Prior to AF cells 3D experiments in Col I hydrogels, cell concentration was optimized, testing 500 and 1000 cells per μL of hydrogel. Viability, cytotoxicity and metabolic activity were assessed at day 2, both in basal and inflammatory conditions. LIVE/DEAD assay showed that, in both cell seeding conditions, the majority of cells remained alive after IL-1 β stimulation (Figure 2B1). Regarding cytotoxicity, the increase on cell density did not lead to an increased cell cytotoxicity, with all conditions tested with values below 15% (Figure 2B2).

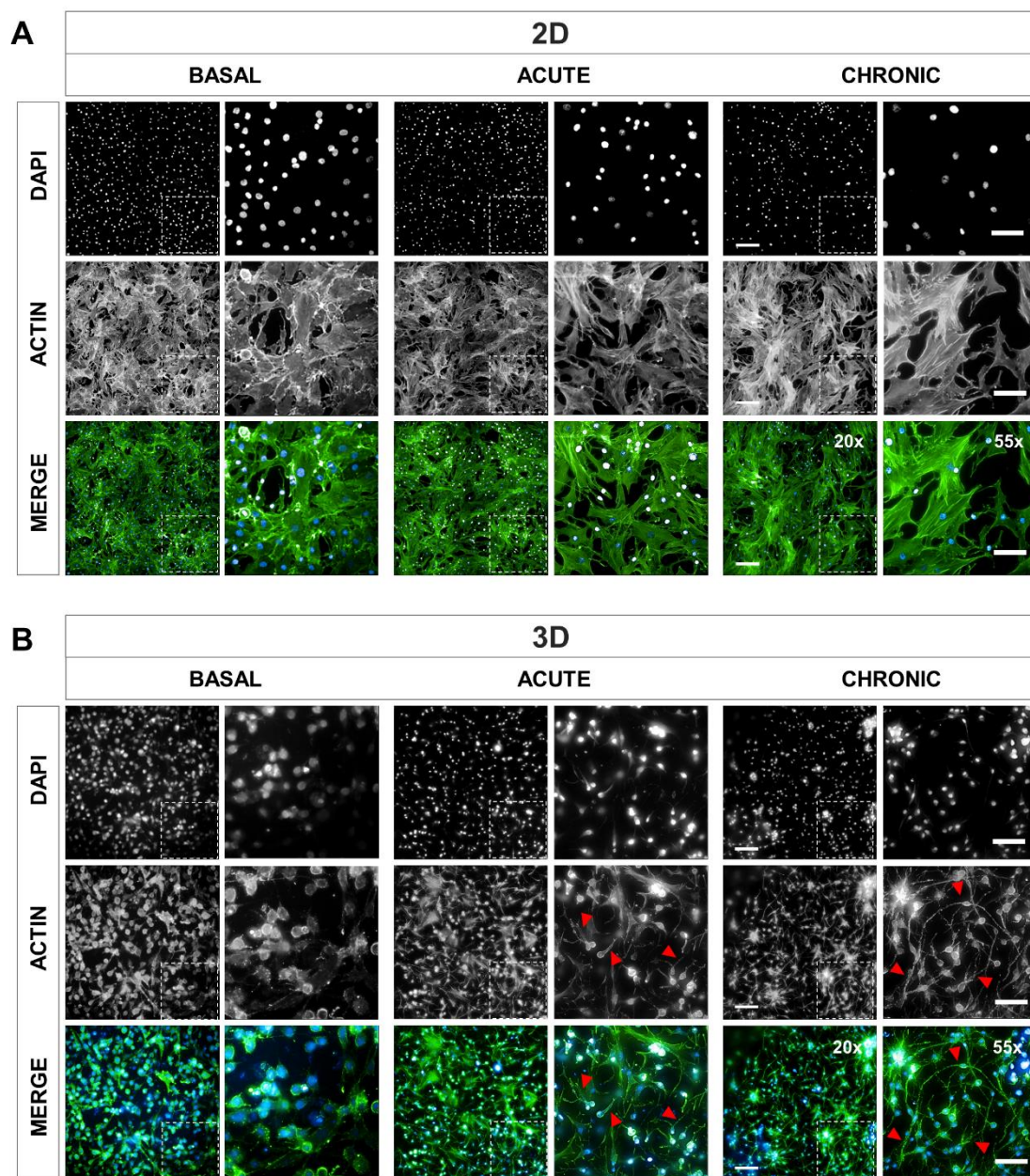


Figure 3 – Cell morphology assessment at day 7 of stimulation. **A:** 2D AF cell culture. 20x magnification, scale bar = 200 μm ; 55x magnification, scale bar = 200 μm . **B:** 3D AF cell culture. 20x magnification, scale bar = 200 μm ; 55x magnification, scale bar = 200 μm

Metabolic activity showed similar levels in both cell seeding conditions, with no significant alterations observed upon inflammation (Figure 2B3). Cell concentration of 1000 cells per μL of hydrogel was selected to further conduct the 3D experiments.

3.2. Alterations in AF cell morphology upon inflammation

Cell morphology was assessed at day 7 using F-actin staining for both 2D and 3D experiments. Actin filaments were not affected by inflammation in 2D AF cell culture (Figure 3A). Nevertheless, when evaluating cell morphology in 3D approaches, alterations were observed, as cell protrusions appeared to be affected by the IL-1 β stimulus. For cells in basal conditions, actin filaments were spread, while a constriction on cell protrusions was observed in the acute and chronic group, as observed in Figure 3B (red arrows).

3.3. ECM characterization of AF cells under inflammation

The role of chronic inflammation in ECM was evaluated after 7 days by immunofluorescence analysis of FN and Col I, commonly altered and associated with IVD degeneration and herniation.

Figure 4A1-B1 show representative images of each staining and condition, for both 2D and 3D approaches. Apart from FN staining in 2D samples, all stainings and conditions exhibited cytoplasmatic expression, without extracellular deposition. Percentage of positive cells expressing the ECM-stained markers was conducted, showing that almost all the AF cells express produce FN and Col I, without alterations between the conditions tested (Figure S1A). Thus, the area of staining per cell was evaluated and data was plotted per experimental group in Figure 4A2-B2.

In general, results showed contradictory trends in 2D vs 3D. While in 2D, ECM expression increased with inflammation, in Col I 3D hydrogels, ECM production by AF cells decreased with inflammation. Regarding quantification of FN stained area, 2D results (Figure 4A2) revealed a significant increase upon inflammation, 1.2-fold and 1.1-fold change to acute and chronic, compared to basal group respectively [1819 μm^2 (1080-2888) vs acute: 2119 μm^2 (1227-3355), **** p value < 0.0001; and vs chronic: 1998 μm^2 (1083-3336), ** p value < 0.01]. Moreover, chronic inflammation promoted a decrease in FN stained area compared to acute (0.9-fold decrease, * p value < 0.05). 3D approaches (Figure 4B2) disclosed a significant decrease in FN stained area upon inflammation, 0.7-fold and 0.9-fold change to acute and chronic, compared to basal groups [807 μm^2 (429.7-1374) vs acute: 581.3 μm^2 (363.4-936.7), **** p value < 0.0001; vs chronic: 686.6 μm^2 (443.6-1055), **** p value < 0.0001]. Still, chronic group showed an increased FN stained area compared to acute (1.2-fold change, **** p value < 0.0001).

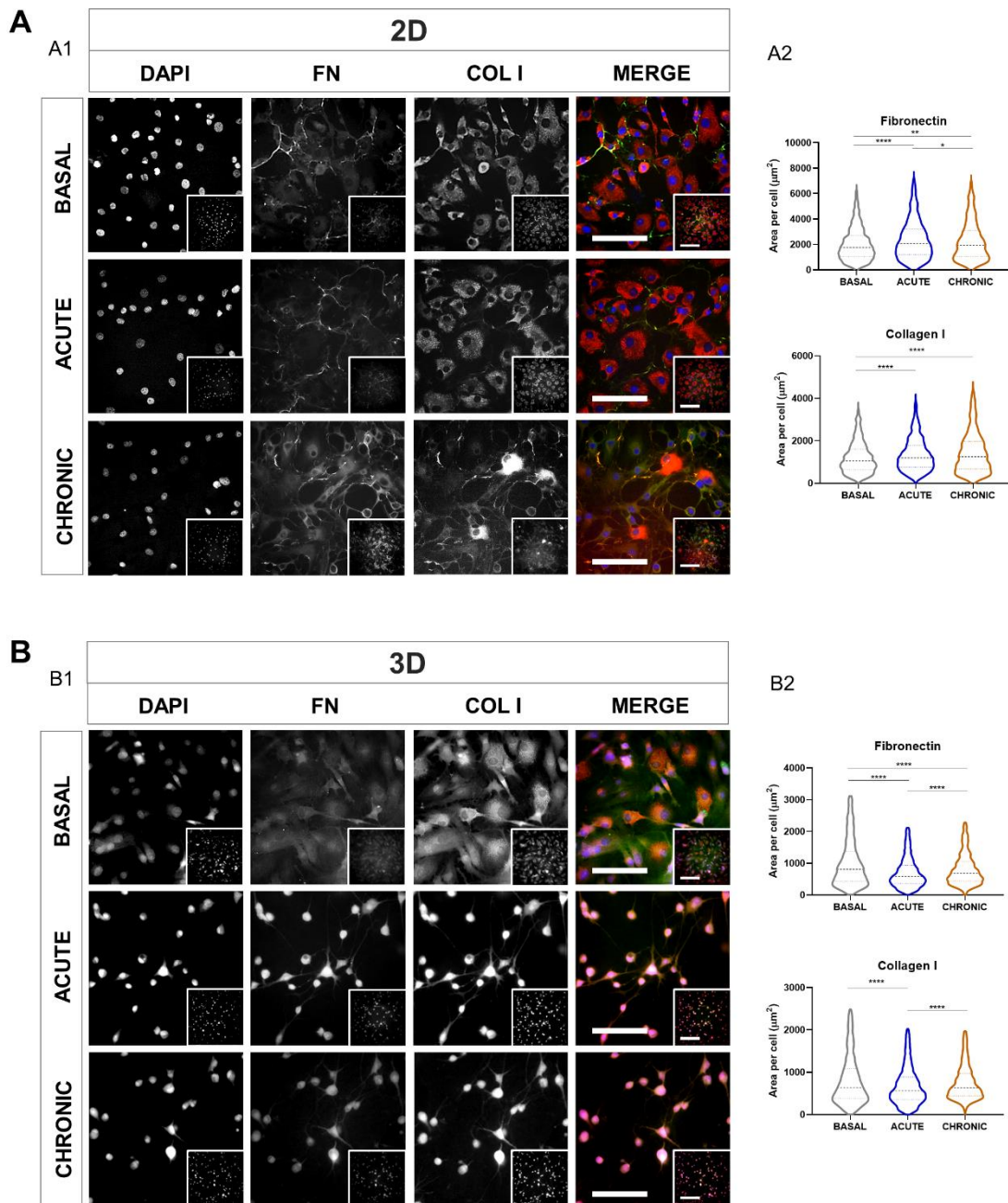


Figure 4 – Extracellular matrix production assessment. **A:** FN and COL I evaluation in 2D culture. A1: Representative images of each staining per experimental condition. 20x magnification, scale bar = 200 μm . 40x magnification, scale bar = 100 μm . A2: Quantification of stainings area per cell. Data presented as violin plot. N= 3 biological replicas. Outliers were identified and cleaned. Kruskal-Wallis test followed by Dunn's comparison was conducted, * $p < 0.05$; ** $p < 0.01$, **** $p < 0.0001$. **B:** FN and COL I evaluation in 3D culture. B1: Representative images of each staining per experimental condition. 20x magnification, scale bar = 200 μm . 40x magnification, scale bar = 100 μm . B2: Quantification of stainings area per cell. Data presented as violin plot. N= 3 biological replicas. Outliers were identified and cleaned. Kruskal-Wallis test followed by Dunn's comparison was conducted, **** $p < 0.0001$

When analyzing Col I stained area, 2D data (Figure 4A2) showed an increase from basal conditions [1082 μm^2 (672.2-1687)] to both inflammatory groups, 1.1-fold and 1.2-fold change, for acute and chronic, respectively [acute: 1214 μm^2 (775.6-1865) **** p value < 0.0001; and chronic: 1296 μm^2 (699-2098) **** p value < 0.0001]. No differences were observed between acute and chronic. Contrarily, 3D experiments (Figure 4B2) showed a significant decrease (0.9-fold decrease, **** p value < 0.0001) in Col I stained area upon acute inflammation [563.5 μm^2 (352.7-888.8)] compared to basal group [638.3 μm^2 (378.1-1088)]. Chronic exposure to IL-1 β led to an increase in Col I stained area [635.1 μm^2 (445.7-969.2)] compared to acute stimulation (1.1-fold change, **** p value < 0.0001).

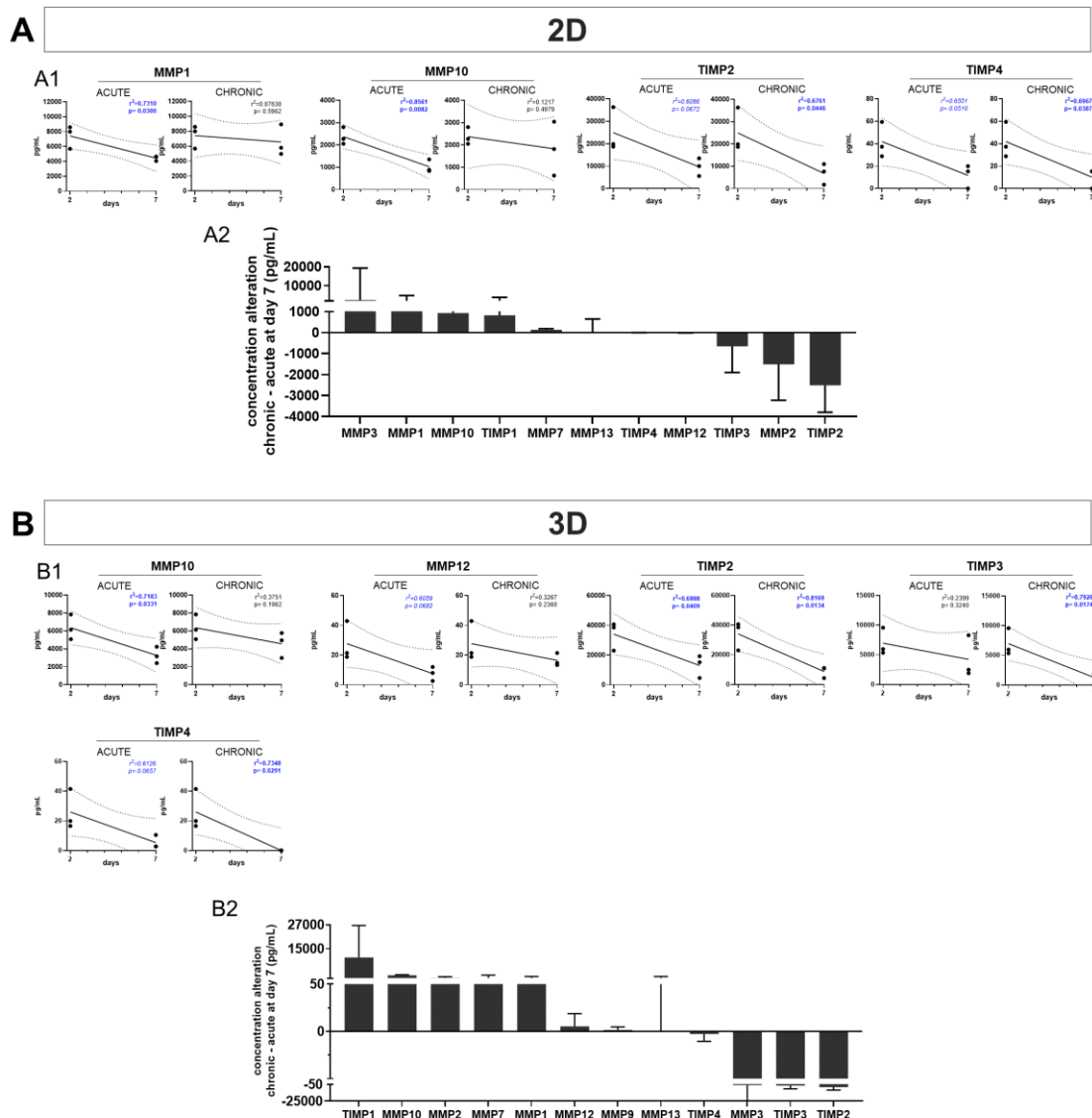


Figure 5 – Evaluation of MMPs/TIMPs in AF cells in 2D (**A**) and 3D (**B**). 1: Correlation analysis of alterations under inflammation with time. Data presented using dot plots, with 95% confidence intervals and indication of r^2 and p value for each linear regression. N= 3 biological replicas. 2: Concentration alterations at day 7 between acute and chronic. Data presented as graph bars, with median and interquartile range. N= 3 biological replicas.

3.4. Secretion of MMPs/TIMPs by AF cells under inflammation

Next, the production of MMPs and TIMPs was evaluated in the conditioned media of AF cells at day 2 and day 7. All the factors tested and produced by AF cells can be found in Figure S1B-C. An analysis with time was performed, considering the inflammatory experimental results at day 2 and 7. 2D approaches (Figure 5A1) showed a decrease in MMP1 and MMP10 decrease upon acute inflammation. In both inflammatory experimental groups, a decrease with time was observed in TIMP2 and TIMP4. Analysis of 3D experiments (Figure 5B1) revealed that MMP10 and MMP12 decreased with time only upon acute stimulation. TIMP3 decreased with time in AF cells exclusively upon chronic exposure to IL-1 β . Considering both inflammatory experimental conditions, a decrease in TIMP2 and TIMP4 was observed. All the statistically significant correlation analysis can be found in Figure 5A1-B1. Focusing on acute and chronic stimulation at day 7, no differences were observed for both approaches (Figure 5A2-B2).

3.5. Secretion of inflammatory mediators by AF cells under inflammation

To address the impact of chronic IL-1 β driven inflammation in the production of inflammatory cytokines/chemokines during 2D and 3D culture was evaluated (at day 2 and 7). For that, a broad panel of cytokines, chemokines and growth factors was selected. The factors produced by AF cells in the different conditions at day 7 are presented in Figure S1B-C. First, an analysis of the dynamics of inflammatory cytokine profile of AF cells with time was performed, by addressing inflammatory conditions at day 2 and 7. By correlation analysis, 2D results showed that when following acute stimulation, with time, there is a reduction in IL-15, interferon-gamma-induced protein (IP-10), G-CSF, fibroblast growth factor (FGF)-2, monocyte chemoattractant protein (MCP)-3, macrophage inflammatory protein (MIP)-1 α , MIP-1 β , regulated on activation, normal T-cell expressed, and secreted (RANTES) and tumor necrosis factor (TNF)- α . Looking into the chronic conditions, from day 2 to 7, data revealed an increase in IL-1 α and monokine induced by interferon-gamma (MIG)/CXC chemokine ligand (CXCL)9 and a decrease in IP-10 and TNF- α . 3D analysis with time also presented alterations in both inflammatory groups. For both acute and chronic conditions, there was an increase with time of IL-1 α and a decrease of MCP-3 and TNF- α . More specifically after IL-1 β suppressing in acute conditions, a decrease on G-CSF, GM-CSF and MCP-1 was also observed. Correlation analysis graphs can be found in Figure 6A1 and Figure 6B1 for 2D and 3D experiments, respectively.

Next, analysis of the concentration alteration between inflammatory proteins in chronic and acute inflammatory groups at day 7 was performed (Figure 6A2-B2). 2D data showed that chronic stimulation promoted an increase (* p value < 0.05) of IL-1RA (5.97-fold change)

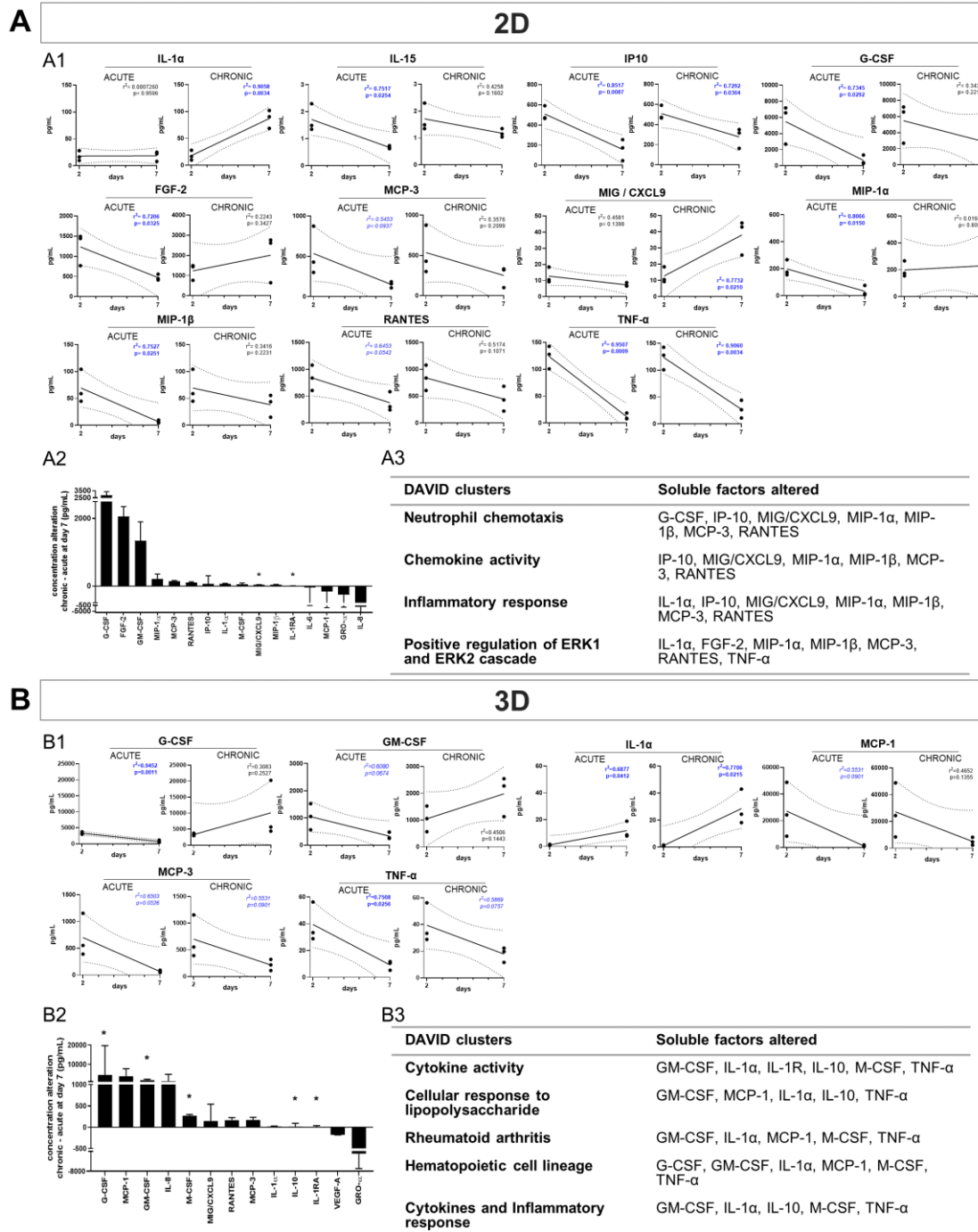


Figure 6 – Evaluation of pro-inflammatory mediators in AF cells in 2D (**A**) and 3D (**B**). 1: Correlation analysis of alterations under inflammation with time, in 2D. Data presented using dot plots, with 95% confidence intervals and indication of r^2 and p value for each linear regression. N= 3 biological replicas. 2: Concentration alterations at day 7 between acute and chronic. Data presented as graph bars, with median and interquartile range. N= 3 biological replicas. Kruskal-Wallis test followed by Dunn's comparison was conducted, * $p < 0.05$. 3 – Summary of DAVID clusters analysis.

and MIG/CXCL9 (6.38-fold change). 3D results showed that chronic inflammation led to a significant (* p value < 0.05) increase of G-CSF (10.23-fold change), GM-CSF (8.41-fold change), IL-1RA (1.99-fold change), IL-10 (6.21-fold change) and M-CSF (2.06-fold change).

Analyzing all the factors altered upon inflammation with the DAVID functional clustering tool, 2D results showed significantly enriched clusters with implications in neutrophil chemotaxis, chemokine activity, inflammatory response and positive regulation of ERK1 and ERK2 cascade, among others. On other hand, 3D results showed significantly enriched clusters with implications in cytokine activity, cellular response to LPS, rheumatoid arthritis, hematopoietic cell lineage, cytokines and inflammatory response, among others. Figure 6A3-B3 summarizes all the details on each cluster and the respective factors altered.

3.6. *In vivo* immune cell recruitment by 3D AF cell culture under inflammation

Taking advantage of the CAM assay, besides evaluating the angiogenic response induced by the hydrogels, we were able to analyze the in-situ response of the tested conditions. This assay allows for the non-invasive visualization of the inflammatory and angiogenic response to bioengineered matrices within a fully intact *in vivo* environment. Specifically, we were able to evaluate the *in vivo* recruitment of the chick immune cells towards the tested 3D AF constructs. The mortality rate observed in the assay was 8.7% (4 eggs out of 46), below the expected value of 10%, suggesting the absence of significant systemic toxicity.

Angiogenic evaluation was performed, by conducting vessel count on the ex ovo representative images (Figure 7A) of the AF 3D constructs. Both inflammatory conditions exhibited similar angiogenic potential (Figure 7B), consistent with similarities observed upon the evaluation of VEGF-A secretion.

Furthermore, by evaluation performing an overview of the H&E CAM slides, in a blind fashion way by two independent observers, we were able to establish distinct inflammatory patterns that were translated into “Inflammatory scores” (Figure 7C-D). The presence of few (<30) heterophils scattered in the CAM was scores as 0, contrasting with score 1, presenting samples with more than 50 immune cells near the hydrogel, frequently in clusters (see black arrows in Figure 7E). Chronic conditions exhibited a higher percentage of samples in score 1 (73%) than the acute (55%), Analysis of the inflammation score revealed a significant increase (* p value<0.05) in immune cell recruitment in the CAM of hydrogels with chronic inflammation, in comparison with hydrogels exposed to acute inflammation (Figure 7D). This result is consistent with previous results on cytokine/chemokine analysis (subsection 3.5).

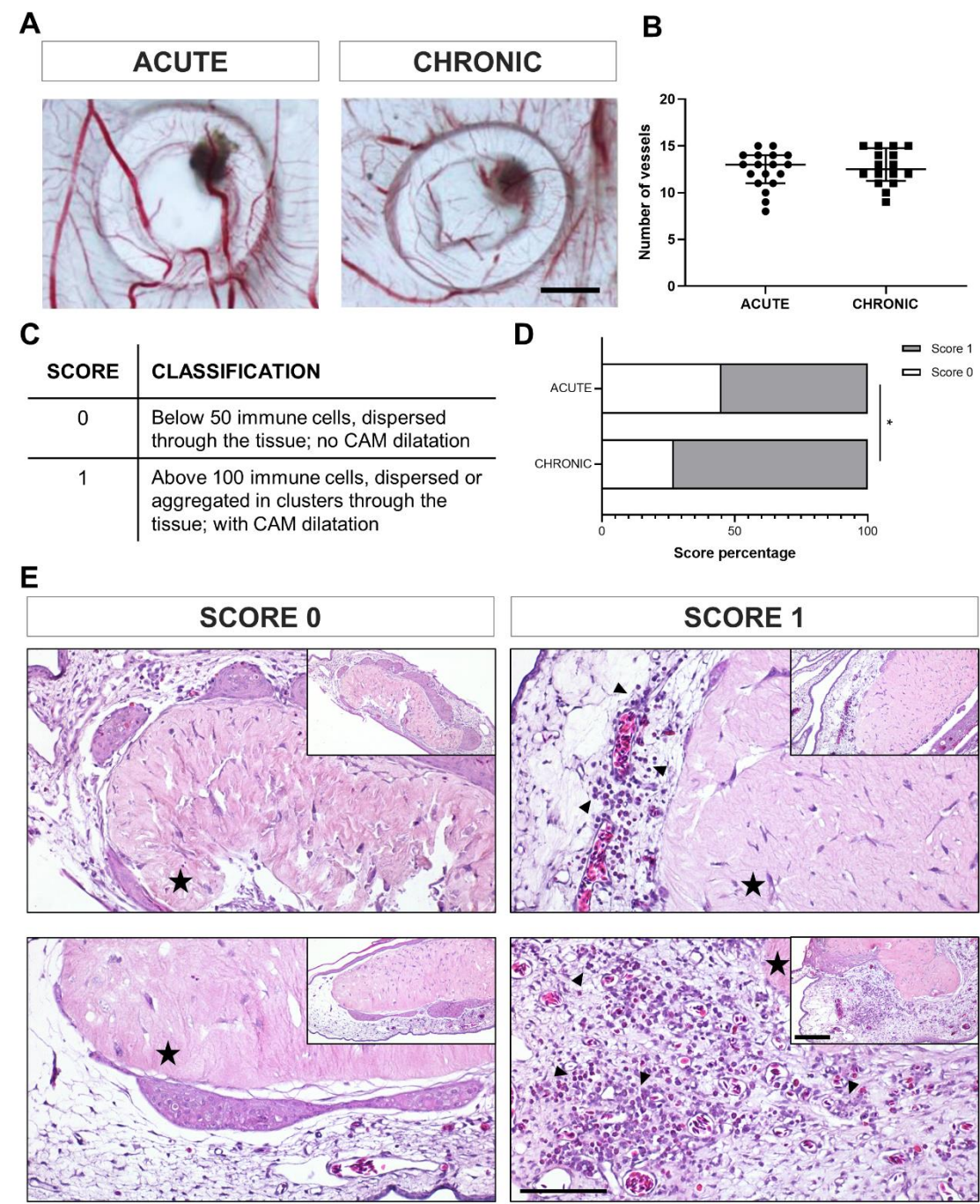


Figure 7 – *In vivo* evaluation of AF 3D culture previously under inflammation. Angiogenic evaluation: **A**: Representative images per condition, scale bar = 1 mm. **B**: Vessel quantification. N=18 eggs for acute and 16 eggs for chronic group. Data presented using scatter plot. Immune cell recruitment evaluation: **C**: Inflammation score classification, based on a first blind overview of all samples. **D**: Inflammation score quantification. N=11 eggs per condition. Data presented using graph bars. Fisher's exact test was conducted. * p value <0.05 ; **E**: Representative images per score. Black arrows indicate examples of recruited immune cells. Black stars indicate AF hydrogels. 10x magnification, scale bar = 200 μ m; 20x magnification, scale bar = 100 μ m

4. Discussion

The AF, playing a crucial role in containing the NP and general IVD homeostasis, is compromised by a pro-inflammatory microenvironment, causing AF rupture and ultimately leading to IVD herniation [17]. The interplay between inflammation and AF failure remains unclear and has yet to be accurately replicated in *in vitro* models.

In this study, a systematic investigation of hAF cells cultured in 2D and 3D (using Col I hydrogels) in acute versus chronic inflammatory conditions, was performed. This stands in contrast to the current studies on IVD research that rely on a short inflammatory stimulation (single pulse) [18-27].

The inflammatory stimuli of hAF cells in both 2D and 3D conditions allows the maintenance of high cell viability and low toxicity. Regarding cell morphology, in 2D no alterations were observed with the inflammatory stimuli, while in 3D, inflammation induced alterations in actin filaments. Scarce information is available on how the cytoskeleton of hAF cells responds to pro-inflammatory stimuli. Molladavoodi et al. have reported reduced F-actin protrusions upon chronic stimulation of rat AF cells with LPS [32]. TNF- α has been reported to increase stress fiber formation in hAF cells, as assessed by F-actin, vinculin and α -tubulin stainings [37]. Maidhof et al. reported alterations in F-actin distribution in NP cells following inflammatory stimuli using LPS and TNF- α [38]. Further studies are required to explore mechanosensitivity upon inflammation on hAF cells and their impact on AF failure.

ECM alterations, particularly in AF tissue, are associated with IVD degeneration and herniation, regarding collagen (type I and II), FN and proteoglycans and sulphated glycosaminoglycans [3]. Here, ECM analysis was conducted, at day 7, focused on stained area per cell, regarding FN and Col I. Notably, contradictory results emerged in inflammation progression between 2D and 3D experiments, with ECM stained area per cell was increasing in 2D conditions and decreasing in 3D hydrogels. Within the IVD, FN levels escalate with degeneration grades (evaluated by Thompson's scale) [15], from protrusion to extrusion [18] and from initial to severe stages of herniation [3]. In the 3D context of this study, results reveal a reduction in FN content in inflammatory conditions compared to basal states. However, the observed interaction between acute and chronic groups, suggesting an increase of FN with inflammation progression, aligns with existing literature documenting increased FN levels as degeneration and herniation advance. In AF tissue, a reduction in Col I has been commonly associated with ageing [1,11,16], with the degeneration level in both goat [44] and human (*in vitro* study) [17], and with human herniation progression [3]. Current literature and the results of this study emphasize the importance of using 3D over 2D approaches, to replicate AF failure and Col I reduction. Nevertheless, acute stimulation

led to a more prominent decrease on Col I area. Further studies addressing with detail the alterations of Col I homeostasis upon different inflammatory studies are needed, potentially unravelling insights into the temporal alterations of Col I in AF tissue during IVD degeneration. Overall, ECM evaluation showed the importance of adopting 3D approaches for studying inflammation in hAF cell culture. A chronic environment allowed the replication of key alterations observed *in vivo* (as for FN) and provided possible insights on temporal ECM alterations (as for Col I).

The MMPs and TIMPs secretion profiles were evaluated, with both 2D and 3D approaches revealed similar temporal alterations upon inflammatory stimulation. Only in acute conditions a decrease over time of MMPs was observed, specifically for MMP1 (2D), MMP12 (3D) and MMP10 (in both). MMP1 expression is reported to increase with degeneration [39] and *in vitro* under inflammatory stimulus [40,41]. MMP12 exhibits an increase with degeneration in rat and human [42, 43], but not herniation progression [3]. MMP10 has been associated with an increase in degeneration (Pfirrmann grades) [44]. Altogether, MMPs analysis highlights the significance of establishing chronic inflammatory conditions in *in vitro* studies to better resemble the MMPs profile over extended time periods. TIMPs evaluation revealed a consistent decrease on TIMP2 and TIMP4 over time, irrespective of dimensional or inflammatory approaches. Studies involving hAF cells exposed to IL-1 β have reported a decrease in TIMP2 [45]. While the expression of TIMP4 in AF tissue has not been addressed, it has been shown to downregulate in tendon tissue [46]. In the current 3D model, TIMP3 also decreased over time upon chronic stimulation. However, past studies have not reported alterations in TIMP3, as it has been found both in non-degenerated and degenerated hAF tissue [47].

The profile of inflammatory cytokines secreted by AF cells under chronic versus acute inflammation was evaluated. Overall, the 2D conditions exhibited a more prominent decrease in inflammatory cytokines over time, particularly following acute stimulation, failing on replicating the inflammatory milieu observed in IVD disorders. This highlights the significance of employing 3D models for investigating IVD degeneration. IL-1 α has been shown to increase from non-degenerated to degenerated tissues, escalating with the severity of disease [48]. In the proposed study, both 2D and 3D approaches have reproduced these alterations, with an increase in cytokine expression with time in both acute and chronic conditions (in 3D). TNF- α , widely used as an inflammation-inducing cytokine in both *in vitro* and *in vivo* studies [37,49], has shown increased expression in degenerated and herniated IVD, but not at gene and protein level [50]. In the current study, results indicate a consistent decrease in here presented have showed that, irrespective of the model or inflammation approach. Moreover, a study correlating acute and chronic

symptomatology in degenerated IVD has reported a decrease of TNF- α with chronic symptoms, hinting at its possible impact on the disease in an acute term and proposing it as a potential biomarker for the disease status [51]. The mentioned study reported a decrease in IL-10 in patients with chronic symptoms, a trend not observed in the present study, where IL-10 increased in chronic compared to acute inflammation. Notably, IL-10 has been demonstrated to rise in AF cells stimulated with IL-1 β [52]. Additionally, previous studies indicated that IL-1RA increases in degenerative discs, without correlation with disease severity [48]. Here, IL-1RA increased from acute to chronic, in both dimensional approaches.

Other important mediators in the inflammatory process in IVD diseased environment include chemokines to promote the recruitment of immune cells [17]. For instance, G-CSF and GM-CSF, reported to increase in symptomatic herniated discs [53], were observed to increase upon chronic inflammation in this study, together with M-CSF, other macrophage colony stimulation factor. Interestingly, MCP-1, responsible for monocyte recruitment and previously shown to be elevated in herniated discs [54] and with degeneration [55], decreased over time under acute stimulation, highlighting the importance of establishing a chronic inflammatory environment. While MCP-3, detected in both scoliotic and herniated IVD samples [54] and shown to increase with degenerative progression [55], demonstrated a contrasting trend in this study, with a decrease observed over time in both inflammatory conditions.

The relevance of using the chick embryo to address the immune system response has previous been validated [56]. The similarity between the immune systems of human and chicken has been addressed and highlighted [57]. Hence, the CAM model is considered relevant not only for angiogenesis analysis but also for immune-focused investigations, particularly after 10 days of chick embryonic development, where lymphoid cells, antigen-present cells and granulocytes are already observed, together with the secretion of several inflammatory cytokines, as IL-1 β and IL-8 [57]. Here, AF 3D constructs that had previously underwent 7 days of inflammation (acute and chronic conditions) were implanted at EDD10 and excised at EDD14. Results showed that chronic stimulation led to increased immune cell recruitment, through the inflammation score analysis. These align with the *in vitro* results on inflammatory mediators, mainly the increase on G-CSF, GM-CSF and M-CSF observed in chronic versus acute stimulated hydrogels. Angiogenic potential evaluation showed no differences on the two types of inflammation, in line with the no differences observed upon VEGF-A secretion analysis.

Despite the findings of this study, we cannot exclude some limitations. Due to scarcity of IVD samples from healthy donors, AF cells were isolated from herniated IVD tissue. Despite this, the expanded AF cells in 2D culture did not exhibit a degenerative profile, as confirmed by cell morphology and pro-inflammatory production (data not shown) and consistent with previous observations by gene expression [35]. Moreover, experiments were conducted under normoxia, 10% FBS and 10 ng/mL IL-1 β , which allowed us to address with accuracy the role of IL-1 β in AF cells but may not fully resemble the *in vivo* disease environment characterized by reduced oxygen and nutrients starvation, along with the inflammation driven by multiple cytokines and for longer periods of time. Further studies should address the complexation of the *in vitro* model, namely considering oxygen reduction, biomaterial-based ECM composition, external inflammatory mediators and extended culture durations.

5. Conclusion

To our knowledge, this is the first study investigating the resemblance of 2D and 3D methodologies and acute vs chronic inflammation in the context of degeneration/herniation of IVD, more specifically in AF tissue. The findings support the importance of 3D culture techniques in mimicking the inflammatory cascade, regarding ECM production and pro-inflammatory cytokines. Chronic stimulation proved to be essential for replicating the *in vivo* diseased microenvironment over extended durations, mainly regarding ECM production (e.g. FN) and chemokines orchestrating immune cells recruitment (e.g., G-CSF and GM-CSF), the latter corroborated by *in vivo* experiments. Moreover, analysis with time revealed interesting insights on inflammatory mediators dynamics (e.g., IL-1 α , MCP-3, TNF- α , TIMP2 and TIMP4), key players in IVD degenerative process. These findings offer critical insights for designing study models of IVD herniation, specifically within the AF tissue, while opening opportunities for specific inflammatory mediators towards new therapeutic targets or biomarkers of IVD herniation.

Acknowledgments

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Ethics Declaration

This study was conducted after patients' informed consent and approval from local ethics committee approval (Centro Hospitalar Universitário S. João, Porto, Portugal, 29-11-2011).

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Supplementary Data**Tables****Table S1:** Donor information regarding gender, age, hernia condition and disc level.

ID	Gender	Age	Condition	Disc Level
1	Female	46	Uncontained	L4-L5
2	Male	21	Uncontained	L4-L5
3	Female	49	Contained	L4-L5
4	Male	42	Contained	L5-S1
5	Male	64	Uncontained	L4-L5
6	Female	55	Protruded	L4-L5
7	Male	35	Contained	L5-S1

Table S2: Antibodies select for IF, optimized blocking dilution and incubation period

Antibody	Permeabilization	Blocking	Incubation	Reference
Fibronectin	0.1% Triton for 10' in 2D cultures and for 40' in 3D cultures	0.5% BSA, 5% FBS and 0.3 % Triton for 1h	1:200, overnight at 4°C	SC-8422 / Santa Cruz Biotechnology
Collagen type I	0.1% Triton for 10' in 2D cultures and for 40' in 3D cultures	0.5% BSA, 5% FBS and 0.3 % Triton for 1h	1:200, overnight at 4°C	72026 / Cell Signaling Technology
Alexa Fluor™ 594 goat anti-mouse	NA	NA	1:1000, 1h	A11020 / Invitrogen
Alexa Fluor™ 647 donkey anti-rabbit	NA	NA	1:1000, 1h	A31573 / Invitrogen

Figures

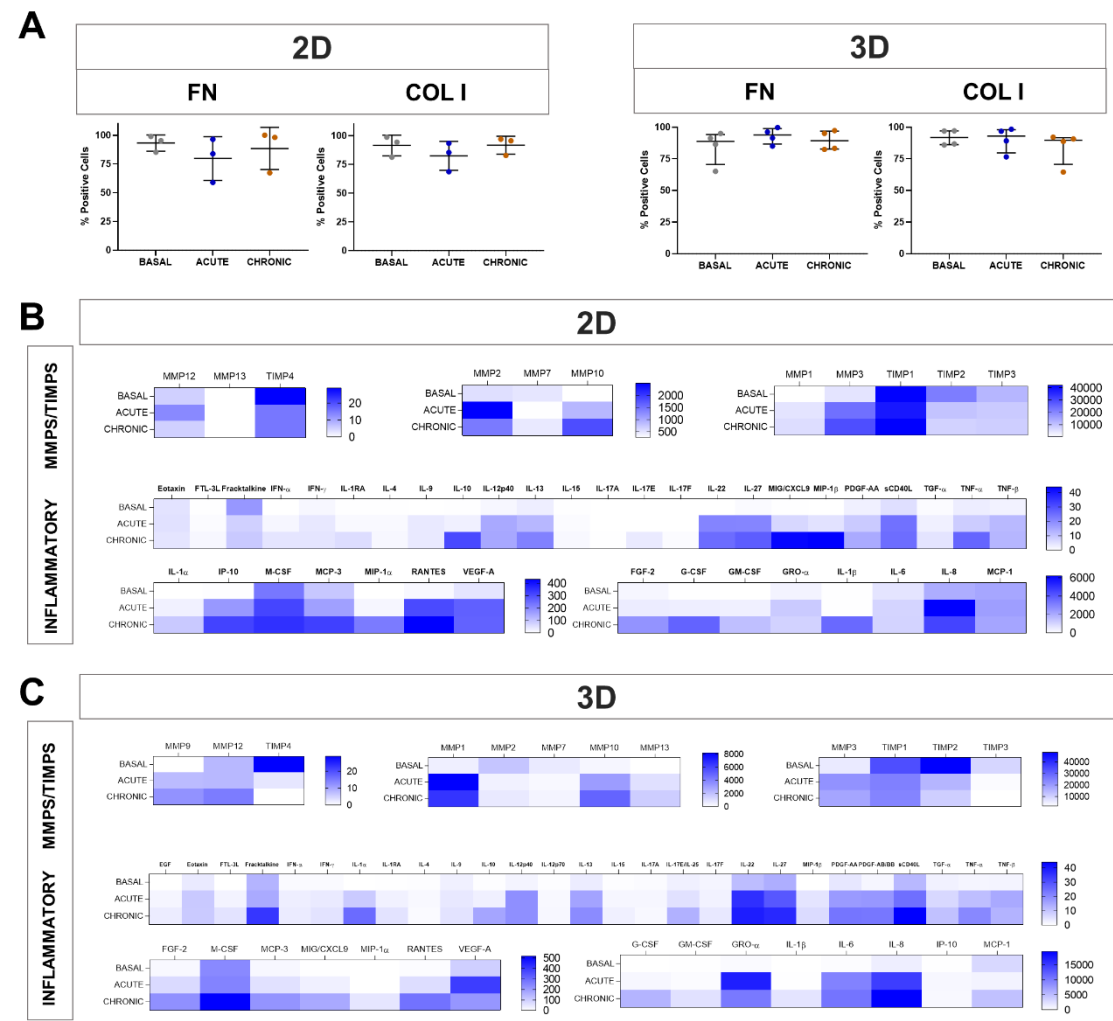


Figure S1 – A: ECM positive cells quantification, per staining, in 2D culture (N= 3 biological replicas) and in 3D culture (N= 4 biological replicas) **B:** Heat map representation of pro-Inflammatory cytokine and MMPs/TIMPs alterations at day 7, in 2D culture. N= 3 biological replicas. **C:** Heat map representation of pro-Inflammatory cytokine and MMPs/TIMPs alterations at day 7, in 3D culture. N= 3 biological replicas.

Chapter V

Engineering Anisotropic Cell Models:

Development of Collagen Hydrogel Scaffolds with Magneto-Responsive PEG Microgels for Tissue Engineering Applications

Engineering Anisotropic Cell Models: Development of Collagen Hydrogel Scaffolds with Magneto-responsive PEG microgels for Tissue Engineering Applications

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Abstract

Mimicking tissue-oriented organization *in vitro* has been extensively studied in recent years, using both natural and synthetic materials in combination with external magnetic fields to establish anisotropic conditions. Here, a new combination between magneto-responsive anisometric PEG microgels and collagen hydrogels is explored to establish anisotropic *in vitro* models. Different sizes of PEG microgels are tested to assess the impact of both width and aspect ratio on the formation and alignment of collagen hydrogels. Results show that the key properties of collagen hydrogels, regarding fibrillogenesis, rheological properties, and fiber diameter are kept consistent upon the combination with PEG microgels. Furthermore, partial collagen fiber alignment is observed when larger (width 10 μm) PEG microgels are employed and magnetically aligned. *In vitro* studies show cell alignment within the anisotropic collagen hydrogels from the first day in culture. Interestingly, PEG microgels with higher width and length tend to induce less hydrogel contraction even after 7 days in culture. The results demonstrate the ability to establish a 3D unidirectional collagen hydrogel by magnetically aligning anisometric microgels during the gelation process, which can be promising for different tissue engineering applications.

1. Introduction

Collagen type I (Col I) is the major organic component of the extracellular matrix (ECM) of mammalian tissue [1]. Together with other ECM molecules, this protein has an important structural action for growth and support of cells, playing a relevant role in different physiological processes [1-3]. Col I acts as an attractive protein for tissue engineering both due its biocompatibility, biodegradability, and antigenicity, but also due to its ability to self-assemble and generate hydrogels [2-5]. Indeed, Col I hydrogels have been broadly used for different applications in tissue engineering, either alone or in combination with other ECM proteins/constituents and biomaterials, as they show great results as support for 3D cell culture [6-8].

In recent years, *in vitro* models have focused on providing a microenvironment to support cell growth that beside having a 3D structure, also mimic features of the *in vivo* architecture [9]. Each tissue has a specific architecture, particularly regarding Col I anisotropy. Tissues, such as tendon, intervertebral disc, muscle, and others, present a specific microarchitecture composed of oriented Col I fibers, which has an impact on tissue homeostasis concerning structural and cellular processes [10].

Several strategies have been developed to recreate the natural anisotropy observed in collagenous native tissues in an *in vitro* environment, which have been extensively reviewed elsewhere [9,11,12]. These strategies include biomaterial processing techniques, such as extrusion, freeze-drying, electrospinning, magnetic alignment, flow-induced orientation, among others [13-17]. Electrospinning-aligned collagen nanofibers has been proposed for different applications in wound healing, bone mineralization, and nerve tissue regeneration [18-20]. Here, an additional crosslinking step is often needed to overcome the reduction of pore size due to dense packing of aligned collagen fibers, impairing cell proliferation within the scaffold [21]. Moreover, collagen denaturation has been reported upon electro-spinning, impairing its fibrillar structure [22]. On the other hand, for several decades, extrusion techniques have been successfully used to produce aligned collagen scaffolds, due to its ease and possibility of scale up, with applications in bone and tendon regeneration, ligament replacement, and wound healing [9,23,24]. Despite the recent advances on the combination of 3D printing with extrusion, this approach still presents limitations regarding production time and cell mortality [9]. Magnetic alignment techniques have risen interest in recent years as they can overcome some of the most common drawbacks associated with the previously mentioned strategies, including reduced fibril formation and cell infiltration. To achieve anisotropic scaffolds, magnetic nanoparticles (e.g., iron nanoparticles) have been used together with a Col I solution to accomplish fibril alignment under an external magnetic field,

both for cartilage and nerve regeneration [25,26]. Anisotropy induced by magnetic approaches has shown promising results, albeit higher amounts of iron nanoparticles might lead to cell cytotoxicity [27]. In this context, magnetic-responsive, rod-shaped microgels have been used to overcome this drawback, using poly(ethylene glycol) (PEG) and fibrin as surrounding hydrogels [28-30]. Briefly, a hybrid hydrogel, named Anisogel, has been established where unidirectional microgel orientation is accomplished in the presence of an external magnetic field (100 millitesla) by incorporating small amounts of superparamagnetic iron oxide nanoparticles inside PEG microgels during their production [29]. This methodology has been thoroughly characterized in prior publications [29,30], including analysis on inter-microgel distances depending on the microgel dimensions and

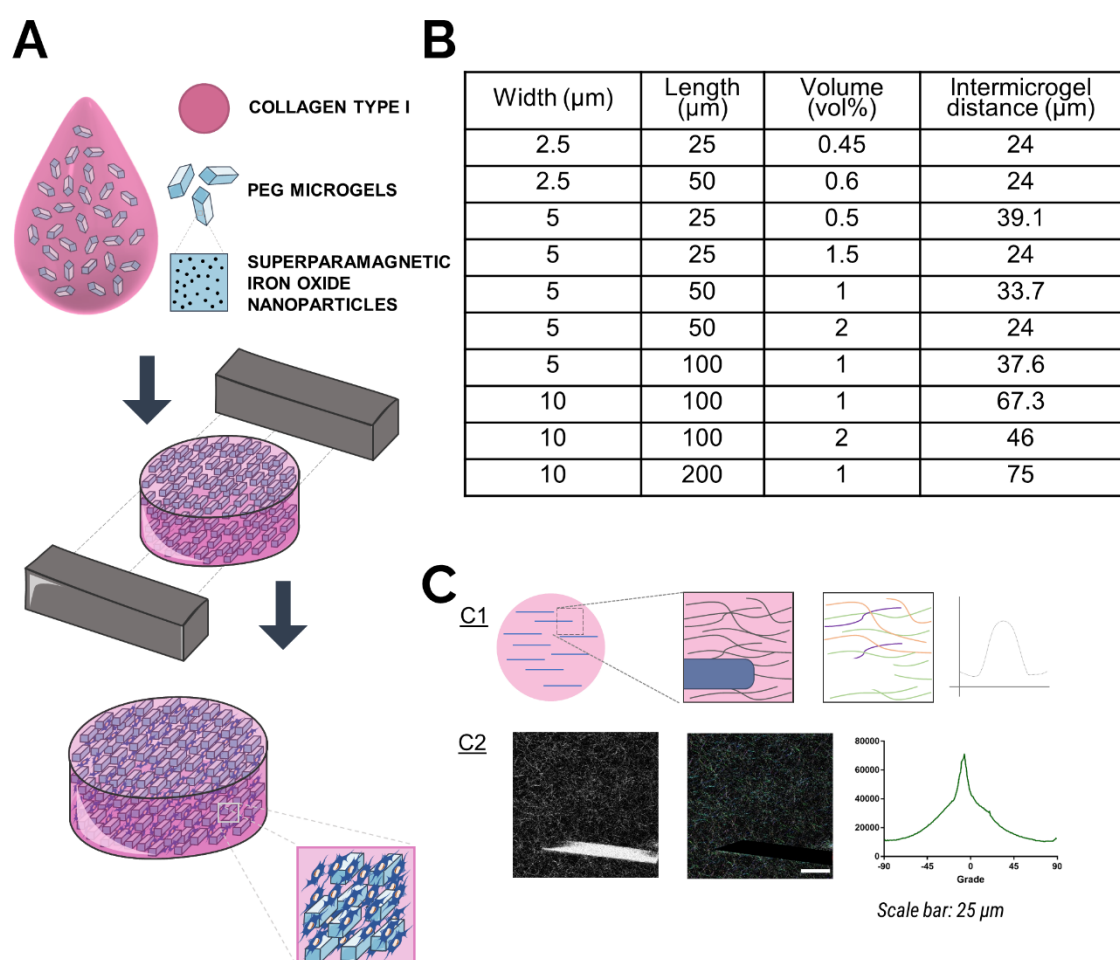


Figure 1 – **A:** Schematic representation of the experimental setup. **B:** Microgel's sizes description for all formulations evaluated. **C:** (C1) Schematic representation of the orientation of collagen fibers surrounding the microgels, which were analyzed analysis using Orientation J. (C2) Example given with fiber orientation analysis on confocal reflection microscopy images.

amount (Figure 1B), and its successful application in various surrounding hydrogels [28-32]. The anisotropy achieved by the aligned microgels is secured upon crosslinking of the surrounding hydrogel with cells encapsulated in 3D microenvironment.

The current work aims to develop a collagen-based Anisogel (collagen-Anisogel), using Col I as the surrounding hydrogel for the oriented PEG microgels (Figure 1A). We hypothesized that the proposed construct would provide anisotropy to growing cells in 3D, while microgel orientation would influence the properties of the Col I hydrogel. Therefore, bovine Col I was combined with PEG microgels of different size combinations (cross-section (width) and length). Collagen fiber formation, diameter, and orientation were analyzed, together with the hydrogels' mechanical properties and the behavior of human dermal fibroblasts in collagen-Anisogels.

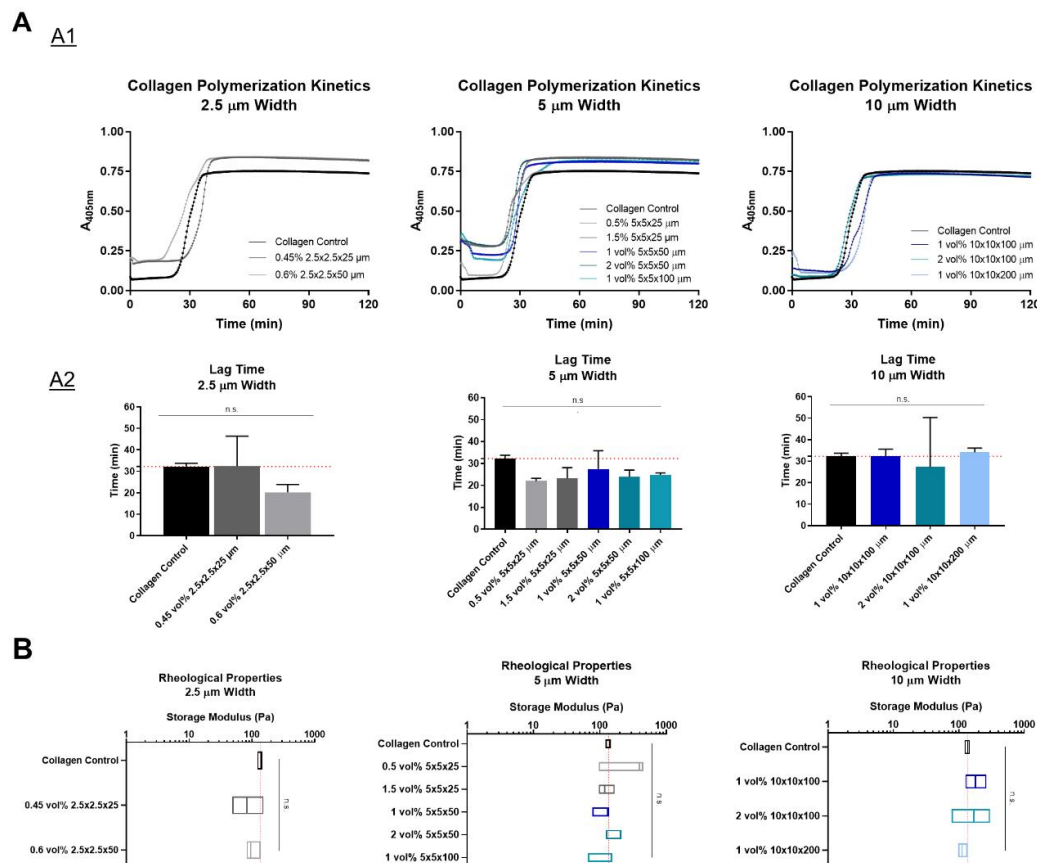


Figure 2 – Collagen-Anisogels formulations characterization in non-oriented conditions. **A:** Fibrillogenesis analysis of collagen-Anisogels formulations: A1 – Polymerization Kinetics profiles; A2 – Lag Time evaluation for different formulations. Data presented using bar graphs, with median and interquartile range. **B:** Rheological properties of collagen-anisogels. Data presented using box plot, with median and interquartile range.

2. Results and Discussion

2.1 Turbidimetric Profile of Non-Oriented Collagen-Anisogel Formulations

Collagen's characteristic fibril features are possible due to a mechanism of nucleation and growth. Initially, self-assembly of collagen molecules occurs by aggregation to form nuclei and later these nuclei grow into fibrils, in a process called fibrillogenesis, evaluated using turbidimetric assays [33,34]. Turbidimetric analysis was used to monitor fibrillogenesis of collagen-Anisogel formulations, without inducing magnetic orientation, to analyze the effect of the microgels' presence (Figure 2A). PEG microgels were prepared using in mold polymerization, as previously described [31], and combined with collagen solution prior to cross-linking. The different microgels sizes and concentrations tested are described in Figure 1B. Results obtained were grouped for microgels with the same width and presented as absorption (405 nm) versus time (Figure 2A1). They showed that absorbance increased with time, indicating fibril formation in the collagen-Anisogel formulations. To study the influence of microgel dimensions and concentrations in fibrillogenesis, the time required for initiation and completion of fibrils was measured (Figure 2A2). For all the tested formulations, the turbidimetric curves observed exhibited a sigmoidal shape, separated in three different stages: initial lag-phase, growth phase, and a plateau, reached after the formation of the hydrogel in an equilibrium of absorbance values [34,35]. Importantly, no distinct polymerization kinetics were observed between the collagen control and collagen-Anisogel formulations, which suggests the absence of alterations in fibril formation and growth mechanisms upon combination with PEG- μ gels [32], with median lag times ranging widely from 20.2 to 34.2 min. Formulations with smaller widths (2.5 and 5 μ m) showed an increase on final A405 values compared to the collagen control, possibly related to the higher amount of PEG microgels (calculated previously [30]) within the formulation. In general, the absorbance spectra showed that the addition of PEG microgels, independently of their size and concentration, preserved collagen's fiber formation.

2.2 Rheological Properties of Non-Oriented Collagen-Anisogels

To investigate possible alterations in basal collagen mechanical properties by the combination with PEG- μ gels, rheological studies were performed. The storage and loss modulus of the collagen-Anisogel formulations were measured upon in situ gelation at 37 °C for a period of 45 min. The storage modulus after reaching the plateau at the end of the analysis of each formulation is presented in Figure 2B, grouped for microgels with the same width. For the control group (collagen alone formulation), the storage modulus observed was 136 ± 13 Pa. The median storage modulus of the different collagen-Anisogel

formulations ranged from 95 ± 53 to 314 ± 190 Pa, with no statistically significant difference between formulations and the control group. These data demonstrated that the addition of PEG microgels does not significantly impact the hydrogel's mechanical properties, in non-oriented conditions, as similar stiffnesses were observed among all the formulations tested. Previous work on rheological studies of PEG hydrogels upon PEG microgels incorporation have also reported similar storage moduli between the control and Anisogel groups [31].

2.3 Collagen Fiber Diameter Characterization in Collagen-Anisogels

As the collagen macroscopic properties were not altered with different PEG- μ gel dimensions and concentrations, we probed the microscopic alterations after magnetic microgel orientation inside collagen-Anisogels. Microgel alignment was observed along the magnetic field lines within the collagen hydrogels (Figure S1, Supporting Information). This is similar to previously reported Anisogels that were formulated with a surrounding fibrin hydrogel stabilized by Ca^{2+} ions [28] or PEG hydrogel that are enzymatically crosslinked

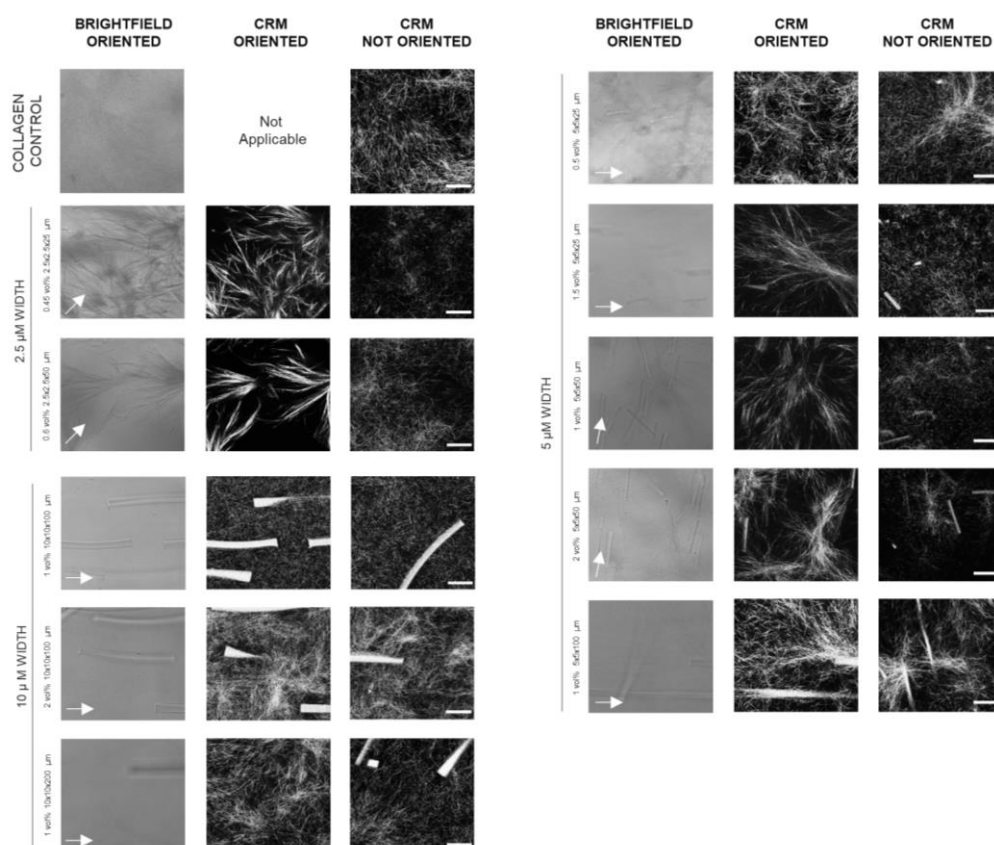


Figure 3 – Fiber characterization in collagen-Anisogels. Representative brightfield and confocal reflectance microscopy images for collagen control and tested conditions of collagen-Anisogel both with and without magnetic stimuli. White arrows indicate the direction of the magnetic field and PEG microgels. Scale bar: 25 μm

[32]. Next, we assessed the influence of aligning PEG- μ gels inside the collagen solution during gelation on the collagen fiber diameters. This can be achieved using confocal reflectance microscopy (CRM) where the collagen fibers are reflected using 488 nm laser, a popular technique to visualize collagen structures [36]. A series of images of collagen control and collagen-Anisogels, both in oriented and non-oriented conditions, were captured at high magnification for fiber visualization (Figure 3). As PEG- μ gels were also reflected, as observed in Figure 3, before proceeding to diameter and orientation evaluation, all images were corrected in Fiji software to remove the artifacts caused by the PEG microgels.

Fibers' diameters were analyzed to evaluate alterations in the presence of oriented PEG- μ gels. A script was designed to evaluate the thickness of the fibers using python. Diameters were calculated by measuring the pixels from the CRM images and grouped considering diameter ranges of 0–400, 400–800, 800–1200, and 1200 nm and above. Figure 4A shows the representation of the collagen control and all collagen-Anisogels tested, grouped again by microgel width. Starting with the 2.5 μ m wide microgels, no differences were observed between the collagen control and collagen-Anisogels for the smallest fiber diameters. When looking into diameters ranging from 400 to 800 nm, we observed a significant decrease in the fiber diameter in 0.6 vol.% 2.5 $\times$ 2.5 $\times$ 50 μ m microgels condition. Interestingly, this condition showed a tendency for enabling fibrils with larger diameter fibers (>1200 nm).

Looking into the 5 μ m wide microgels, for the longer length (5 $\times$ 5 $\times$ 100 μ m microgel condition), we observed a similar trend compared to the collagen control for all diameter ranges. For the other tested conditions within this group, a tendency for higher frequency in smaller diameters (<400 nm) and a decreased frequency in intermediate diameters (400 to 800 nm) was observed, when compared with collagen control. However, statistically significant differences were only observed for 1.5 vol.% 5 $\times$ 5 $\times$ 25 μ m microgels condition. For the larger fiber diameters (>800 nm), no differences were observed between the collagen control and collagen-Anisogels.

Regarding the 10 μ m wide microgels, the 1 vol.% 10 $\times$ 10 $\times$ 200 μ m microgels condition showed significant alterations in fiber diameter, compared to the collagen control, namely with an increase in <400 nm diameter and decrease in 400–800 nm. The other two conditions, both with a length of 100 μ m, showed structures similar to collagen control for all the fiber diameters analyzed. Overall, by comparing with collagen control, the significant alterations mainly involved an increase in thinner fibers (diameter <400 nm) accompanied by a decrease in middle size diameter (diameter between 400 and 800 nm), as seen in 1.5 vol.% 5 $\times$ 5 $\times$ 25 μ m and 1 vol.% 10 $\times$ 10 $\times$ 200 μ m microgels conditions. For 0.6 vol.% 2.5 $\times$ 2.5 $\times$ 50 μ m microgels condition, a decrease on middle size diameter and a tendency for

an increase of larger fibers (diameter >1200 nm) was observed. Previous studies using combinations of collagen and other biomaterials have shown alterations in fiber diameter. For instance, when combining a high chondroitin sulfate concentration with collagen, increase or decrease in fiber bundling has been reported [37]. The impact of fiber diameter

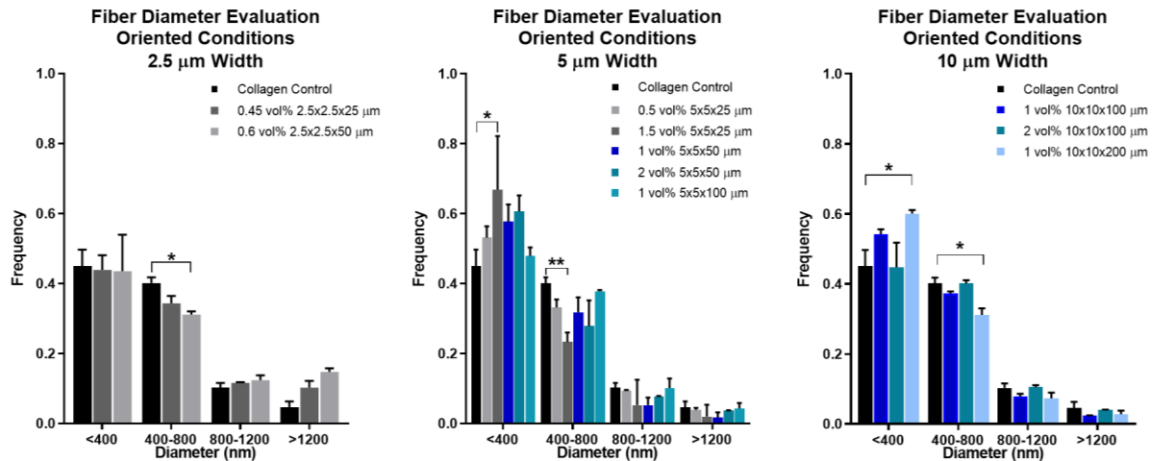
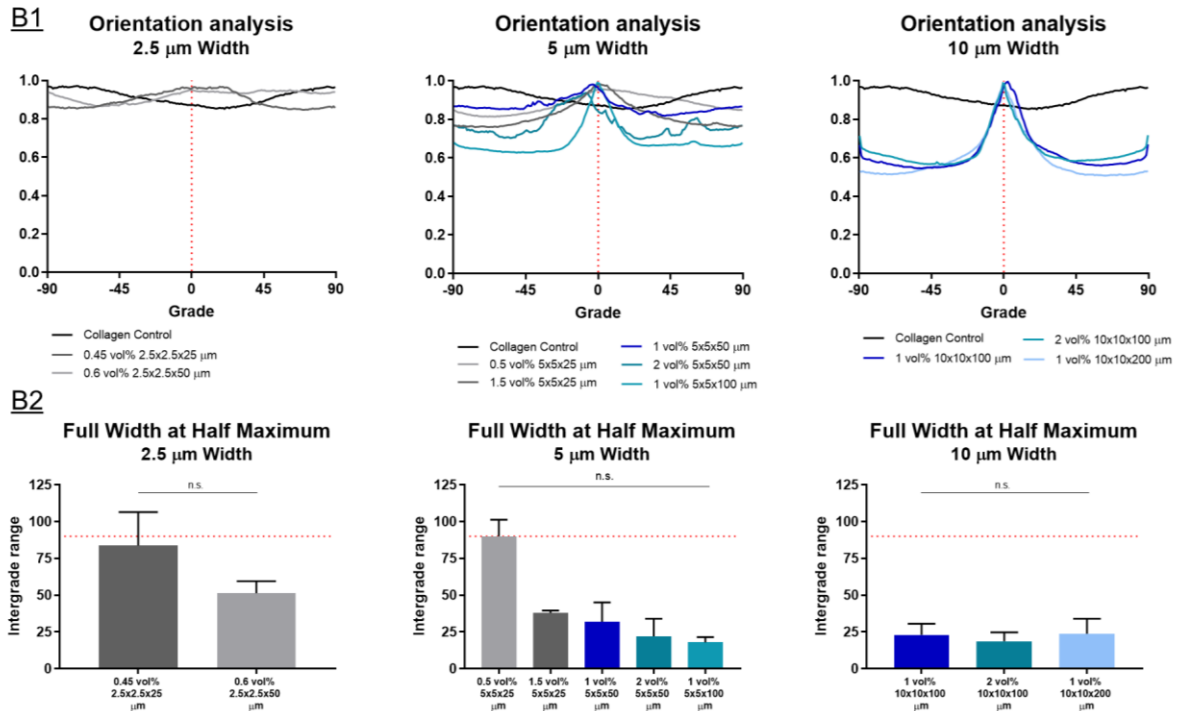
A**B**

Figure 4 – Collagen fiber characterization under oriented conditions. A: Diameter analysis regarding microgel cross-section. Data presented using bar graphs. with median and interquartile range. Kruskal-Wallis followed by Dunn's comparisons test was performed, * $p < 0.05$; ** $p < 0.01$. **B:** Orientation analysis. B1 – Orientation characterization of collagen fibers compared to microgel alignment. B2 – Full Width at Half Maximum evaluation. Data presented using bar graphs. with median and interquartile range.

on cell morphology has been analyzed as well in previous studies. For example, in poly Lactic-co-Glycolic Acid (PLGA) meshes cultured with NIH3T3 fibroblasts, an increased fiber diameter (>1400 nm) was shown to enhance aspect ratios and projected cell areas, while cells cultured in meshes with smaller fiber diameters (<400 nm) exhibited reduced cell spreading [38]. Additionally, the impact of fiber diameter on human dermal fibroblasts has been assessed in electrospun bioresorbable poly(desamino tyrosyl-tyrosine ethyl ester carbonate) scaffolds, showing that smaller fiber diameters (≈ 600 nm) exhibited less focal adhesion complexes compared to larger diameters (>2000 nm). The increased diameters also allowed for higher long-term (above 2 weeks) cell proliferation [39]. As most of the tested collagen-Anisogels retained the diameter profile similar to the collagen control, the addition of PEG microgels is likely to not affect the collagen hydrogel function during 3D cell culture.

2.4 Collagen Fiber Orientation Analysis in Collagen-Anisogels

The Anisogel strategy has been extensively studied and characterized regarding microgel dimensions and aspect ratio [30,40]. In collagen-Anisogels, collagen fiber orientation was evaluated, using CRM images analyzed with Orientation J, an established Fiji plug-in. Figure 4B1 shows the orientation curves, from -90 to 90° for each width group and collagen control, with the direction of microgel orientation normalized to 0° . CRM images showed partial collagen fiber orientation along the magnetic field inside the collagen-Anisogel for all microgel sizes. To evaluate the differences between the partial alignments in each collagen-Anisogel and compare it to the random fiber orientation of the collagen control, we conducted an isolated analysis of full width at half maximum (FWHM) between the maximum and minimum values for each condition (Figure 4B2). Among the partially oriented collagen fibers, the microgels with a larger width ($10\text{ }\mu\text{m}$) showed the highest collagen fiber orientation, with the small FWHM values (mean ranging from 18.67 to 23.75°) and no significant differences between the different lengths were observed. The broad distribution was achieved with the $2.5\text{ }\mu\text{m}$ wide microgels exhibiting high FWHM, while $5\text{ }\mu\text{m}$ wide microgels at $0.5\text{ vol.}\%$ $5 \times 5 \times 25\text{ }\mu\text{m}$ microgel condition showed a higher FWHM ($90 \pm 11.3^\circ$) however no statistical differences were observed among conditions with longer or higher density of microgels formulations, as also seen in Figure 4B1. Interestingly, considering $5\text{ }\mu\text{m}$ wide microgels, specifically with a 25 or $50\text{ }\mu\text{m}$ length, we observed a decreasing FWHM, therefore an increase in fiber orientation, when the concentration of microgels is increased. These results were consistent with previous studies conducted by our group showing higher cell orientation in Anisogel conditions with higher concentrations of microgels [31]. Yet, such tendency was not observed in the case of larger microgels (10

$\times 10 \times 100 \mu\text{m}$, at 1% and 2%) inside collagen-Anisogels, as both microgel concentrations showed low FWHM values.

Comparing different microgels widths, results revealed that an increase in collagen fiber orientation, characterized by a smaller FWHM was achieved with larger microgels with a width of $10 \mu\text{m}$ and lengths equal or larger than $100 \mu\text{m}$. This partial alignment of the collagen fibers will act together with the oriented PEG microgels to provide anisotropy to the collagen-Anisogels. Nevertheless, caution should be taken as a full fiber orientation was never achieved and discussion can only be done regarding partial orientation.

2.5 Normal Human Dermal Fibroblasts Cell Behavior in Collagen-Anisogels

Considering the results obtained so far, we performed cell *in vitro* studies with three types of collagen-Anisogels, using the conditions of: 0.6 vol.% $2.5 \times 2.5 \times 50 \mu\text{m}$ (no collagen fiber orientation), 1 vol.% $5 \times 5 \times 50 \mu\text{m}$ (intermediate partial collagen fiber orientation) and 2 vol.% $10 \times 10 \times 100 \mu\text{m}$ (high partial collagen fiber orientation). Figure S2 (Supporting Information) illustrates the control evaluation for collagen fiber orientation in collagen gels containing random microgels that were not magnetically aligned in the specified conditions, demonstrating no collagen alignment. Normal human dermal fibroblasts (NHDF) were employed, as they are majorly producing and inhabiting in collagenous tissue [41]. Cells were encapsulated in these formulations and cultured for 7 days. Collagen-Anisogels were analyzed for culture periods of 1, 5, and 7 days.

At each time point, cell orientation was measured by staining the actin filaments in all samples. On visual inspection, the actin micrographs (Figure 5A) showed cell orientation already from day 1 for all the three collagen-Anisogels conditions, as seen by the orientation distributions of the stained cytoskeleton (F-actin) (Figure 5B,C) and subsequent FWHM analysis (Figure 5D). A clear distinction of cell orientation was observed between the aligned collagen-Anisogels and collagen gels containing non-aligned microgels for each condition after 1 day of culture, as shown in Figure S3 (Supporting Information). At day 1, all collagen-Anisogels conditions showed significant reduced FWHM values compared to collagen control (**** $p < 0.0001$). Moreover, collagen-Anisogel condition using $10 \times 10 \times 100 \mu\text{m}$ microgels appeared to induce a higher cell orientation compared to smaller microgels ($2.5 \times 2.5 \times 50 \mu\text{m}$, $p = 0.0869$). With time in culture (Figure 5D1), the $10 \times 10 \times 100 \mu\text{m}$ collagen-Anisogels demonstrated consistency in providing cell anisotropy, always showing significant differences to cells encapsulated in collagen control regarding FWHM values (at day 1, **** $p < 0.0001$; at day 5, * $p < 0.05$; at day 7, ** $p < 0.01$). $2.5 \times 2.5 \times 50 \mu\text{m}$ collagen-Anisogels exhibited significant higher cell orientation compared to collagen control (characterized by

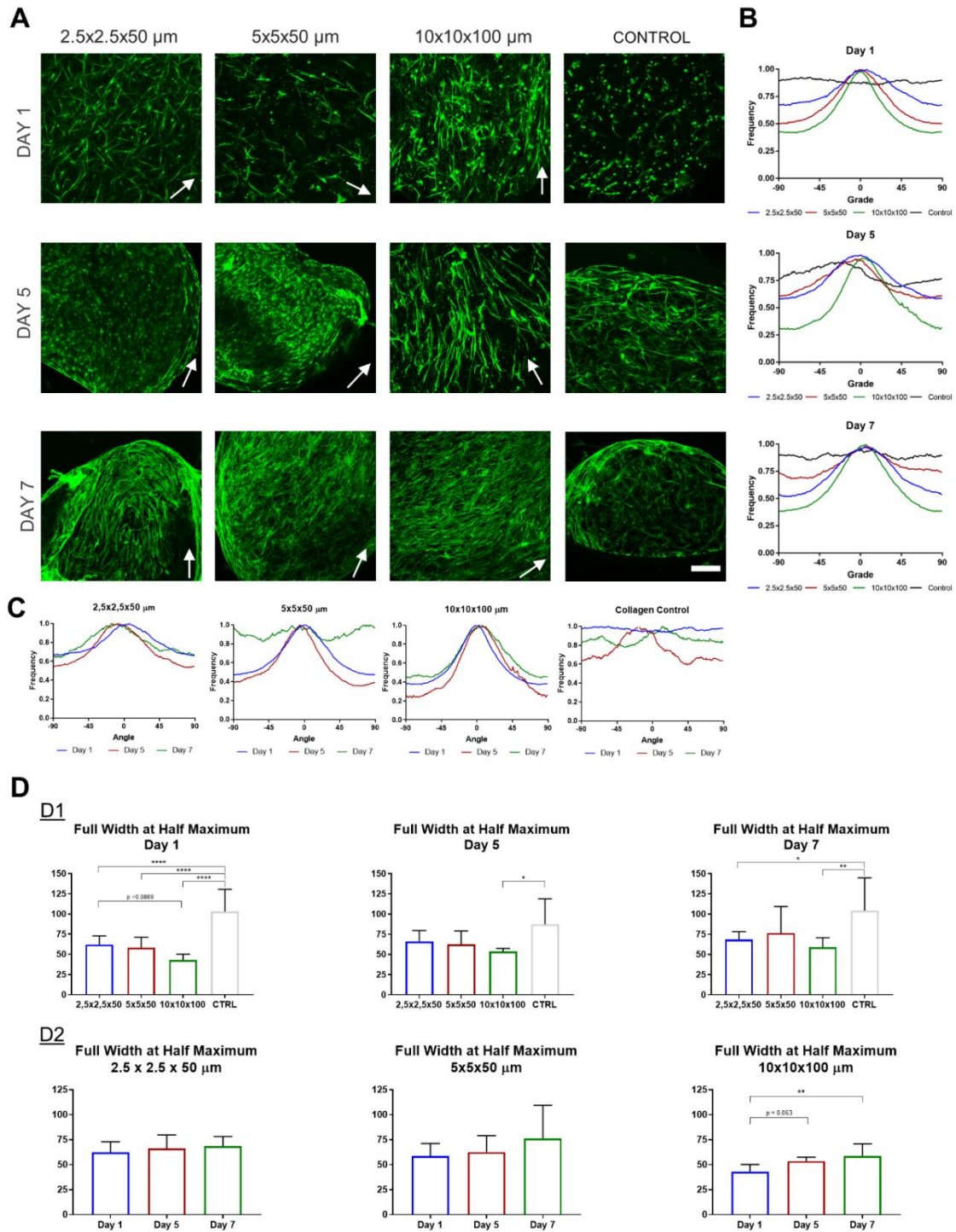


Figure 5 – Fibroblasts (NHDF) behavior in collagen-Anisogels. **A:** Cell orientation in collagen-Anisogels through actin staining evaluation at day 1, 5, and 7. White arrows indicate the direction of the magnetic field and PEG microgels. Scale bar = 100 μm . **B:** Actin orientation characterization per day in culture. **C:** Actin orientation characterization per microgel condition. **D:** Full Width at Half Maximum evaluation. Data presented using bar graphs. with mean and standard deviation. Ordinary one-way ANOVA followed by Tukey's multiple comparisons test was performed, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$

a reduced FWHM) only for day 1 and day 7 (**** $p < 0.0001$; and * $p < 0.05$, respectively). Although $5 \times 5 \times 50 \mu\text{m}$ collagen-Anisogels showed significant alignment at day 1 compared to collagen control, this was not consistent with time in culture. Cell orientation results are according to the orientation of the collagen fibers inside these collagen-Anisogels (Figure 4B). When analyzing the behavior of the cells in each collagen-Anisogel throughout time (Figure 5D2), we observed a tendency for an increase of FWHM values with time, and therefore a reduction in cell orientation. This increase was more significant in $10 \times 10 \times 100 \mu\text{m}$ collagen-Anisogels (day 1 versus day 5, $p = 0.063$; day 5 vs day 7, ** $p < 0.01$) and may be due to the increased number of cells over time, which influences the cell material interaction causing strain-induced stiffening in collagen-Anisogels. Moreover, cells sense collagen substrate topography [42] through mechanical forces and ECM interactions, which might be influencing the cells' ability to maintain their anisotropy. Further studies addressing the influence of cell proliferation on their orientation and its mechanobiological implication on collagen ECM should be conducted. Nevertheless, for 2 vol.% $10 \times 10 \times 100 \mu\text{m}$, cell orientation remained more aligned than in the control collagen gel throughout the period of culture, exhibiting the most favorable conditions for establishing a stable long-term anisotropy in collagen-Anisogels (Figure 5B–D).

Fibroblasts have been previously reported to align in anisotropic conditions [43, 44]. Efficient cell adhesion and proliferation has been reported in a collagen/PEG hydrogel using human fibroblasts [45]. Similarly, our results demonstrated that NHDF cells efficiently grew within collagen-Anisogels, exhibiting alignment with the unidirectional orientation of the PEG microgels established during the initial gelation process and up to 7 days in culture. Mimicking native cell anisotropy is a key aspect for accurate design of *in vitro* models that recapitulate tissue architecture and organization to resemble physiological conditions [46, 47]. In addition, these models are of interest to understand disease models in anisotropic collagenous tissues, which are sensitive to cellular forces and cell population, such as in musculoskeletal and cardiovascular research, allowing for a deeper understanding of the cellular alterations and for a better therapeutics design, among others [48, 49].

2.6 Hydrogel Shrinkage Evaluation upon Cell Culture

The collagen hydrogels experienced shrinkage due to stress-induced matrix stiffening as expected in all hydrogels conditions [50,51]. This was observed visually at macroscopic level in Figure 6A1. To comprehend the effect of PEG- μgels in collagen-Anisogels, hydrogel contraction was evaluated by measuring the area revealing a reduction to near 20% at day 7 compared to day 0 (Figure 6B1). No macroscopical differences were observed when comparing shrinking between the collagen-Anisogels and collagen gels with random

microgels (data not shown). At day 1 (Figure 6B2), the collagen control had a greater area reduction compared to collagen-Anisogels (*, $p < 0.05$ for $5 \times 5 \times 50 \mu\text{m}$; $p = 0.0506$ for $2.5 \times 2.5 \times 50 \mu\text{m}$; $p = 0.0556$ for $10 \times 10 \times 100 \mu\text{m}$). At day 7 (Figure 6B3), there was a clear reduction in hydrogel contraction in $10 \times 10 \times 100 \mu\text{m}$ collagen-Anisogels compared to all other conditions, especially compared to $2.5 \times 2.5 \times 50 \mu\text{m}$ (*, $p < 0.05$) and to collagen control (**, $p < 0.01$), revealing that this condition not only promoted long-term oriented cell growth, but also limited strain-induced collagen contraction, which is often one of the key problems in collagen-based biomaterials development [52,53].

An improvement of the shrinkage process has also been reported in a Col I hydrogel combined with PEG as a crosslinker, for skin applications [54], however in our case the PEG microgels were likely not covalently linked to the collagen. Tuning collagen hydrogel shrinkage is of paramount importance for a better development of *in vitro* models and implantable biomaterials, regarding cell culture time and therapeutic potential [53]. Indeed, depending on the specific application, research has focused on both restrain shrinkage, as in for muscle tissue engineering [55], and in increasing shrinkage, to develop denser tissues and better resemble heart tissue [56].

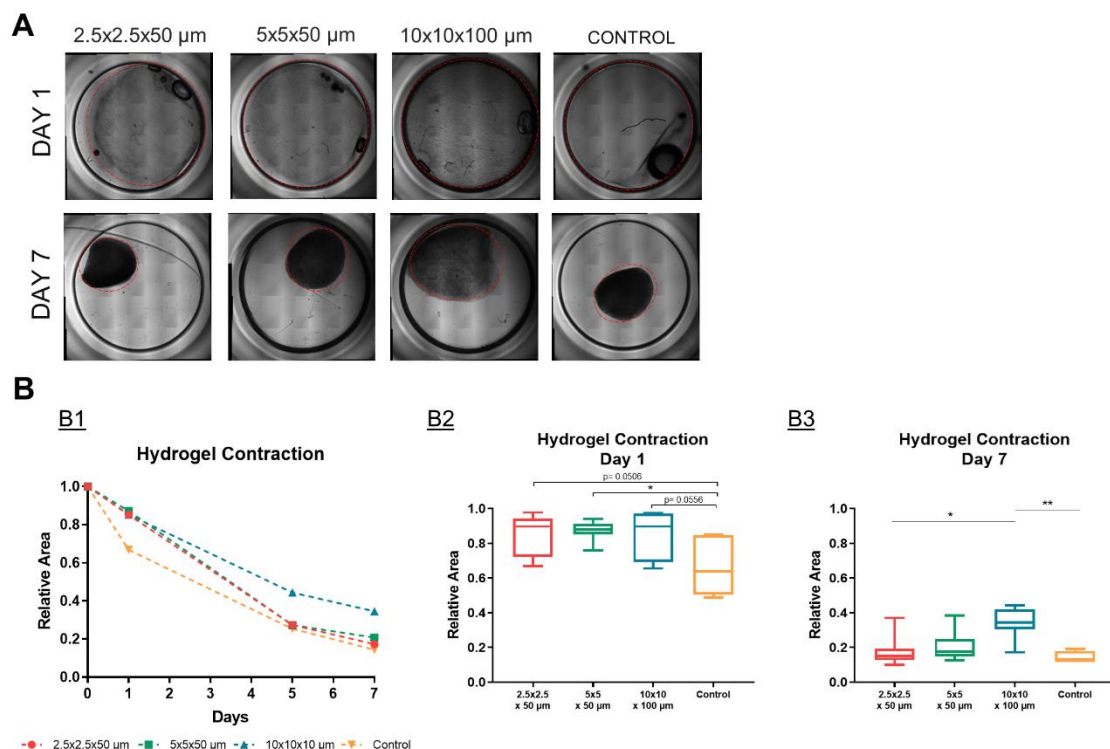


Figure 6 – Cellular Hydrogels Analysis. **A:** Evaluation of hydrogel contraction. A1 – Brightfield mosaic images of cellular hydrogels. **B:** Hydrogel contraction analysis by area evaluation (B1); Detailed comparison at day 1 (B2) and day 7 (B3). For B2, ordinary one-way ANOVA test was conducted, with Tukey's multiple comparison, * $p < 0.05$. For B3, Kurskal-Wallis test was conducted, with Dunn's comparison, * $p < 0.05$, ** $p < 0.01$.

Collagen-Anisogels have shown not only to induce collagen fiber orientation without impairing their mechanical properties, but also demonstrated the potential of *in vitro* collagen-based anisotropic models, allowing oriented cell growth and sustaining cellular orientation for several days. In collagen-Anisogels with larger microgels ($10 \times 10 \times 100 \mu\text{m}$), low hydrogel shrinkage was also observed, while for smaller size microgels ($2.5 \times 2.5 \times 50 \mu\text{m}$ and for $5 \times 5 \times 50 \mu\text{m}$) profound shrinkage was present, leading to opportunities for controlled collagen scaffold shrinkage using Anisogel-based models. This technology, therefore, shows promising opportunities in the bioengineering of *in vitro* functional tissues.

Anisotropic formulations, here applied with Col I, are a key component of several organs in the human body, having well-established oriented structures in homeostatic conditions in several tissues, such as tendon, intervertebral disc, bone, among others. Therefore, the collagen-Anisogels developed here can be applied as a potential platform for different applications in tissue engineering, ultimately contributing to a better comprehension of anisotropic-related pathomechanisms.

3. Conclusion

To our knowledge, this is the first study reporting an Anisogel composed by PEG rod-shaped microgels and Col I as surrounding gel. Besides evaluating the orientation properties of the hydrogel, we started by evaluating the impact on the collagen fibril when adding PEG microgels. Here, we demonstrated the maintenance of the key characteristics of collagen hydrogels considering fiber formation and mechanical properties. Moreover, we explored different dimensions and concentrations of PEG microgels to induce anisotropy by magnetically aligning the microgels during collagen gelation and studied the effect of this alignment on collagen fiber diameter and orientation. Microgels with larger width and length showed promising results in regulating collagen fiber orientation, without impairing fiber thickness. Human fibroblasts oriented along the microgels and collagen fibers, especially for the larger microgels, which also led to a reduction in hydrogel contraction, serving as a promising feature for further experiments. Future studies will include deeper analysis on other cell types and tissues, to recapitulate *in vivo* mechanisms and diseases and reassuring the potential of collagen-Anisogels as a platform for anisotropic studies.

4. Experimental Section

4.1. Production of PEG Microgels

PEG microgels were prepared as previously described [31]. Briefly, microgels were produced by an in-mold polymerization method. The mold was prepared by mixing

polydimethylsiloxane (PDMS, Sylgard184, Dow Corning) with a thermo initiator (10:1 ratio), followed by pouring the mixed solution on silicon wafers (AMO GmbH) with different size patterns. The wafer with the liquid PDMS solution was cured at 110 °C for 4 h, while the PDMS mold was peeled off the wafer after curing. Next, a prepolymer solution (20 wt.% PEG di-acrylate (PEG-DA, Sigma Aldrich) and photoinitiator (Irgacure 2959, Sigma Aldrich) upon sonication was diluted in 200 Da PEG-OH (Sigma Aldrich) with 400 µg mL⁻¹ superparamagnetic iron oxide nanoparticles (SPIONs; EMG-700, Ferrotec)). The solution was poured on top of the PDMS mold and laminated with a polyethylene terephthalate (PET) foil (Goodfellow). By delaminating the PET film slowly, excess prepolymer solution was removed. The mold with PEG-DA solution was then cured in a nitrogen atmosphere for 60 min under UV light. Subsequently, the molds were cut in smaller sizes and glued on glass slides using a 50% polyvinylpyrrolidone glue (360 kDa, Sigma–Aldrich) layer, allowing the harvesting of the dried microgels by peeling the mold after 5 days at 37 °C. Microgels were harvested by dissolving the dried glue in water, purified multiple times in water by centrifugation, UV-sterilized, and disinfected in 70% ethanol. Last, microgels were washed in PBS (Lonza), quantified using a Neubauer chamber, and stored for a maximum of 4 weeks at 4 °C.

4.2. Production of Collagen-Anisogels

Type I collagen (Bovine skin, Sigma-Aldrich) working solutions were prepared following the manufacturer's instructions. Briefly, to achieve a 0.32 w/v% concentration, eight parts of collagen G (Sigma-Aldrich) were mixed with one part of Dulbecco's modified Eagle medium (DMEM) and one part of deionized water. The final solution was neutralized by adding 2 v/v% NaOH (1 M). When PEG microgels were mixed within the collagen solution, the microgels were part of the one part of deionized water, without altering the final collagen concentration and maintaining the neutralization step. Different dimensions and concentrations of PEG microgels were used leading to several collagen-Anisogels formulations, as described in Figure 1B.

4.3. Analysis of Fibrillogenesis for Different Collagen-Anisogels Formulations

The polymerization kinetics of collagen-Anisogels formulations were measured through a turbidimetric assay. The described collagen-Anisogels solutions were prepared and neutralized as previously described and transferred to 96-well plates (glass bottom, Corning), which were previously cooled to 4 °C to inhibit an early onset gelation. Plates were loaded into a microplate reader (BioTek Synergy HT), which was pre-heated to 37 °C. Absorbance at 405 nm was measured for 2 h, at 30 s intervals. Kinetic parameters were

calculated from the sigmoidal-shaped curves, which included lag time and maximum absorbance value.

4.4. Analysis of the Rheological Properties of Collagen-Anisogels Formulations

All collagen-Anisogels formulations were prepared as described in 2.2 and kept on ice prior to rheological measurements. A rheometer (TA instruments, DHR) with a cone-plate geometry (20 mm, 2.0°) was used. Time sweep analyzes were conducted at 37 °C for 45 min for all tested formulations, after which a frequency (0.25–1 Hz) and amplitude (0.15–1%) sweep were performed, without magnetic stimulation, to confirm that the formulations were in the linear viscoelastic regime at the used conditions described in Table S1 (Supporting Information).

4.5. Preparation of Magnetically Oriented Collagen-Anisogels

Collagen-Anisogels formulations were prepared as described in 4.2. and kept on ice until casting on the ibidi chambers. Samples were casted in volumes between 10–20 µL in a well plate, in the presence of a uniform magnetic field of ≈100 mT, applied by using a pair of neodymium rectangle magnets (Magnosphere, Germany) (Figure 1A). Samples were incubated at RT for 5 min to allow for the orientation of the microgels within the hydrogels, and then transferred to an incubator at 37 °C for 60 min to trigger and complete the crosslinking of the collagen fibrils. Fresh media / PBS was added after the complete hydrogel crosslinking.

4.6. Analysis of Collagen Fibers

Collagen-Anisogels were produced as described in 4.2, together with control samples (not subjected to magnetic field), and stored in phosphate buffer saline (PBS 1x, pH 7.4). CRM was used to acquire high-resolution images of the collagen fibers at high magnification (63x) in their native and hydrated state. Confocal imaging was performed using an SP8 Tandem Confocal (Leica Microsystems Inc.), with an argon-ion laser adjusted to 488 nm emission as a light source, used in reflection mode with a 63x oil immersion objective. Images were collected at 8–12 positions within three independent hydrogels per each different collagen-Anisogel. The 3D fiber network was evaluated considering fiber diameter and orientation, both quantified from images with Python and Fiji (free software (v2.0.0)) software, respectively. The reflection of PEG-µgels in the samples were removed prior to analysis (Figure 1C). Diameter analysis was written in python (<https://www.python.org>) using the libraries: numpy (<https://numpy.org>), matplotlib (<https://matplotlib.org>) and ImageP and BatchAnalyzer libraries developed by team, both available at github under

<https://www.github.com/tomio13/ImageP> and <https://www.github.com/tomio13/BatchAnalyzer>. Images were first blurred through convolution with a Gaussian kernel (standard deviation of 100 pixels, window size 801 pixels). This blurred image was subtracted as background, resulted negative values set to zero. Then the image was smoothed using another Gaussian kernel with standard deviation of 1 pixel, window size 11 pixels. For increasing the detail content, the dynamic range of the image was compressed setting the intensity values to a power of 0.5. Fibers were detected then applying a dynamic threshold calculated with the method of Otsu [57]. The width of the fibers was established employing a distance transform on the binary images (based on the work of [58]). Then a local maxima search was applied to collect the center points indicating the local radii of the fibers. The diameter values calculated from these local radii were then used to calculate a thickness histogram for each image. OrientationJ, a Java plugin for Fiji [59], was used to map the directionality in the images (gradient: Gaussian; local window: 2 pixel), where each color is assigned to an orientation angle (Figure 1C).

4.7. Cell Encapsulation and 3D Cell Culture in Collagen-Anisogels

NHDF (Promocell) were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Gibco) at 37 °C under 5% CO₂. Cells from passages below 10 were used for the *in vitro* experiments. Briefly, collagen-Anisogels were prepared as described in section preparation of magnetically oriented collagen-anisogels, with cells being part of the eight-part collagen prior to mixing the other components. The casting of the precursor solutions in the presence of a uniform magnetic field was performed as above mentioned. Collagen-Anisogels with NHDF, and collagen hydrogels with NHDF as control, were kept in culture for 7 days, and culture media was replaced on day 5. Brightfield mosaic images with magnification of 10x were captured for each collagen gel at time day 1, 5, and 7 days of culture, using the confocal microscope. The hydrogel contraction was evaluated by area measurements using Fiji software.

4.8. Immunofluorescence Staining and Imaging

Hydrogels were fixed in 4% paraformaldehyde (PFA, AppliChem) in PBS for 40 min after 1, 5, and 7 days of culture. The samples were washed twice with PBS, permeabilized using 0.1% Triton X-100 (Sigma–Aldrich) in PBS for 40 min and washed twice again with PBS. Actin staining was performed by incubating the samples with phalloidin-iFluor 488 and 555 (1: 1000, Abcam) for 3 h, followed by PBS wash (2x) for 30 min. All samples were incubated during staining and washing steps in an elliptical plate shaker at 30 rpm. Samples were stored in PBS at 4 °C until imaging.

Z stack images of collagen-Anisogels with encapsulated cells were imaged using a 10x magnification air objective in a laser scanning confocal microscope (SP8 Tandem Confocal, Leica Microsystems Inc). The light source used was an argon-ion laser adjusted to 488 and 555 nm emission (fluorescence from microelements). Z-stack images were collected from 2–3 random locations in the middle of the hydrogels, with a stack size between 50 and 150 μm for the three biologically relevant replicas for each independent time point (1, 5, and 7 days of culture). Actin orientation was quantified by superimposing the z-stack images, using Fiji software. Cell orientation was evaluated following the same procedure described before for fiber orientation analysis.

4.9. Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (v5). A Shapiro Wilk normality test was used to assess data normality. When a normal distribution was verified, one-way ANOVA analysis followed by Tukey's multiple comparison test was performed, and results were presented using mean and standard deviation. For analysis not following a normal distribution, a Kruskal–Wallis test followed by corrected Dunn's were performed, and results were presented using median and interquartile range. For evaluations regarding paired experiments, upon normality assessment, Friedman test followed by Dunn's comparison were conducted. Statistical significance was considered for * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

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Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Data**Tables****Table S1.** Rheological parameters (Strain and Frequency) tested for all formulations evaluated.

Formulation	Strain (%)	Frequency (Hz)
Collagen Control	1	1
0.45 vol% 2.5x2.5x25	0.25	0.5
0.6 vol% 2.5x2.5x50	0.25	0.5
0.5 vol% 5x5x25	0.15	0.5
1.5 vol% 5x5x25	0.15	0.5
1 vol% 5x5x50	0.15	0.5
2 vol% 5x5x50	0.15	0.5
1 vol% 5x5x100	0.2	0.25
1 vol% 10x10x100	0.2	0.25
2 vol% 10x10x100	0.2	0.25
1 vol% 10x10x200	0.2	0.25

Figures

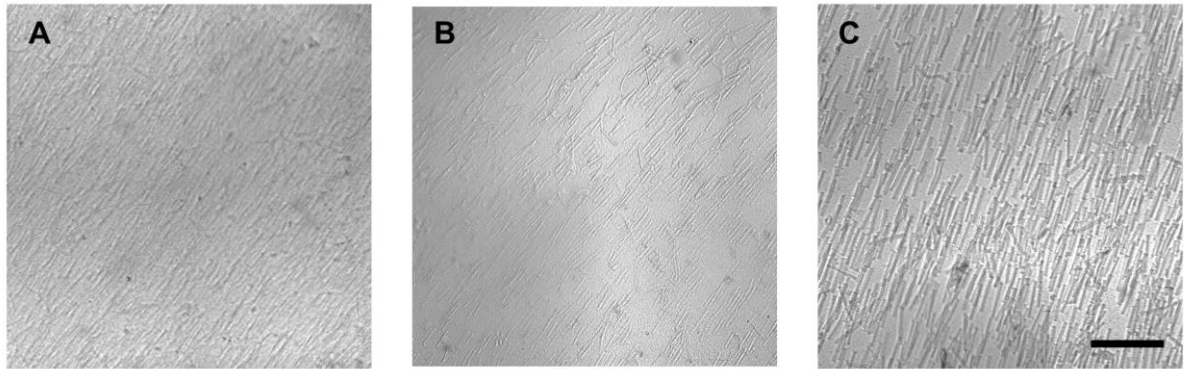


Figure S1 – Brightfield images of collagen-Anisogels. A) 0.6 vol% 2,5x2,5x50 μm ; B) 1 vol% 5x5x50 μm ; C) 2 vol% 10x10x100 μm . Scale bar: 200 μm

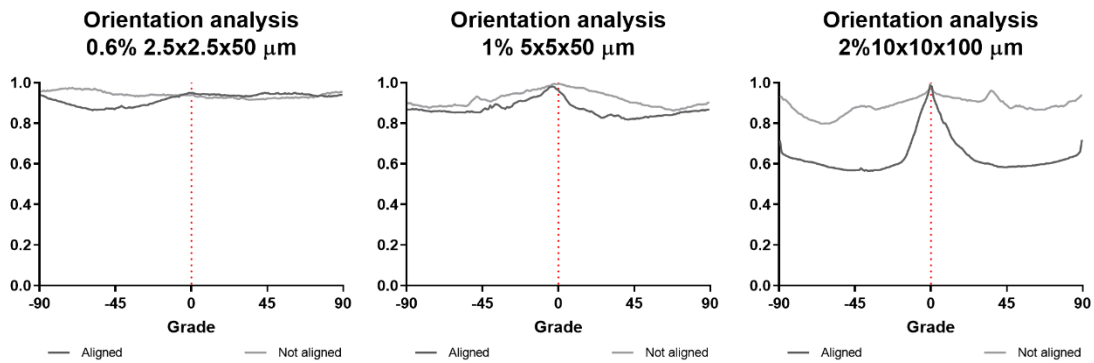


Figure S2 – Orientation characterization of collagen fibers in collagen gels containing magnetically aligned and not aligned microgels for different collagen-Anisogel formulations: 0.6 vol% 2.5x2.5x50 μm ; 1 vol% 5x5x50 μm ; and 2 vol% 10x10x100 μm .

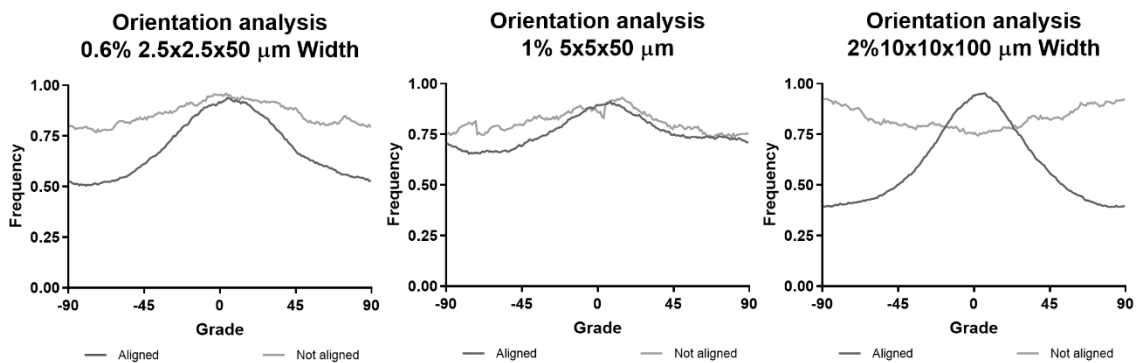


Figure S3 – Cell orientation in collagen-Anisogels and collagen gels consisting of randomly oriented microgels through actin orientation characterization at day 1 in culture.

Chapter VI

General Discussion and Future Perspectives

1. General Discussion

LBP is continuously affecting the population worldwide, without effective solutions that can restore the IVD and consequently, patient's health and well-being [1]. Among LBP contributors, LDH arises as one of the most significant contributors to the current economic and social burden, since it is pointed as the main cause of spine surgery [2]. The shortage of therapeutic approaches that target the IVD and the lack of understanding the pathomechanisms of LDH, creates a central obstacle towards a better outcome for LDH.

AF tissue is one of the major players in LDH, as IVD degeneration leads to the loss of integrity of this tissue, impairing its ability to restrain the NP and promoting IVD herniation [3]. As researchers have improved the knowledge of IVD disorders in the last years, AF tissue is often neglected, with much more studies being conducted in the NP. Therefore, it is urgent to better understand the pathomechanism behind AF tissue failure during LDH to ultimately develop more efficient strategies to regenerate the herniated IVD.

Fibrosis has been associated with IVD disorders, mainly IVD degeneration and NP tissue [4]. Alterations in Col I, II and FN have been reported to be associated with aging and degeneration [4-6]. Moreover, other key players in fibrosis, as the presence of cells such as myofibroblasts and macrophages, have also been identified in diseased IVD [7, 8]. In this work, a systematic characterization of the fibrotic alterations in AF failure and their respective correlations with hernia progression was performed. Specifically, alterations were observed considering disorganization of collagen fibers, sGAG, Col I, FN and Col II content and α -SMA, expression. While a systematic characterization was performed, we cannot exclude other markers that might be associated with herniation progression. For that, it would be important to expand this characterization to a broader analysis using big data techniques, as proteomics, to, for example, characterize the presence of inflammatory mediators in IVD herniation. In fact, recently, Compte et al. characterized the relationship between five circulating inflammatory mediators and IVD degeneration and Modic change classification and found no correlation [9]. The characterization of hernia progression should also be investigated, to disclose potential inflammatory targets. Moreover, it would also be relevant to expand the performed characterization considering the spatial distribution of AF fibrotic markers. AF tissue is often distinguished between inner and outer AF as different cell phenotypes and ECM composition have been reported [10, 11]. Lu et al. have shown that inner and outer AF cells cultured in 3D scaffolds exhibit distinguished Col I, Col II, versican and aggrecan expression [11]. Maeda et al. have addressed the impact of inflammation on rabbit AF cells, showing that a greater inhibition of PG synthesis was observed in the inner AF [12]. Moreover, the AF ECM is organized in lamellae which are

separated by the TLBN, a 3D elastic network connecting the layers of the AF, research has focused on understanding the impact of this network on IVD homeostasis and disease. TLBN has been structurally characterized and is proposed to prevent IVD damage and consequent herniation upon extreme mechanical stress, preventing the bulging of the disc [13-15]. Saggese et al. have investigated the impact of mechanical loading and pro-inflammatory stimulation on TBLN integrity using a bovine AF organ culture, showing a reduction in annular mechanical strength and an increase of pro-inflammatory mediators specifically within this network [16]. Yet, human studies addressing thorough spatial analysis of AF tissue have not been addressed, posing as an important path to better characterize and treat human IVD herniation.

Different study models have addressed inflammation by using stimulation with cytokines or LPS, as described in Chapter I [17-20]. However, current study models of AF tissue still lack resemblance to native tissue in many parameters, as biochemical composition, architecture, homeostatic and diseased cues, among others. In general, the bioengineered 3D AF model (BIO3D-AF) developed in this work endorses the relevance of 3D approaches when studying inflammation in AF tissue, considering ECM and pro-inflammatory mediator production. The results obtained in the first contribution of this work, Chapter III, and previously described were important to access the closeness of the ECM alterations observed upon induced inflammation compared to the alterations observed in AF tissue during IVD herniation. The BIO3D-AF model based on Col I hydrogels was able to recapitulate *in vitro* the ECM alterations observed in AF failure upon IL-1 β stimulation, while replicating key alterations in inflammatory mediators, upon chronic inflammation. Temporal evaluation also contributed to the disclosure of interesting insights on the time-related dynamics of several proteins, which have all been previously correlated with IVD degeneration and herniation [21-24]. The *in vitro* results observed regarding the inflammatory mediators were consistent with the *in vivo* observations, from the analysis of immune cell recruitment in CAM assay, however, the discrimination of immune cell types was not addressed. Macrophages have been found within AF tissue in the first contribution of this work and in previous studies [8]. Moreover, macrophage polarization in the presence of bovine IVD has also been studied [25]. The presence of mast cells was also confirmed in painful human IVD [26]. Panebianco et al. have also described immune cell populations with hematopoietic markers in bovine IVD tissue [27]. Therefore, and considering that immunotherapy might enhance the current treatment of IVD degeneration and herniation, further studies with different human cells should be addressed [28].

Nevertheless, the optimal AF study model should also take into account more parameters, as architecture, biomechanical properties and microenvironment cues, while extending the

time of chronic inflammatory stimuli. Anisotropy is an important characteristic of AF tissue, acquired during embryonic development, as explored in Chapter I. Nevertheless, as also described in that chapter, out of the current AF study models only Chong et al. have used anisotropic techniques, yet using a biomaterial choice that does not resemble AF tissue [29]. Different strategies have been explored to align collagen, as reviewed elsewhere [30]. Nevertheless, cytotoxicity and collagen denaturation have been critical to the successful outcomes of these strategies. Among the tissue engineering strategies to induce anisotropy, magnetic approaches have been explored, namely with the development of a novel technology called Anisogel. Briefly, this technique uses magnetic-responsive rod-shaped PEG microgels that, in the presence of an external magnetic field, align. These microgels are embedded in a surrounding hydrogel, forming a hybrid hydrogel (Anisogel) [31]. Previously to the current work performed in this thesis, this Anisogel technique, developed by Laura De Laporte's team at Aachen, Germany, had only been employed in fibrin and PEG hydrogels [32, 33]. Through a new collaborative project between Portugal and Germany, a collagen-based anisotropic model was developed, using Anisogel technology. However, when translating this know-how to align human AF cells, this was not observed, making evident the tissue-specificity of Anisogels. *In vitro* tests were performed for up to 14 days using several PEG microgels (distinct sizes and concentrations) in collagen-Anisogels. Nevertheless, in all the conditions tested, AF cell alignment was not achieved, as evidenced by the f-actin staining performed (Annex 1). As discussed in Chapter I, AF cells elongate during embryonic development, by forming concentric rings around the NP tissue, a process that researchers propose to precede the aligned collagen deposition [34-36]. To replicate this phenomenon *in vitro*, external factors, such as AF tissue growth factors like TGF- β and IGF, might need to be explored, to better recapitulate the microenvironment favorable to AF cell anisotropy. In fact, Peredo et al. have highlighted the importance of growth factors as TGF- β 3, PDGF-BB, CTGF, or IGF-1 on the guidance of iPSCs towards AF cell fate [37]. Moreover, mechanical forces cannot be neglected, as AF embryonic development is intrinsically connected with movement and mechanical stimulation of the spine [38]. Overall, further studies must be conducted, sophisticating the AF cell microenvironment to achieve cell anisotropy. However, this does not devalue the contribution of this part of the work, as the collagen-based anisotropic platform can not only be used to better study how AF cell anisotropy can be achieved *in vitro* but also extends its potential to other collagenous tissues that are characterized by their anisotropy, as muscle, tendon and others.

2. Future Perspectives

Altogether, the work in this thesis has unequivocally enhanced the current study approach to AF failure and IVD herniation by enhancing the current knowledge on AF tissue of herniated samples and by providing a novel AF study model to dissect the role of inflammation together with a collagen-based anisotropic platform to be further combined with the study model. Moreover, the work here described has been conducted with a fully humanized approach, highlighting its relevance and translational potential for clinical research. Future perspectives, as also described individually in each contribution to this thesis, can encompass different approaches and directions.

Human herniated samples can be used to perform other studies, as the evaluation of the impact of external factors. In the abovementioned study, Compte et al. have shown a correlation between circulating TNF- α , IL-6 and IL-8 and body mass index, although no correlation with Modic changes and IVD degeneration was found [9]. Other correlations should be explored, as this could improve the patient's diagnosis and treatment, by defining possible risk factors.

The BIO3D-AF model can serve as an opportunity for deeper cellular studies as no standard panel of cell markers has been established for AF cells. Different cell studies have been conducted in IVD, exploring different subpopulations essentially in NP tissue. Molinos et al. have characterized alterations in cell markers within human IVD and correlated them with age [39]. Ferreira et al. have thoroughly sorted and characterized CD44 and CD45 positive cells within NP tissue [40]. The BIO3D-AF model can be of great value conducting similar studies exploring these and other markers to further enhance the current knowledge on AF tissue and IVD herniation. As current research has been advancing on biofabrication tools, the developed AF study model can also be used to incorporate organ-on-a-chip methods, for example. In recent years, different organ-on-a-chip technologies have been proposed to study IVD degeneration. Hwang et al. have developed a spine-on-a-chip, using AF and THP-1 cells to address the impact of concentrations of inflammatory cytokines [41]. Also, Hwang et al. have proposed a study combining both NP and AF cells and endothelial cells to access the angiogenic potential upon inflammatory stimulation [19]. A disc-on-a-chip was proposed by Dai et al. using mouse organ culture to refine current study models, revealing increased cell viability, structure integrity and metabolism [42]. Monocyte extravasation has also been addressed using organ-on-a-chip methods and inflammatory stimulation of NP cells [43]. Furthermore, recent advances in bioreactors are also relevant in the scope of this work and can be combined with the current AF study model of inflammation. Several studies have been conducted to replicate the mechanical loading of the human spine. Lazaro-

Pacheco, D. et al. have developed a protocol to faithfully replicate the loading of standard daily activities, aiming to include it in further *in vitro* tests [44]. A physiological and pathophysiological environment was established using mechanical loading by Salzer et al., revealing alterations in metabolic activity in NP tissue [45]. Alterations in ECM homeostasis with different hydrostatic pressures have also been addressed in NP and inner AF tissue, by Wang et al. [46]. Moreover, from a therapeutic point of view, the proposed AF study model can also be used to disclose more knowledge on LDH therapies, enhancing our understanding of how the current drugs used to treat LDH patients (e.g. NSAIDs) act. Previous work has successfully addressed the use of anti-inflammatory chitosan/poly- γ -glutamic acid nanoparticles in bovine NP tissue, by decreasing inflammation and up-regulating ECM proteins [47]. Other anti-inflammatory drugs, namely Etanercept and Tofacitinib, were tested in a bovine organ culture, highlighting their potential for attenuating the pro-inflammatory microenvironment [48]. Novais et al. have addressed the use of senolytic drugs in IVD degeneration, using mice in an *in vivo* model, showing their ability to reduce senescent and inflammatory markers [49]. Also, as AF fibrotic alterations were disclosed in this work, the model here developed can also serve for fibrotic drug testing. Lu et al. have explored the effect of Physalin A, an anti-inflammatory drug, on IVD degeneration using a rat *in vivo* model, revealing its anti-fibrotic potential, through the SMAD2/3 pathway [50]. Nevertheless, all of the abovementioned therapeutic strategies have been employed in the context of IVD degeneration, but without targeting AF tissue alterations.

Considering the anisotropic platform developed in this work, although it has not been successful in providing AF cell anisotropy, it opens opportunities to deeply dissect the mechanism through which AF cells align. Approaches such as growth-factor supplementation and longer studies can be explored using collagen-Anisogels which can reveal important key players in anisotropy establishment in AF tissue, relevant to human LDH. Moreover, it also has a transversal potential to other tissues, essentially fibrocartilaginous tissues as meniscus, tendon and others, as collagen anisotropy is common for several diseases and organs.

Last, it is important to highlight that although the focus of this work was IVD herniation, more specifically AF tissue and failure, the other IVD tissues should not be neglected in a future analysis. Additional studies with the integration of the anisotropic and inflammatory models proposed here with relevant NP and/or CEP models should be implemented [17, 29]. For this, techniques as bioprinting and organ-on-a-chip, as mentioned above, can be of interest to develop a more robust IVD model. A full human based IVD model will be an important step in having a broad view of the impact of inflammation on the process of IVD herniation, disclosing novel and more accurate therapeutic targets and providing new opportunities for

drug testing. Together, this will certainly have an impact on the future direction of LDH management.

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Annex

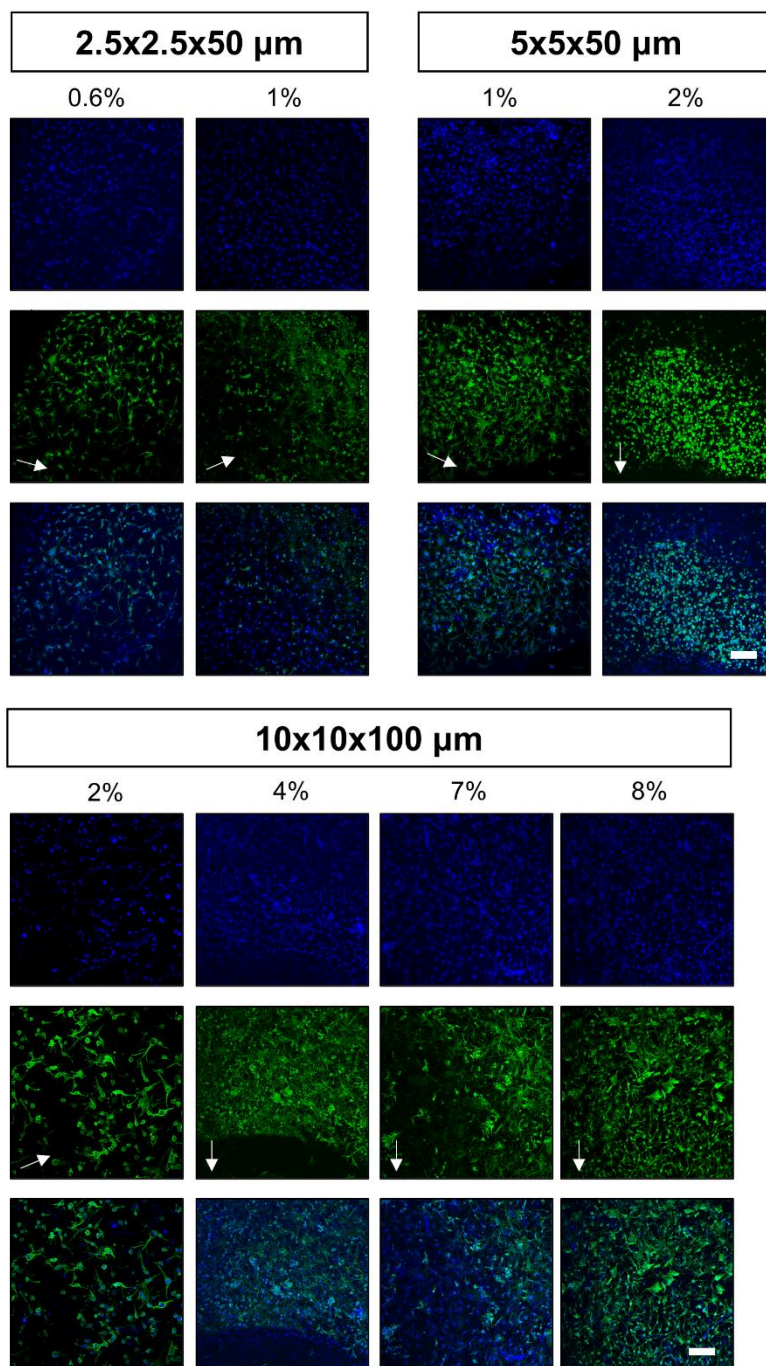


Figure 1 – AF cells alignment evaluation in collagen-Anisogels with varying PEG microgels size and concentrations. Representative images of F-actin and DAPI staining, at day 14 of culture, were obtained using a 10x magnification air objective in a laser scanning confocal microscope (SP5 Tandem Confocal, Leica Microsystems Inc). White arrows indicate the direction of magnetic field. Scale bar = 200 μm