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BIOMARCADOR INFLAMATÓRIO E CARACTERIZAÇÃO HISTOLÓGICA PARA DISTINGUIR GRAUS GRAVES DE DISCOPATIA DEGENERATIVA LOMBAR

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Bruna Lins

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Orientador: Prof. Dr. Asdrubal Falavigna

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BIOMARCADOR INFLAMATÓRIO E CARACTERIZAÇÃO HISTOLÓGICA PARA DISTINGUIR GRAUS GRAVES DE DISCOPATIA DEGENERATIVA LOMBAR

Bruna Lins

Dissertação de Mestrado submetida à Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em Ciências da Saúde da Universidade de Caxias do Sul, como parte dos requisitos necessários para a obtenção do título de Mestre em Ciências da Saúde, Linha de Pesquisa: Farmacologia e Biomarcadores.

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“Ciência e Arte jorram da mesma fonte”

Christian Albert Theodor Billroth

(Áustria, 1829-1894)

Sumário

Esta dissertação de Mestrado Acadêmico Stricto Sensu é apresentada no formato exigido pelo Programa de Pós-Graduação em Ciências da Saúde da Universidade de Caxias do Sul. É constituída da “introdução com referências bibliográficas”, do artigo original submetido/publicado em periódico e das “considerações finais e perspectivas”.

A submissão do artigo foi realizada no periódico *Annals of Anatomy*, com fator de impacto 2.2 e qualis A2. Escolheu-se esse periódico por ele ter grande abrangência e por o público-alvo ter interesse em estudos da área da saúde, o que possibilita maior divulgação e alcance desta pesquisa.

1 INTRODUÇÃO

A lombalgia é um grande problema de saúde pública, tendo consequências socioeconômicas que afetam 619 milhões de pessoas em todo o mundo.[1] Estima-se que 84% da população mundial tenha, ao menos, um evento de lombalgia ao longo de suas vidas e que algumas delas sofrerão de forma crônica, o que impactará significativamente não apenas a economia, mas a perda de produtividade social.[2,3] A etiologia da discopatia degenerativa lombar (DDL) é multifatorial, atribuída ao envelhecimento, fatores genéticos, lesões, obesidade, tabagismo e carga mecânica anormal, levando a alterações celulares e teciduais no disco intervertebral.[4,5,6]

Durante a degeneração do disco intervertebral (DIV), várias alterações fenotípicas, incluindo senescência celular, apoptose, degradação da matriz e desarranjo estrutural, estão envolvidas.[7] Além dessas modificações, a inflamação desempenha um papel importante na DDL.[8] O avanço da degeneração está associado a níveis elevados de citocinas pró-inflamatórias, aumento da atividade enzimática de degradação da matriz, sensibilização dos neurônios e crescimento neurovascular no DIV, além da infiltração de células imunes, incluindo macrófagos no disco.[9,10] O recrutamento de células imunológicas para o disco, amplifica a resposta inflamatória.[9]

Os macrófagos participam da degradação e remodelação da matriz extracelular através da produção de metaloproteinases (MMPs) e desintegrina A e metaloproteinases com motivos de trombospondina (ADAMTSs) assim os macrófagos podem participar de mecanismos patológicos críticos durante a degeneração do disco devido à importância da integridade da MEC para a homeostase.[9] Déficits estruturais no núcleo pulposo (NP) e anel fibroso (AF), e a formação de rupturas e fissuras nesses tecidos, bem como hérnia de disco em alguns casos, permitem a ativação e infiltração de células imunológicas.[11] Os diferentes efeitos produzidos pela infiltração de macrófagos dependem da sua polarização no DIV.[12] Os macrófagos sofrem diferenciação fenotípica e funcional sob a influência de citocinas teciduais locais, caracterizados pelo tipo M1 que produz altos níveis de citocinas pró-inflamatórias (IL-1B e TNF-a) capazes de modular a inflamação, e tipo M2 que secretam fatores com função

antiinflamatória (IL-4 e IL10), ambos encontrados recentemente em DIVS's (Figura 1) .[12,13]

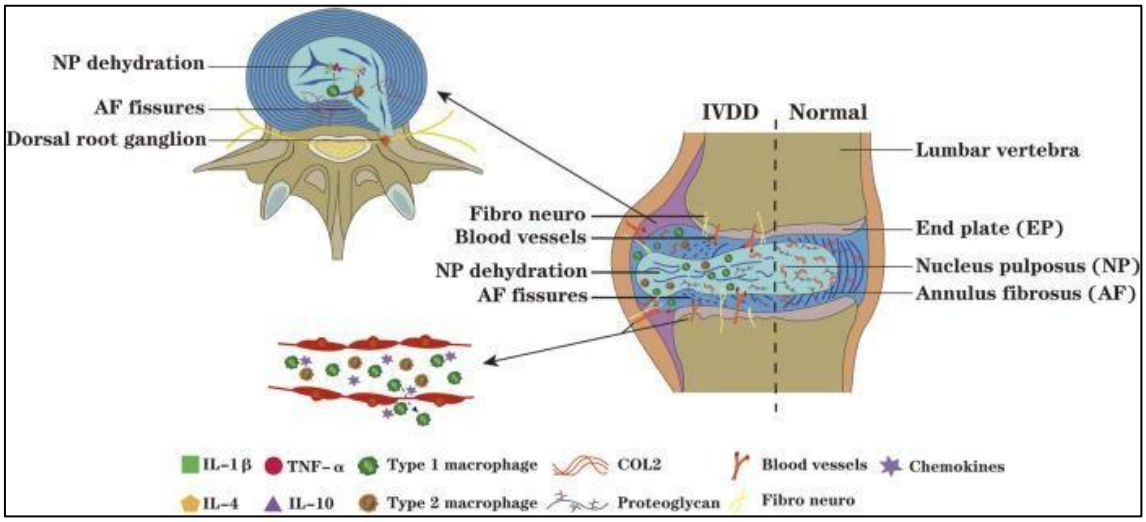

Figura 1. Macrófagos na degeneração do disco intervertebral

Estudos indicam que os macrófagos exercem efeitos biológicos principalmente em seus estados polarizados, e não como os próprios macrófagos.[14] A fraca resposta de cura no DIV torna-o vulnerável a desafios inflamatórios, com pouca capacidade de recuperação de lesões.[10]

A DDL é um processo crônico que resulta em modificações histológicas, incluindo o aumento do número de fendas e fissuras, presença de material granular, neovascularização no AF, proliferação celular e formação de aglomerados de células dentro do NP.[15,16] Além disso, observa-se o rompimento da estrutura lamelar das fibras colágenas e o aumento do grau de vascularização e inervação.[17]

Para diagnosticar a degeneração do DIV, a ressonância magnética por imagem (RMI) é a modalidade médica mais amplamente utilizada, por meio do sistema de graduação semiquantitativo da Escala de Pfirmann.[18,19] Esse diagnóstico poderia ser complementado por outras análises, como a histológica e imunohistoquímica, que permitem identificar alterações estruturais e morfológicas relevantes que ocorrem durante a degeneração do DIV humano.[19]

Apesar da sua importância, os estudos sobre as características histológicas do DIV degenerado não são conclusivos. A falta de uma avaliação detalhada e metodologias padronizadas dificultam o entendimento da degeneração dentro de cada componente do disco, além de ser uma avaliação

fundamental para auxiliar a Escala de Pfirmann, visto que alterações sutis no tecido não são identificadas através da RMI.

Morfologicamente Thompson et al., (1990) foram os primeiros a propor uma classificação para a doença degenerativa do disco intervertebral usando estudo histológico.[20] Em seguida, a classificação histológica foi introduzida para determinar as alterações no CEP, AF e NP, celularidade e matriz do NP.[21] Recentemente, o sistema foi padronizado com foco abrangente na taxonomia de classificação para recursos de NP, AF e CEP, incluindo celularidade, lesões e estrutura da MEC.[15] Peletti-Figueiró et al., (2016), correlacionaram alterações histológicas com dados clínico-radiológicos e apresentaram uma nova proposta de escores de degeneração discal, adaptando os escores propostos em trabalhos anteriores.[19]

Desse modo, esta pesquisa teve como objetivo definir as características degenerativas do DIV e inferir possíveis biomarcadores de severidade da doença que possam através da sua modulação contribuir para um futuro tratamento e atribuir auxílio para a escala de Pfirrmann.

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3 ARTIGO

Auxiliary inflammatory biomarker and histological characterization for distinguishing severe degrees of Lumbar Degenerative Discopathy

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Abstract

Background: Intervertebral disc degeneration is a multifactorial process resulting from cellular, biochemical, and structural changes. This study aimed to elucidate possible biomarkers that contribute to the definition of the Pfirrmann scale, mainly in distinguishing between the most severe degrees of Lumbar Degenerative Discopathy.

Methods: The intervertebral disc (IVD) was used in a standardized way to evaluate numerous degenerative characteristics using HE, Safranin O/FCF, Masson's Trichrome, Verhoeff and PAS stains. The expression of Macrophages was analyzed in different degrees of severity of the disease and the extracellular matrix's structure was evaluated by scanning electron microscopy (SEM).

Results: The stains allowed us to define the tissue degenerative characteristics of IVD. As it is simpler and less expensive than special stains, the data determined that HE staining can be standardized without compromising fidelity and interpretation. The overexpression of the macrophage showed that it could be an interesting biomarker of the severity of the disease, as it statistically differentiated grades IV and V. The evaluation of elastic fibers by SEM can also be seen as an essential factor to be considered as a marker of degenerative scale.

Conclusions: HE staining efficiently determines degenerative processes if applied competently by a qualified professional and could replace special stains. Macrophages could be a future biomarker of disease severity and check the quantity and integrity of elastic fibers by SEM.

Keywords:
Lumbar Degenerative Discopathy
Intervertebral Disc
Histology
Immunohistochemistry
Macrophages
Scanning electron microscopy

1. Introduction

It is estimated that low back pain is one of the leading causes of disability, morbidity, and deterioration in quality of life and represents a significant burden not only for the individual but also for Society (Basanta et al., 2022). It is estimated that 84% of adults have low back pain at some point in their lives, while 10% are chronically disabled (Qingshen et al., 2019). This serious problem affects the world population, involving around 619 million people globally, with a projection of 843 million prevalent cases by 2050 (Ferreira et al., 2023). As the population ages, the number of people with low back pain increases substantially, affecting work performance and quality of life (Qingshen et al., 2019).

Lumbar degenerative disc disease (LDDD) is a clinical condition of persistent and severe pain, which worsens with usual activities and is usually progressive (Teles et al., 2019). Most low back pain occurrences are treated through conservative interventions, physiotherapy and administration of analgesics (Dowdell et al., 2016). It is estimated that 20% of cases will be considered refractory to conservative treatment, causing LDDD with more severe and advanced clinical stages, where surgical intervention is considered the main form of therapy (Schol and Sakai, 2019; Yoshihara and Yoneoka, 2015).

Magnetic resonance imaging (MRI) is the most helpful modality to characterize disc lesions to diagnose intervertebral disc (IVD) degeneration (Takashima et al., 2012). Changes in the IVD signal on images make determining the degree of degeneration possible. Pfirrmann et al. (2001) proposed an MRI-based grading system for degeneration, providing a semiquantitative morphological assessment of IVD degeneration (Pfirrmann et al., 2001; Blumenkrantz et al., 2010).

Intervertebral disc degeneration is a multifactorial process resulting from cellular, biochemical, and structural changes, among which degradation of the extracellular matrix, inflammation, production of ROS, cellular catabolism, release of chemokines and cytokines are some of the most common conditions found in the degenerative process (Isma et al., 2022). During the development of these degenerative mechanisms, several histological findings are observed, including an increase in the number of slits and fissures, the presence of granular material, neovascularization in the AF, cell proliferation, and the formation of

clusters of cells within the NP (Takeshi et al., 2020; Le Maitre et al., 2021). The disruption of the lamellar structure of collagen fibers and an increase in the degree of vascularization and innervation are also observed (Boss et al., 2002).

IVD degeneration is characterized by dysregulated remodeling of the ECM (Phillips et al., 2015). Elevated levels of molecular mediators of inflammation have been described in pathological IVD tissue (Shamji et al., 2010). Pro- inflammatory cytokines and chemokines characterize the degenerative process in various tissues and diseases. These inflammatory cytokines promote a catabolic response, resulting in ECM loss, cellular apoptosis, neurotrophin production, and infiltration of immune cells, including macrophages, into the intervertebral disc during LDDD (Koroth et al., 2023; Kirmaz et al., 2022).

Structural and morphological changes that occur during IVD degeneration that cannot be visualized through MRI can be identified through histological investigations (Walter et al., 2015). The ability to describe degenerative characteristics associated with degenerative conditions is fundamental to devising new treatment approaches and aligning clinical practice with evidence (Veroutis et al., 2021). Despite much research, there are still no conclusive studies that provide a detailed, comprehensive, qualitative, and quantitative assessment of the histological and immunohistochemical biomarker changes exhibited during the process of senescence and degeneration of the IVD in LDDD (Boss et al., 2002). The present work attempted to elucidate possible biomarkers that could contribute to the definition of the Pfirrmann scale, mainly distinguishing between the most severe degrees of pathology.

2. Materials and Methods

2.1. Ethics statement

This study complied with ethical standards, and the patients were invited after informed consent. This research was approved by the Ethics Committee of the University of Caxias do Sul (CEP/UCS 2.503.156 and 2668520.0.0000.5341).

2.2. Patient selection and Radiological and clinical evaluation

Symptomatic patients with LDDD were initially assessed and treated conservatively. When the symptoms persisted, magnetic resonance imaging (MRI) was performed for evaluation. Surgical intervention was considered in

worsening pain with a clinical and radiological agreement. The exclusion criteria included a history of prior lumbar spine surgery, spinal tumors, or infections. The severity of the lumbar spine IVD was assessed using magnetic resonance with the Pfirrmann scale, which is categorized according to the severity of disc degeneration into grades I (normal) to grade V (severe degeneration (Pfirrman et al., 2001)

2.3. Samples

A total of 26 patients was selected for the study. Among these, the total number of biological samples collected was 33 IVD from the lumbar spine. It stans out, some patients underwent decompression in adjacent segments, making it possible to study more than one biological sample per patient (Table 1). The IVLD fragments excised during the surgical procedure underwent a dual-phase cleaning process within sterile containers holding a physiological solution to eliminate potential contamination from blood cells. Subsequently, the material was immersed in a sterile alkaline phosphate buffer (PBS 1X with 0.8% NaCl, 0.02% KH2PO4, and 0.088% Na2HPO4, with a pH of 7.4 – sourced from LGC Biotecnologia in São Paulo, Brazil) and was then transported to the cell therapy laboratory.

The biological samples were standardized with an approximate size of 2cm x 1cm, each containing a 1:1 ratio of annulus fibrosus (AF) and nucleus pulposus (NP). These standardized samples were then immersed in 10% buffered formalin at a pH of 7.4 for a 24-hour fixation period. A neurosurgeon carried out verification of the specific IVD regions. To ensure reliable methodological assessments, two-grade I Pfirrmann samples were utilized for the analyses (Carazzo et al., 2023 - subject).

2.4. Histological Evaluation

To determine the degenerative factors to be proposed on a degenerative histological scale, the following stains were performed: HE (Sigma-Aldrich®, St. Louis, Missouri, USA) (Peletti-Figueiró et al., 2017; Saciloto et al., 2021), Safranin O/Fast Green (Sigma-Aldrich® St. Louis, Missouri, USA) (Peletti-Figueiró et al., 2017), Masson Trichrome (Sigma-Aldrich® St. Louis, Missouri, USA) (Peletti-Figieró et al., 2017; Masson, 1929; Lillie, 1940), Verhoeff (EasyPath, São Paulo,

Brazil) (Lillie, 1944; Berg, 1953) and PAS (Periodic Acid-Shiff – EasyPath, São Paulo, Brazil) (Hotchkiss, 1948; McManus, 1948).

The results were obtained through optical microscopy evaluation by two independent researchers, and the means of the analyses were calculated for each degenerative factor evaluated in the total tissue captured on the slide and standardized for all stains (Leica DM2500 optical microscope, 200x magnification, Wetzlar, Germany).

2.5. Immunohistochemistry – Evaluation of Macrophage Inflammatory 1α

Sections of IVLD samples were deparaffinized, dehydrated, incubated in 3% hydrogen peroxide (H_2O_2 - VETEC, São Paulo, Brazil), and subsequently in 2.5% bovine serum albumin (BSA in house) diluted in PBS 1X (0.8% NaCl, 0.02% KCl, 0.02% KH2PO4 and 0.088% Na2HPO4, pH 7.4 - LGC Biotecnologia, São Paulo, Brazil). The samples were then incubated with monoclonal antibodies anti-macrophage Inflammatory 1α (ab259372, Abcam – Cambridge, UK) and prepared per the manufacturer's recommendations. Samples underwent treatment with Amplifier (HiDef Detection Amplifier Mouse & Rabbit, Cell Marque - Sigma-Aldrich®, St. Louis, Missouri, USA) and Detection Polymer (HiDef Detection HRP Polymer Detector, Cell Marque - Sigma-Aldrich®, St. Louis, Missouri, USA). Subsequently, the material was incubated with the chromogen 3,3'-diaminobenzidine (DAB - Cell Marque - Sigma-Aldrich®, St. Louis, Missouri, USA) and counterstained with Harris Hematoxylin (Sigma-Aldrich®, St. Louis, Missouri, USA). The slides were dehydrated, fixed, and mounted with Eukitt quick hardening mounting (Sigma-Aldrich®, St. Louis, Missouri, (USA) (Iwashina et al., 2006; Saciloto et al., 2021; Peletti-Figueiró et al., 2017; Carazzo et al., 2023 - subject).

2.6. Optical and Digital Microscopy Immunohistochemical Evaluation

The results were obtained through optical microscopy evaluation by two independent researchers, and the averages of the analyses were calculated (Leica DM2500 optical microscope, 200x magnification, Wetzlar, Germany). The evaluated proteins are expressed in the cell nucleus. The assessment of MAC1α protein expression was conducted by determining the percentage of at least one positive nucleus within the clusters of chondrocytes in the NP about the total

number of chondrocyte clusters in the entire tissue (Xu et al., 2020; Carazzo et al., 2023 - subject).

The protein expression results were digitally assessed by capturing 20 random fields from the study samples using the LAS V4.4 software at 300 dpi (Wetzlar, Germany). The digitized images were transferred, and the staining intensity was determined using the Image J software (1.43μ, Wayne Rasband, National Institute of Health, USA, http://rsb.info.nih.gov/ij, java 1.6.0_12, 64-bit) to obtain the pixel averages and arbitrary units of protein expression based on RGB colors. Two positive control macro images were utilized to duplicate all assessments (Saciloto et al., 2021).

2.7. Scanning Electron Microscopy (SEM) - ECM and Elastin

Tissue sections captured on silanized slides (4μm thick) were deparaffinized, diaphanized and dehydrated. Then, they were incubated at 37°C to dry the tissue completely.

Afterward, the samples were assembled, and gold was deposited by sputtering. Positive ions were produced by the ionization of argon and injected into the chamber (Denton Vacuum, Moorestown, USA). The images and micrographs were evaluated (SEM- Tescan Performance in Nanospace Mira 3LMH – 20Kx magnification, Czech Republic) (Castro, 2001; Mannheimer, 2002; Goldstein et al., 2003).

2.8. Statistical analysis

Data storage was performed using the Excel 2007 program. Statistical analysis was performed using the IBM SPSS® 22.0 for Windows program (IBM, Chicago, IL, USA). Mac1α protein expression was evaluated using the T-Test coefficient (Sig. $p \leq 0.05$).

All histological biomarkers were verified statistically with Tukey, ANOVA, ROC Curve, Kruskal-Wallis, Chi-square, Mann-Whitney, T-tests, and Spearman (Sig. $p \leq 0.001$ or $p \leq 0.05$).

3. Results

3.1. Demographic Assessment of Patients and Analysis of IVD Distribution

The present study featured a predominance of female patients (14:12). Age distribution exhibited heterogeneity, ranging from 27 to 72 years, with a mean age

of 49.6 years. The evaluated levels showed a prevalence in L4/L5, L5/S1, and L3/L4, respectively. Regarding Pfirrmann's grades, the distribution of IVLD samples showed a higher prevalence in severe grades (V and IV – 26 samples) compared to mild grades of the disease (II and III – 7 samples), as shown in Table 1.

3.2. Optical Microscopic Histological Evaluation

Microscopic evaluation of histological stains identified a positive correlation using the Spearman test between assessing the diameter of chondrocyte clusters in the NP in HE and Safranin O/FCF stains (Sig. 0.019). The number of chondrocyte clusters in the NP was also statistically correlated in these stains (Sig.0.003). The collagen disorganization positively correlated with HE and Masson's Trichrome staining (Sig. 0.006). Despite the evaluation of many variables and histological biomarkers, the representative number restricted by radiological degrees of degeneration prevented statistical strength and the definition of a scale with more precise tissue markers, making it impossible to distinguish between mild and severe degrees according to the Pfirrmann grading, an inherent characteristic of the surgical biological sample. However, the data showed that although special stains show more precise markings, routine staining applied in clinical practices (HE) allows accurate and statistically significant identification compared to the special stains of histological biomarkers proposed in the research. We, thus, present the image of the characteristics with statistical representation between different staining (Fig 1).

3.3. Optical Microscopy and Digital Immunohistochemical Evaluation – Mac1α

Microscopic assessment of protein expression revealed that the Mac1α protein expression is predominantly positive in the clusters of NP chondrocytes (Fig. 2A e 2B). Therefore, it can be statistically observed, using the Tukey test, that Mac1α was less expressed in Grade IV compared to the Pfirmann grade V at Sig of 0.022 (Fig. 2C). This shows that the Mac1α protein can be considered a biomarker to be associated with radiology so that the medical team knows how inflammation and the present biomarker behave in degrees that are difficult to

interpret radiologically. The digital analysis of the Mac1α protein revealed no statistically significant differences or distribution trends.

3.4. SEM Evaluation

Evaluation of the photomicrographs allowed us to observe the MEC framework and the distribution of collagen in the different samples. Furthermore, it made it possible to verify that the quantity of elastic fibers is more abundant in samples with a lower degree of degeneration according to the Pfirmann scale and increases, even showing fiber rupture, in the most severe degrees of the disease. The average thickness of the elastic fibers was approximately 84.44nm (Fig. 3).

4. Discussion

The etiology of IVD degeneration is multifactorial. At the cellular level, it includes the formation of cell clusters in the NP, an increase in the number of senescent cells, apoptosis, degradation of the extracellular matrix, collagen disorganization, formation of cracks and fissures, formation of granules, internal enlargement of blood vessels and nerves, and infiltration of inflammatory cells that can be determined histologically (Le Maitre et al., 2021).

This study was able to delimit a possible biomarker that helps differentiate the most severe degrees of the disease (IV and V on the Pfirrmann scale). Due to the low sample number in mild degrees, verifying whether there was a relevant statistical difference between these and severe degrees was impossible. Our data are corroborated with those found by other researchers about the importance of the Macrophage and how it is overexpressed in the patient's advanced age and the degree of severity on the Pfirrmann scale (Richardson et al., 2009; Purmessur et al., 2013; Koroth et al., 2023).

According to Feng et al. (2017), as degeneration increases, the degradation of the extracellular matrix increases. There is also an increase in the secretion of cytokines and chemokines by senescent cells and the infiltration of immune cells. Macrophages are highly capable of capturing and detecting signals and stimuli from the microenvironment. When the NP loses the immunological barriers lacking the proteccion, it is immediately detected by the immune system, and signaling macrophage chemotaxis and aggregation, triggering macrophage

and NP interaction (Feng et al., 2017). In this way, macrophages polarize towards the M1 type under the action of chemokines, triggering an inflammatory response. This interaction between macrophages and NP forms a positive feedback system, which can lead to persistent disc degeneration and chronic pain (Yan et al., 2022). Many studies also indicate that excessive apoptosis, together with the abundance of the inflammatory response of NP cells, causes cellular catabolism and the entire cellular cascade of degeneration, making the macrophage a possible therapeutic target (Bao et al., 2021).

Other investigators have also reported the predominant infiltration of macrophages in herniated discs and determined that such agents play a central role in tissue repair and remodeling (Le Maitre et al., 2005). Our research showed that macrophages are more active in the most severe stages of the disease. Despite that, M2 polarized macrophages efficiently promote wound healing and tissue repair. Their pro-inflammatory and anti-infectious capabilities are weaker than those of M1 polarized macrophages and therefore cannot help with tissue remodeling (Kawaguchi et al., 2002; Mosser et al.,2008). Further studies are needed to understand the exact extent of the influence of the macrophage associated with Pfirrmann degrees and its action as a biomarker of LDD.

The data presented in this study showed that special stains with specific macromolecules' staining patterns are efficient and vital in LDD as they provide the researcher with a clear contrast for observing the degenerative characteristics determined by the differential dyes. However, the positive non-parametric Spearman correlation with p=0.001 (Sig) (statistical test also used by Rutges et al., 2013) in some degenerative factors between Safranin O/FCF and HE staining and staining Trichrome of Masson and HE determines the importance of basic HE staining, widely used in clinical practice.

This definition of positive correlation tells us that we can determine the critical degenerative factors of the disc through simple, quick and inexpensive staining. This factor makes it easier to understand degenerative factors without applying more sophisticated, costly and complex histological techniques. It should be clarified that to use only the HE technique in attributing degenerative factors, the researcher must have extensive knowledge of the tissue in question and the pathology. Peletti-Figueiró et al. (2017) determined that the union of

stains would be ideal for accurately addressing the degenerative characteristics of IVD in humans. Our study corroborates that combined stains can lead to better results. Still, it determines that due to the high level of statistical relevance attributed to Safranin O/FCF and Masson's Trichrome staining with HE, we can efficiently and reliably apply the most basic methodology in a practical way and with reliable results, as described by Rutges et al., (2013).

Using HE staining, it was possible to evaluate fissures, microfractures, collagen misalignment, and the quantity and diameter of chondrocyte clusters in the NP and granule formation. Studies were also able to verify (Rutges et al., 2013); however, unlike Rutges et al. 2013 and Boss et al. 2002, defining an auxiliary scale for Pfirrmann's degrees of degeneration was impossible. This may be because we use quantitative variables, which determine the most precise statistics, and because we have a small sample size and few patients representing the mildest degrees of LDD (an intrinsic difficulty in the study and obtaining the material).

Chan et al., 2014 defined that it is extraordinary that Hematoxylin and Eosin (HE) staining that combines simple and inexpensive dyes can reveal remarkable cellular details, in which ultrastructural characteristics can also be deduced. The interaction of colors can also provide considerable clues about the functional state of cells. According to Wick 2019, the HE technique, if well conducted in the laboratory in all its stages, from collection to fixation, to macroscopic evaluation, to inclusion, to microtomy, to staining and mounting, is still the best staining technique for the assessment of human tissue.

According to Le Maitre et al. (2021), surgically obtained tissues are typically fragments of the IVD structures of patients with various diagnoses. They may not represent the reality of the population with low back pain. To avoid this type of problem in analysis, all patients had the same diagnosis for the surgical procedure, and the neurosurgeon and the researchers were careful to have a standardized representation of the IVD structures in all samples.

The same authors determined that many histopathological methods for IVD and classification systems are used to define the severity of the disease. They also report that the development of these factors can make it challenging to arrive at conclusions about the tissue and limit standardization and direct

comparison between studies (Le Maitre et al., 2021). Our research used different stains to verify the various aspects of IVD degeneration. It was concluded that standardized HE staining can be used for IVD without deviations in interpretation or conclusion, facilitating the histopathological evaluation of the injured tissue.

Verhoeff staining allowed the visualization of elastic fibers in the extracellular matrix of the samples, although there is some difficulty in doing so. The data presented will enable us to infer the use of SEM to define elastic fibers about their quantity and structure, as it is possible to observe that in Grade I, there is a greater quantity and a tendency to severe degrees of the disease.

According to Tanoren et al., 2023, the structure of the disc's elastic fiber can be viewed histologically, mainly by staining containing Hematoxylin and Eosin, Weigher's Resorcin-Fuchsin, Verhoeff's Iron-Hematoxylin, orcein and immunology techniques. Through SEM, the presence of elastic fibers distributed in parallel networks in the AF could be verified more efficiently. The density and concentration of these fibers in the NP were more challenging to determine due to the intralamellar structure of the fibers (Tanoren et al., 2023; Tavakoli et al., 2017).

Using SEM, the definition of the quantity and stability of elastic fibers can be a possible biomarker attributed to the Pfirrmann scale and radiological evaluation. By analyzing future biomarkers in IVDs, such as macrophages and elastic fibers, it will be possible to quantify the severity of degeneration, predict progression, monitor treatment efficacy, and inform the development of new therapies (Le Maitre et al., 2021).

5. Conclusion

The study established that HE staining can be applied to obtain IVD degenerative factors, such as fissures, microcracks, granule formation, collagen misalignment, and NP chondrocyte clusters' quantity and diameter. This routine staining is simple and inexpensive and can replace special stains if processed correctly and by a researcher experienced in the routine and knowledge of the tissue being evaluated.

The most striking finding was the macrophage's overexpression in the pathology's most severe degrees, statistically differentiating grades IV and V on

the Pfirrmann Scale. This may contribute to the macrophage being considered, after further studies, as a future biomarker of the severity of the disease. It was possible to verify that the elastic fibers visualized by SEM decrease with increasing severity of the disease and can rupture, altering the extracellular matrix.

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NOTES:

Conflicts of interests

The authors certify no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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Authors' contributions/Conceptualization

Authors Bruna Lins, Milena Bassanesi, Sergio Kato, Manuela Peletti-Figueiró and Asdrubal Falavigna have contributed substantially to the conception or design of the manuscript. Authors Manuela Peletti-Figueiró and Asdrubal Falavigna contributed to the acquisition, analysis, and interpretation. Author Sergio Kato helped with results and statistics. All authors participated in drafting the manuscript and revising it critically. All authors read and approved the final version of the manuscript.

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Ethics approval and consent to participate

This study was conducted and approved by ethical standards (CEP 2.503.156 and 2668520.0.0000.5341). The patients were invited through an informed consent form.

Patient consent for publication

Not applicable.

Figures

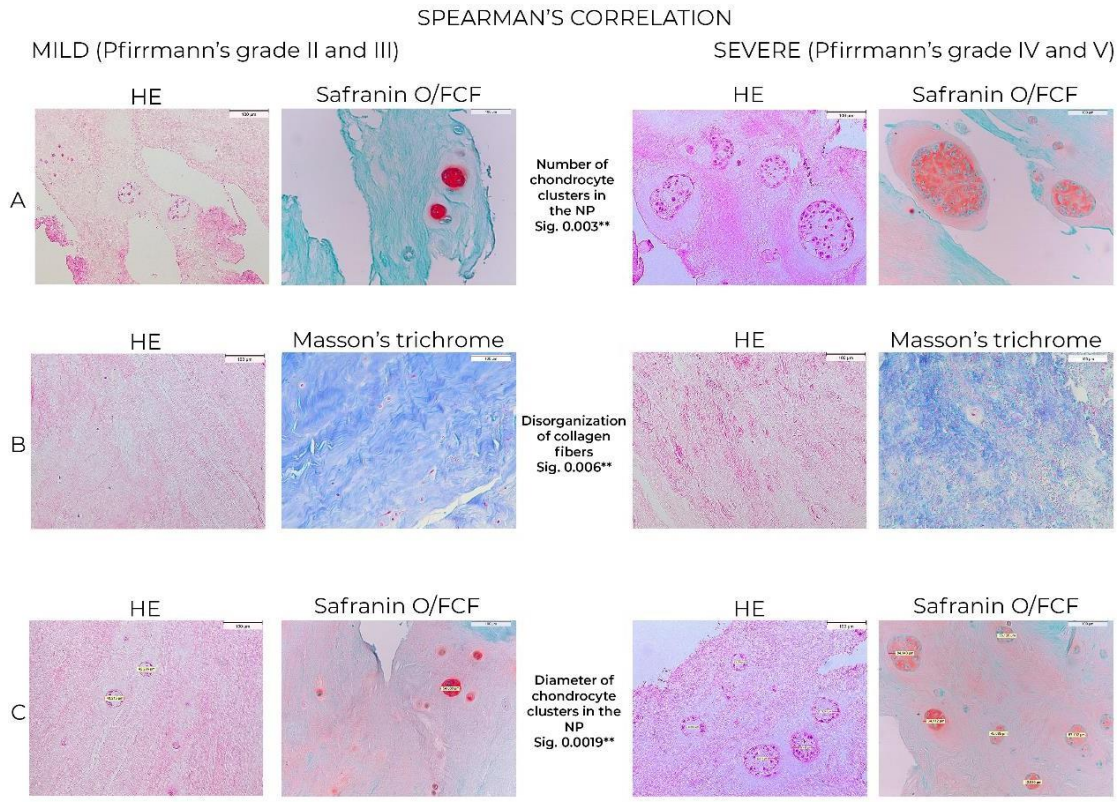

Figure 1. Histological characteristics were evaluated with positive statistical correlation using Spearman's coefficient in mild and severe samples (*p≤0.05 and **p≤0.001). A) Number of chondrocyte clusters in the NP - HE and Safranin O/FCF staining (Sig. 0.0003**). B) Collagen disorganization - HE and Masson Trichromic stains (Sig. 0.0006**). C) Diameter of chondrocyte clusters in the NP – HE and Safranin O/FCF staining (Sig 0.0019**). Scale bar: 100µm (200 x magnification).

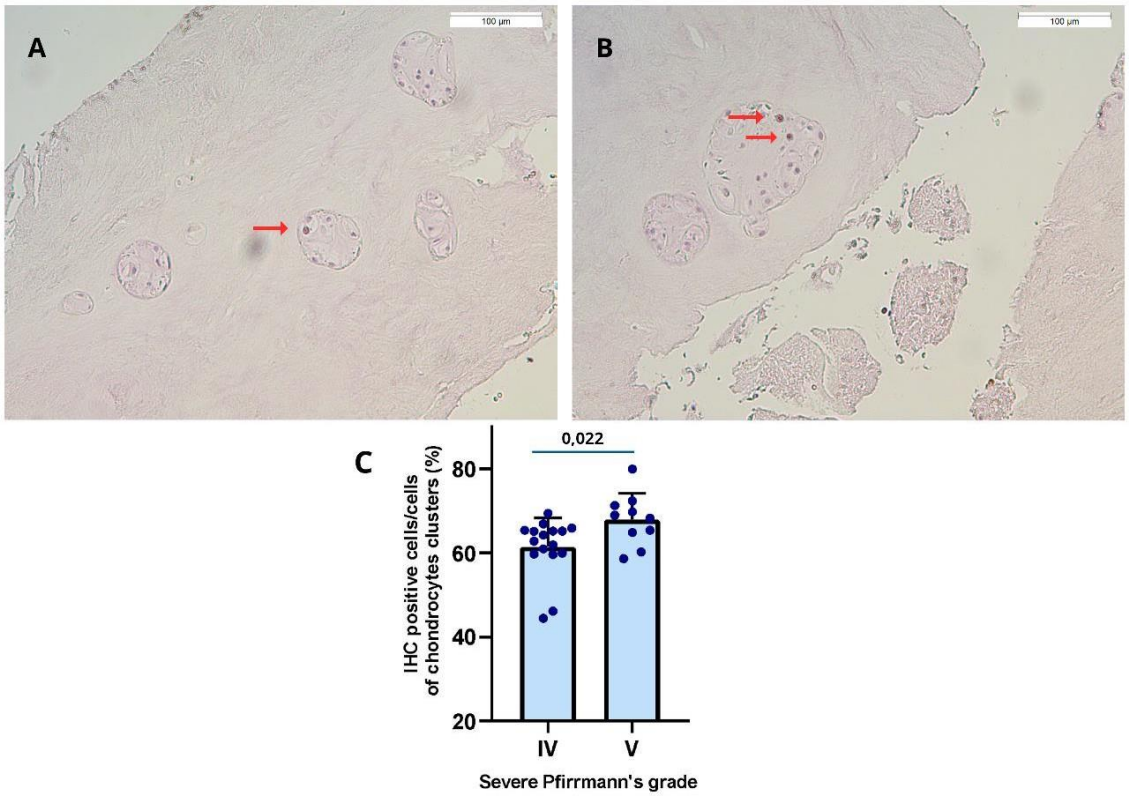

Figure 2. Assessment of invasive macrophage expression and its definition as a biomarker for differentiating grades IV and V of IVDD (Severe grades of the disease). A) Grade IV on the Pfirrmann scale. B) Grade V on the Pfirrmann scale. Black arrows indicate positive nuclei for the expression of the proteins evaluated. Scale bar= 100µm (200X magnification). C) Quantification analysis of Mac1α positive cells in DIV samples (analyzed by the Tukey, $P \leq 0.05$*).

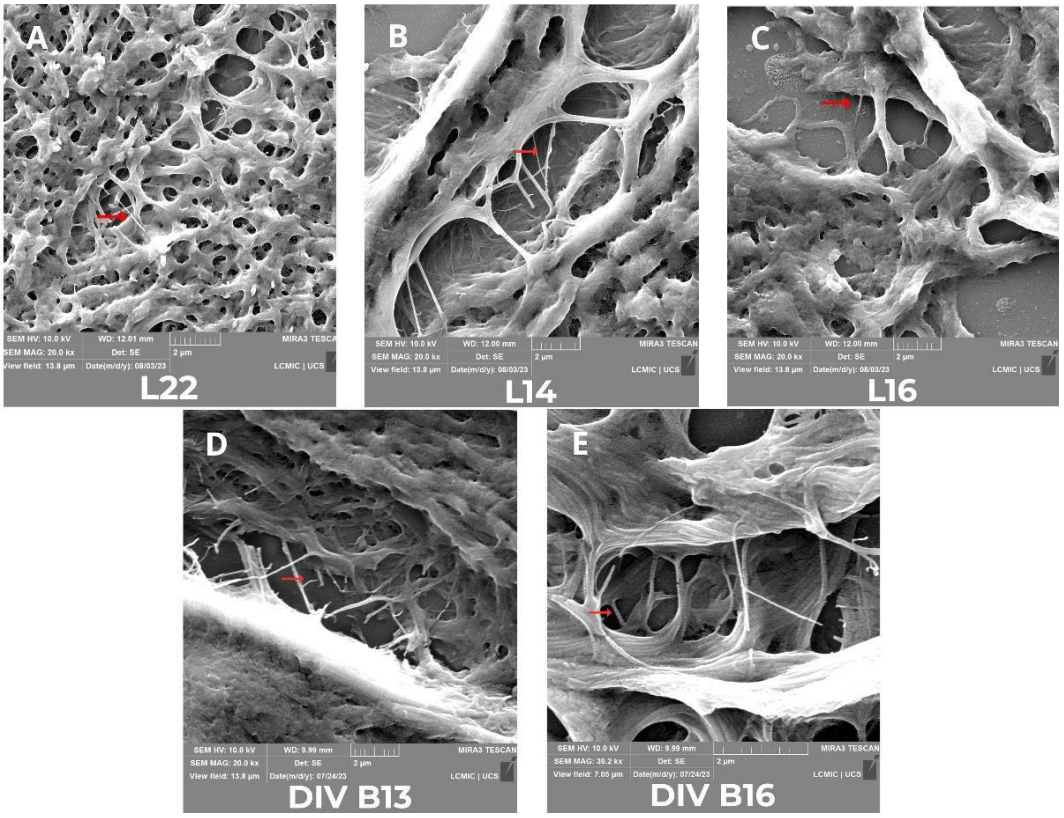

Figure 3. Evaluation of photomicrographs of the ECM (collagen and elastic fibers) in Intervertebral Disc samples evaluated using SEM. A) Grade I in the Pfirmann classification. B) Grade II in the Pfrrmann classification. C) Grade III in the Pfirrmann classification. D) Grade IV in the Pfirrmann classification. E) Grade V in the Pfirrmann classification. The red arrows highlight the elastic fibers. Scale bar: 2µm (20.0 Kx magnification).

Tables

Table 1: Clinical-radiological data of the study patients.

Patients	Level of IVD	Pfirrmann's grade	Age	Gender
P1	L4-L5	Severe (IV)	46	F
P2	L5-L5	Severe (IV)	46	M
	L5-S1	Severe (V)		
P3	L4-L5	Severe (V)	68	F
P4	L4-L5	Severe (IV)	46	F
P5	L4-L5	Severe (IV)	49	M
P6	L4-L5	Severe (IV)	39	M
P7	L5-S1	Severe (IV)	35	F
P8	L3-L4	Severe (IV)	72	F
	L5-S1	Severe (V)		
P9	L5-S1	Severe (IV)	51	M
P10	L4-L5	Severe (V)	57	M
	L5-S1	Severe (IV)		
P11	L3-L4	Severe (V)	64	F
	L4-L5	Severe (V)		
P12	L5-S1	Severe (V)	39	M
P13	L4-L5	Severe (IV)	66	F
P14	L4-L5	Severe (IV)	43	F
	L5-S1	Severe (IV)		
P15	L4-L5	Severe (IV)	44	M
P16	L5-S1	Severe (V)	38	M
P17	L4-L5	Severe (IV)	48	F
P18	L4-L5	Severe (IV)	46	F
P19	L4-L5	Severe (IV)	59	M
	L5-S1	Severe (V)		
P20	L3-L4	Severe (V)	71	M
P21	L5-S1	Mild (III)	29	F
P22	L5-S1	Mild (III)	54	F
P23	L5-S1	Mild (II)	27	M
P24	L5-S1	Mild (III)	40	F
P25	L3-L4	Mild (III)	53	F
P26	L3-L4	Mild (III)	60	M
	L4-L5	Mild (III)		

Annals of Anatomy

Auxiliary inflammatory biomarker for distinguishing severe degrees of Lumbar Degenerative Discopathy and histological characterization

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research Article
Keywords:	Keywords: Lumbar Degenerative Discopathy Intervertebral Disc Histology Immunohistochemistry Macrophages Scanning electron microscopy
Corresponding Author:	Manuela Peletti-Figueiró, Ph.D University of Caxias do Sul Caxias do Sul, BRAZIL
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	Sergio Kato
	Manuela Peletti-Figueiró, Ph.D
	Asdrubal Falavigna
Abstract:	Abstract Background: Intervertebral disc degeneration is a multifactorial process resulting from cellular, biochemical and structural changes. This study aimed to elucidate possible biomarkers that contribute to the definition of the Pfirrmann scale, mainly in distinguishing between the most severe degrees of Lumbar Degenerative Discopathy. Methods: The intervertebral disc (IVD) were used in a standardized way to evaluate numerous degenerative characteristics using HE, Safranin O/FCF, Masson's Trichrome, Verhoeff and PAS stains. The expression of Macrophages was analyzed in different degrees of severity of the disease and the extracellular matrix's structure was evaluated by scanning electron microscopy (SEM). Results: The stains allowed us to define the tissue degenerative characteristics of IVD. As it is simpler and less expensive than special stains, the data determined that HE staining can be standardized without compromising fidelity and interpretation. The overexpression of the macrophage showed that it could be an interesting biomarker of the severity of the disease, as it statistically differentiated grades IV and V. The evaluation of elastic fibers by SEM can also be seen as an essential factor to be considered as a marker of degenerative scale. Conclusions: HE staining efficiently determines degenerative processes if applied competently by a qualified professional and could replace special stains. Macrophages could be a future biomarker of disease severity and check the quantity and integrity of elastic fibers by SEM.
Suggested Reviewers:	Fuxin Wei weifuxin@mail.sysu.edu.cn Researcher with important publications on the study disease.
	Guohua Xu xuguohuamail@smmu.edu.cn Researcher with important publications on the study disease.

4 CONSIDERAÇÕES FINAIS PERSPECTIVAS FUTURAS (PROPOSIÇÕES)

A lombalgia é um problema de saúde mundial que causa incapacidade, morbidade e perda da qualidade de vida e impacta a sociedade não só no campo da econômia, mas, também, do trabalho e pessoal, impedindo as pessoas de exercerem suas atividades.

Devido a alta prevalência dos casos de lombalgia, são necessários estudos sobre esse tema. Considerando isso, este estudo teve como objetivo elucidar possíveis biomarcadores que podem contribuir para a definição da Escala de Pfirrmann. A partir dos resultados obtidos, percebeu-se que os macrófagos estão superexpressos nos graus mais graves da patologia e que as fibras elásticas diminuem e apresentam rupturas com a gravidade da doença. Isso demonstra a importância da definição desses biomarcadores para o entendimento da DDL, contribuindo para monitorar a eficácia do tratamento e para o desenvolvimento de novas terapias.

A aplicação de colorações histopatológicas adicionais e correlacionadas com as deste estudo poderiam contribuir com outros resultados, dentre elas: Alcian PAS/Shiff, Picro Sirius, Reticulina e Orceína. Elas auxiliariam na determinação de importantes processos da fisiopatologia da degeneração. Além disso, apesar de o número amostral reduzido ser inerente da fonte de obtenção do material biológico (processo cirúrgico que visa ao tratamento de uma doença), o aumento do número de amostras e a equidade de graus de degeneração trariam maior definição e força estatística. A atribuição de outros marcadores inflamatórios importantes, como IL-1β e IL-6, poderiam agregar na definição de possíveis marcadores moleculares prognósticos para a DDL.

Apesar de este estudo ter limitações em decorrência do pequeno número de amostras, ele possibilita a criação de um projeto base para contribuir com a determinação da coloração ideal para determinar o grau de degeneração com a Escala de Pfirmann na RMI em *software* que serão propostos para essa finalidade.