



## Innervation and Inflammation Correlate with Structural and Mechanical Changes in a Large Animal Model of Intervertebral Disc Degeneration

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### Disclosures

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### Introduction

Disc degeneration (DD) and associated lower back pain, a leading cause of chronic pain worldwide, is characterized by a cascade of structural and biological changes which ultimately compromise disc mechanical function and height, often resulting in discogenic pain.<sup>1</sup> Potential biological drivers of DD include inflammatory cytokines (IL6, IL1 $\beta$ , TNF $\alpha$ ) and increased innate immune cell presence (phagocytic macrophages), which are persistently observed within the IVD during degeneration.<sup>2,3</sup> Current clinical treatments for DD, including spinal fusion, are limited in that they do not restore healthy disc structure or function, and further, do not target the inflammatory drivers that likely lead to symptomatic pain. Large animal models of DD are essential for translating new therapies to the clinical population, as they recapitulate the human IVD in morphology.<sup>4</sup> We previously developed a chondroitinase ABC (ChABC) model of goat lumbar and cervical disc degeneration, which enzymatically degrades the nucleus pulposus (NP), leading to decreased disc height, water content, histological evidence of degeneration, and increased NP inflammatory cytokine and catabolic enzyme expression.<sup>5-7</sup> However, innervation and presence of immune cell, commonly identified in human DD, has yet to be explored within this large animal model. **We hypothesized that structural and functional changes observed in our large animal model of DD would correlate with increased innervation, inflammation, and immune cell presence.**

### Methods

#### Induction of Disc Degeneration

Degeneration of the cervical C2-C3 or C4-C5 intervertebral discs of large frame goats (~3

years of age, equal distribution of male and female) was induced via intradiscal injection of 2U or 5U ChABC in 200  $\mu$ L of buffer (sterile PBS, 0.1% BSA), following IACUC approval. Intradiscal injection of ChABC was performed percutaneously via an anterior approach using a 22G needle with fluoroscopic guidance.<sup>5,6</sup> C3-C4 discs were utilized as controls.

#### Immunohistochemical Analysis (n = 4-6)

Bone-disc-bone segments of cervical IVDs were prepared from explanted motion segments 12 weeks post-ChABC injection. Spine segments were fixed, decalcified, and paraffin embedded. Mid-sagittal sections were cut and used for immunohistochemical analysis of inflammatory cytokine (IL6, TNF $\alpha$ ), nerve (PGP9.5, 1:1 phosphorylated & non-phosphorylated NFH), and monocyte (Ly6C) and macrophage (CD68) cell markers. Sections underwent antigen retrieval and overnight incubation with primary antibodies, followed by incubation with fluorescent secondary antibodies, and were cover-slipped with mounting medium containing DAPI nuclear dye before imaging (Zeiss Axio Scan.Z1).

#### Protein expression analysis (n=4-6)

Immunofluorescent images were thresholded and expression and localization of targets was assessed via percent of fluorescent area within hand drawn regions of interest (ROIs) containing the NP or annulus fibrosus (AF).

#### Statistical analysis

To assess relationships between inflammation, innervation, and IVD structure and function (NP T2 relaxation times, histology score, Toe and Linear Moduli), study outcomes were analyzed using a Pearson correlation test. Pearson r correlation values (rp) between measured variables were interpreted as either low (rp < 0.29), medium (0.3 < rp < 0.49), or high (rp > 0.5) effect size between measured variables. Significance was defined as correlations with p < 0.05 (GraphPad Prism).

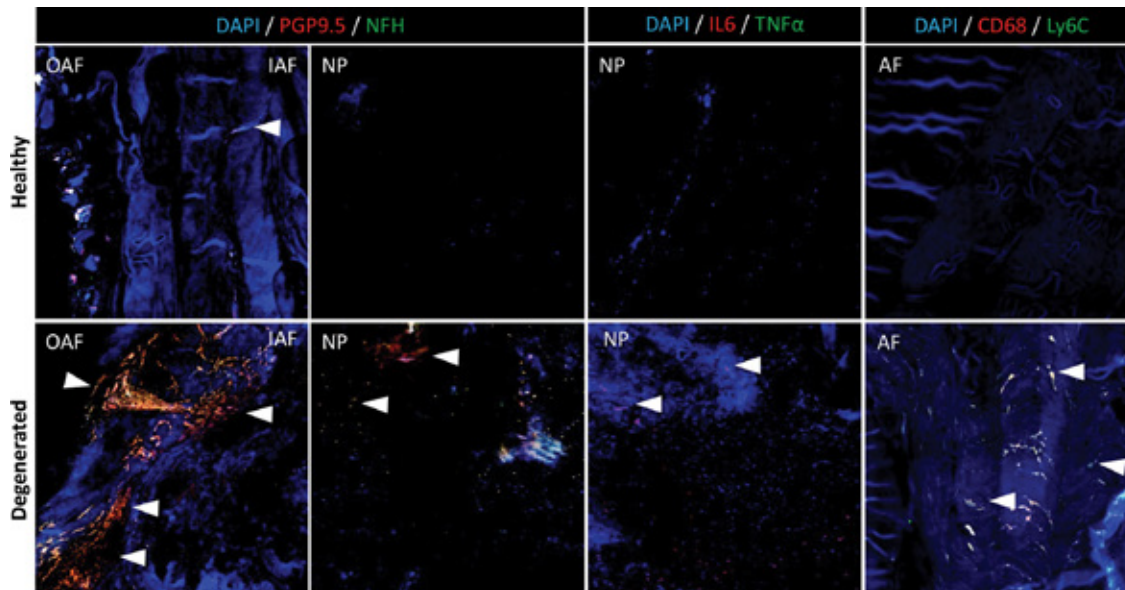
**Results**

Analysis of neuroinflammation revealed a notable presence of innervation along the outer and inner AF and NP regions of degenerated IVDs ( $T2 < 60ms$ ), as indicated by positive PGP9.5 and NFH staining (Figure 1). Further, increased presence of inflammatory cytokines, IL6 and  $TNF\alpha$ , and naïve monocyte (Ly6C) and mature macrophage (CD68) markers was also observed within the NP and AF regions of degenerated discs (Figure 1). Evaluating relationships between structural and functional IVD outcomes revealed a high correlation ( $r_p = -0.7138$ ,  $p = 0.0091$ ) between increased disc histology score and decreased T2 (Figure 2). Neuroinflammation analysis revealed significant high positive correlations between innervation markers PGP9.5 and NFH with each other (NP & AF) and with degenerative structural and functional changes. This included increasing NFH (NP) presence with lower T2 ( $r_p = -0.817$ ,  $p = 0.0039$ ) and increasing AF

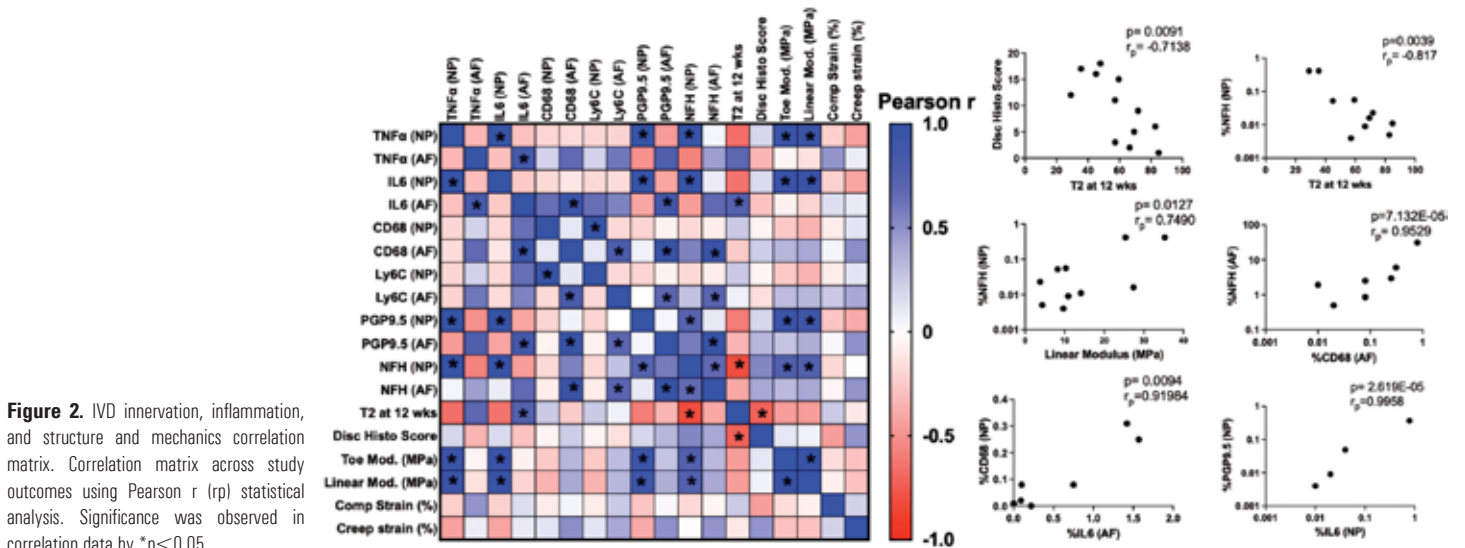
PGP9.5 and NFH presence with increasing Toe and higher Linear moduli ( $r_p = 0.749$ ,  $p = 0.0127$ )(Figure 2). Comparing correlations between innervation, inflammatory, and immune cell markers revealed significant high correlations between increased PGP9.5 and NFH expression with IL6, and CD68 (Figure 2).

**Discussion**

This study sought to determine whether biological drivers of human inflammation and pain commonly associated with DD are observed in a large animal model of ChABC-induced DD. Innervation was identified throughout the AF and NP of degenerated IVDs, as indicated by colocalization of PGP9.5 and NFH. These nerve markers were highly associated with both reduced NP T2, indicative of a loss of NP water and proteoglycan content, and increased Toe and Linear moduli. High positive correlations between inflammatory cytokine expression (IL6,  $TNF\alpha$ ) with both innervation markers (PGP9.5,



**Figure 1.** Innervation and inflammation with chABC induced DD. Fluorescent images of healthy ( $T2 > 60$ ) and degenerated ( $T2 < 60$ ) IVDs stained for nerve, inflammation, and immune cell markers. Positive staining is indicated by white arrows. IVD region is indicated (outer annulus fibrosis AF, inner AF (IAF), and nucleus pulposus (NP)).



**Figure 2.** IVD innervation, inflammation, and structure and mechanics correlation matrix. Correlation matrix across study outcomes using Pearson r ( $r_p$ ) statistical analysis. Significance was observed in correlation data by  $*p < 0.05$ .

NFH) and a mature macrophage marker (CD68) provide a possible mechanism through which local inflammation may be initiating or synergistically promoting immune cell infiltration and innervation to degenerated IVDs, suggesting neuroinflammatory pathways, which have been identified in other chronic inflammatory joint diseases (e.g. OA).<sup>8</sup> Ultimately, these results provide new evidence of immune cell presence and innervation within a large animal DD model, supporting the use of such a model in evaluating both structural and neuroinflammatory therapies.

### Significance

This work improves our understanding of the nociceptive and inflammatory responses within a large animal model of disc degeneration, allowing for the rationale design and

application of therapeutics targeting these pathways to reduce disc inflammation and pain.

### Acknowledgements

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