INTRODUCTION: Shear is an important loading configuration for the annulus fibrosus (AF) of the intervertebral disc, particularly in torsion. Unfortunately, the AF anisotropic shear material properties and the structure-function relationships providing for shear function are not fully established. In this study we define shear modulus for samples within the lamellar plane as $G_{r\theta}$ and perpendicular to the lamella as $G_{\theta \theta}$, where subscripts represent disc orientations of $r = \text{circumferential}$, $\theta = \text{radial}$, and $z = \text{axial}$, and $r = \text{radial}$ (Fig 1). Fiber-reinforced constitutive models suggest very high shear anisotropy, with shear modulus $G_{r\theta}$ being 100X greater than $G_{\theta \theta}$ [1]. However, previous measurements using cubic or cylindrical samples report a difference between $G_{r\theta}$ and $G_{\theta \theta}$ of only 2X [2] and the magnitude of the modulus as very low $G_{r\theta} = 0.06-0.4$ MPa [2,3] and $G_{\theta \theta} = 0.03$ MPa [2] compared to recent measures using AF planar strips, where $G_{r\theta} = 4$ MPa at high tensile preload to engage the fibers and high shear strain [4]. Novel imaging has recently shown that shear strain $\gamma_{r\theta}$ for intact and elastase treated tissue does not involve interlamellar sliding and is not governed by elastin [5], however loads and moduli were not measured in that study. The goal of this study is to quantify AF anisotropic shear properties $G_{r\theta}$ and $G_{\theta \theta}$, and determine the contribution of glycosaminoglycan (GAG) and elastic fibers them via enzymatic digestion.

METHODS: Sample Preparation: Bovine caudal discs were harvested and AF sectioned in $r$, $\theta$ and $z$ orientations (Fig 1). Several planar rectangular samples 3x10 mm and 1.5 mm thick were prepared from each disc. Samples perpendicular to the lamellar plane ($r\theta$) were prepared with $r$ in the long axis; sample within the lamellar plane ($\theta\theta$) were prepared from the outer AF with $\theta$ in the long axis. Samples were assigned to 3 groups: control (no treatment), chondroitinase-ABC (ChABC), and elastase $(r\theta$: n=5/group paired and $\theta\theta$: n=3/group unpaired). ChABC and elastase groups were placed in a 1 ml solution containing buffer, protease inhibitors, and either 1 U ChABC or 3 U elastase and incubated at 37°C for 36 hr. Following incubation, samples were placed in 1 ml of PBS and washed at 4°C for 30 min.

Testing: Cross-sectional area was measured and samples were placed into an Instron 5848 mechanical testing system and equilibrated unloaded for 30 min. Tensile pre-strain in the long axis was applied and held for 15 min ($r\theta$: ε$_{r\theta} = 2\%$ and $\theta\theta$: ε$_{\theta\theta} = 10\%$). Shear strain was applied ($r\theta$: 3/min along the $r$ direction and $\theta\theta$: along the $\theta$ direction, pre-conditioned to 10$^{-2}$ for 20 cycles at 0.05 Hz followed by 3/min ramp to +/- 10$^{-2}$). After testing digestion were verified by DMMB biochemical analysis for GAG and by histology for elastic fiber.

Analysis: The linear-region shear modulus was calculated. For $G_{r\theta}$, the moduli from positive and negative shear strains were averaged. A one way ANOVA with Bonferroni post-hoc analysis was used to evaluate the effect of enzymatic digestion on shear modulus. Significance was set at $p<0.05$ and trends at $0.05<p<0.1$.

RESULTS: There was no difference in GAG between control (following shear testing) and adjacent in situ tissue (untested). Treatment with ChABC reduced GAG by 86% and with elastase reduced GAG by 97%. Histology demonstrated that elastase successfully removed virtually all visible elastic fibers (Fig 2). For samples perpendicular to the lamellar plane, the $r\theta$ stress-strain response was linear (Fig 3) and $G_{r\theta}$ was 18.0 ± 14.9 kPa (mean ± SD), Fig 4. With ChABC treatment $G_{r\theta}$ was 3.5 ± 0.73 (p=0.03 compared to control) and elastase treatments reduced $G_{r\theta}$ by 93% compared to control (p=0.02) to 1.42 ± 1.04 kPa. For samples within the lamellar plane, the $r\theta$ stress-strain response was linear for control, but nonlinear for digested groups (Fig 3). The control $G_{\theta\theta}$ was 133 ± 46 kPa. With ChABC treatment $G_{\theta\theta}$ increased by 450% (p=0.005 compared to control) to 467 ± 137 kPa. With elastase treatment $G_{\theta\theta}$ was 208 ± 81 kPa and not significantly different than control or ChABC. The control $G_{\theta\theta}$ was significantly lower than $G_{r\theta}$ (unpaired t-test) and the ratio of $G_{r\theta}/G_{\theta\theta}$ was 7X, indicating very high anisotropy.

Figure 1 Geometry of $G_{r\theta}$ and $G_{\theta\theta}$ samples showing: control (circle), ChABC (square), elastase (triangle) as well as $G_{rr}$ control (circle).

Figure 2: Representative histology of control and elastase treated samples

Figure 3(Left): Representative plots of $G_{\theta\theta}$ stress-strain curves showing: control (diamond), ChABC (square), elastase (triangle) as well as $G_{rr}$ control (circle).

Figure 4(Left): Bar chart of $G_{r\theta}$ and $G_{\theta\theta}$modulus values, * indicates significance from control

DISCUSSION: In this study $G_{r\theta}$ was an order of magnitude higher than $G_{\theta\theta}$, suggesting that collagen fibers, as opposed to nonfibrillar matrix, are the predominate factor in this loading orientation and confirming strong anisotropy in AF shear. For samples perpendicular to the lamellar plane, $G_{r\theta}$ modulus showed a clear decreasing trend from control to ChABC to elastase treatment. This is consistent with previous reduction in radial tensile moduli under the same treatments [6]. These findings may suggest that radial shear is governed largely by matrix properties and is therefore highly influenced by GAG content. The dependence of shear modulus on GAG corroborates recent observations that long shear strains occur intralamellarly in the form of matrix deformation, as opposed to interlamellarly in the form of straining along the lamellar-interface. Additionally, elastase treatment was more effective than ChABC at removing GAG, with almost all detectable GAG removed. This is likely due to the non-specific nature of GAG removal with elastase, while ChABC does not remove keratin sulfate. The reduction in modulus between ChABC and elastase treatments may be due to further reduction in GAG as opposed to loss of elastic fibers.

For samples within the lamellar plane, $G_{\theta\theta}$ was increased when treated with ChABC. This was surprising and likely represents collagen fibers that can more easily rotate through the nonfibrillar matrix when the GAG is depleted. Samples treated with ChABC and elastase exhibited a nonlinear stress response, similar to that seen in native AF tissue during uniaxial tension, while controls exhibited a linear response. Preliminary studies showed that the transition strain between toe-and linear-region modulus in uniaxial tension was 10%. Thus, by applying a 10% prestain prior to shear, we expected to fully engage the majority of the collagen fibers and commence shear within the linear region of the tissue. In uniaxial tension, treating AF tissue with ChABC or elastase significantly increased their transition strain [6]. For treated samples, it is likely that the 10% prestain applied in this study did not fully engage the collagen fibers, giving rise to nonlinearity in these groups.