In vivo Diffusion MRI Detects Early Changes in Spinal Cord Axonal Pathology in a Mouse Model of Amyotrophic Lateral Sclerosis

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Abbreviations used: DTI, Diffusion Tensor Imaging**;** ALS, Amyotrophic Lateral Sclerosis**;** ADC, apparent diffusion coefficient; MD, Mean Diffusivity; FA, Fractional Anisotropy; AD, Axial Diffusion; RD, Radial Diffusion; SC, Spinal Cord; WM, White matter; GM, Grey Matter; MN, Motoneurons; UMN, Upper motoneurons; LMN, Lower motoneurons; G93A-SOD1: transgenic mice with the overexpression of human mutant gene copper zinc superoxide dismutase identified in familiar forms of ALS patients. YFP: Yellow fluorescent protein.

Abstract:

Diffusion MRI exhibits contrast that identifies macro and microstructural changes in neurodegenerative diseases. Previous studies have shown that MR diffusion tensor imaging (DTI) can observe changes in spinal cord white matter in animals and humans affected with symptomatic amyotrophic lateral sclerosis (ALS). The goal of this preclinical work is to investigate the sensitivity of DTI to detect signs of tissue damage before symptoms appear. High field MRI data was acquired using a 9.4 T animal scanner to examine the spinal cords of ALS mice model at pre- and post-symptomatic stages (day 80 and 120, respectively). The MRI results were validated using yellow fluorescent protein (YFP) via optical microscopy of spinal cord tissue slices collected from the YFP, G93A-SOD1 mouse strain. DTI maps of diffusion weighted imaging (DWI) signal intensity, mean diffusivity (MD), fractional anisotropy (FA), axial diffusivity (AD), and radial diffusivity (RD) were computed for axial slices of the lumbar region of the spinal cord. Significant changes were observed in FA (6.7 % decrease, $p<0.01$), AD (19.5% decrease, $p<0.01$) and RD (16.1 % increase, $p<0.001$) at postnatal day 80 (P80). These differences were correlated with changes in axonal fluorescent intensity and membrane cellular markers. This study demonstrates the value of DTI as a potential tool to detect the underlying pathological progression associated with ALS and may accelerate the discovery of therapeutic strategies for patients with this disease.

1. Introduction

The number of patients diagnosed with Amyotrophic Lateral Sclerosis (ALS) is estimated to be more than 70,000 worldwide and this number is projected to increase by approximately 70% by the year 2040[1,2]. ALS neuropathological evidence points to the lower motor neurons of the spinal cord (SC) as the earliest and the most vulnerable neuronal population affected by this disease[3,4]. Most patients with ALS, have a sporadic form with no obvious family history[1]. However, in approximately 5%–10% of patients, the disease is inherited, and in 20% of these familiar cases, there is a mutation of the SOD1 gene[5]. Thus, studies of ALS using transgenic animal models provide a natural way to identify potential molecular markers and test novel therapeutic approaches at earlier stages of the disease[6,7].

One of the critical features of ALS neuropathology is the presence of early axonal alterations[8]. New fluorescent labeled animal models of neurodegenerative diseases, such as the G93A-SOD1 mutation, have gained momentum among many research groups[9,10]. However, the high resolution morphological information obtained using postmortem cross-section optical fluorescence imaging techniques cannot be applied to preclinical and clinical longitudinal therapeutic studies. Diffusion tensor imaging (DTI) is a novel MRI technique to evaluate early changes in neural structure and axonal connectivity degeneration from white matter (WM) fiber bundles[11] . In the central nervous system (CNS), diffusion of water in structures such as the cerebrospinal fluid or grey matter (GM) is isotropic[12], while in (WM nervous tissue diffusion occurs preferentially along principal directions of fiber bundles. DTI is an imaging technique based on the tensor model, which can be fitted from diffusion measurements along six or more directions from which anisotropy maps may be linked to "within-voxel" axonal organization, whereas "inter-voxel" axonal organization is reconstructed using both FA and orientation information via tractography[13-15]. This technique has been previously applied to the analysis of longitudinal changes in the spinal cord (SC) of ALS patients[16,17]. Medical

studies have shown that diffusivities are correlated with clinical measurements of disease severity and with the ultrastructural presence of axonal loss and reactive gliosis at microscopic level[18-20]. High spatial resolution is beneficial and theoretically easier to achieve at high magnetic fields (e.g.at 7 and 9.4T MRI), where it has been able to identify axonal degeneration in spinal cords from ALS animals following the onset of symptoms[21,22]. However, the underlying connections between diffusivity parameters and biological structural changes at early stages of the disease remain to be investigated.

While the use of *ex vivo* tissues from ALS animal models in high magnetic fields represented a substantial opportunity to correlate and understand the sub voxel changes in the neuropathological environment, this approach does not provide cellular level resolution nor does it allow study of the physiological variables presented in intact biological systems. Thus, the purpose of this study is to show the potential of MRI as a non-invasive technique for the analysis of structural SC changes in a live presymptomatic mice model of ALS. Combining *in vivo* DTI with fluorescent histological techniques, we validate DTI as a way to acquire new tissue biomarkers that reflect the axonal and cellular degeneration characteristic of ALS progression.

2. Experimental Details

2.1. Transgenic mice

C57BJ6 background mice, overexpressing the SOD1 transgene with the G93A mutation were obtained from the Jackson Laboratory (Bar Harbor, ME, JAX # 004435). The phenotype of the G93A-SOD1 mice have been extensively characterized[23-25]. In this genetic background, mice develop motor symptoms at approximately 110 days of age and dying around 160 days of age. To evaluate axonal connectivity anomalies in the context of ALS, a homozygous mouse reporter gene encoding a yellow fluorescent protein (YFP) transgene specifically associated with a neuronal Thy1 promoter was chosen (JAX#003709). A total of 22 animals were used to complete this work, a group of 10 mice were used for MRI imaging scans and 12 mice for histological studies. The first group of YFP-G93A-SOD1 (n=5) and YFP control group mice (n=5) were scanned in the presymptomatic stage of the disease, postnatal day 80 (P80) and later on at their symptomatic age, postnatal day 120 (P120) (total mice =10). In addition, our histological experiments analyzed YFP, G93A-SOD1 mice and YFP littermate mice control (n=3 per each time point P80 and P120) (total mice for histology n=12). Animals were checked daily to assess their level of well-being and health. During our MRI studies, animals were properly anesthetized and no signs of pain or distress were noted during the entire scanning sessions. All procedures used to obtain tissues followed approved protocol of animal care at the University of Illinois at Chicago (UIC).

2.2. Animal preparation

Animals were anesthetized by isoflurane inhalation with vital signs monitored by an MRI-compatible small animal gating system (SA Instruments, Model 1030 Monitoring & Gating System NY) permit free-breathing acquisition during quantitative MRI measurements and the animal was

placed on a heat pad ~~were~~ and the temperature was monitored to maintain the body temperature at 37 degrees Celsius. In order to reduce motion artifacts, animals were positioned in the supine position securing the posterior segments of the spinals cord towards the coil and allowing ventral thoracic and abdominal respiratory movements occurs distant to ROIs. In additions, an air pillow placed under the belly of the animal was connected to the animal gating system to allow respiratory triggering during MRI. The respiratory rate of the animal was kept between the ranges of 35-40 beats per minute by manually adjusting the isoflurane level to migrate effect of repetition time variations. Gating signals were derived from the respiratory motion sensors using a physiological motion gating device. Specifically, changes in respiratory rate on each animals were ~~clinically~~ monitored and proper levels of isoflurane was administered[26,27]. Mice were inserted into the bore of the MR imaging system, and gating signals were used to time the acquisition of MR data. After MRI, the animals were removed from the magnet and placed in a warm environment with frequent checking of their vital signs.

2.3. MRI imaging and calculation of diffusion values

Scans were performed with a 9.4 T Agilent MRI scanner system (St. Clara, CA) using a 31-cm diameter horizontal bore magnet, a 600 mT/m gradient coil, an actively decoupled 72 mm birdcage quadrature coil for transmission and a two-channel mouse brain phase array as receiver. DTI data were acquired using a diffusion weighted fast spin echo sequence with the following parameters: TR = 2100 msec, TE = 36.4 msec, echo train length (ETL) = 8, field of view (FOV) = 19.2 mm x 25.6 mm, acquisition matrix = 192 x 256, slice thickness = 0.8 mm, number of average = 16. Diffusion setting gradients were applied with b= 734 s/mm^2 (the b value used for this study was determined from previous experiments in order to optimize the signal and contrast-to-noise ratio), pulse duration (δ) = 4 msec. and separation (Δ) = 11 milliseconds. The acquisition time was 2 hours per animal. A total of six axial images covering the lumbar spinal cord enlargement were collected

for further analysis **(Figure 3a)**. Images were processed using a diffusion toolkit and Trackvis software (Version 6.0.1, Massachusetts General Hospital, Boston, MA).

Eigenvalues and eigenvectors were determined from images acquired with diffusion gradients oriented in 6 diffusion gradient directions: (1, 1, 0); (1, 0, 1); (0, 1, 1); (-1, 1, 0); (-1, 0, 1); (0, -1, 1). In this analysis, the largest eigenvalue determined the axial diffusivity (AD) (2) and the radial diffusivity (RD) was calculated using the average of the second and third eigenvalues (3). The sum of all three eigenvalues divided by 3 is the mean diffusivity (MD), which corresponds to an isotropic ADC[28]. Therefore Fractional anisotropy (FA) was calculated using Eq. (4),

$$MD = (\lambda_1 + \lambda_2 + \lambda_3) / 3 \quad (1)$$

$$AD = (\lambda_1) \quad (2)$$

$$RD = (\lambda_2 + \lambda_3) / 2 \quad (3)$$

$$FA = \sqrt{\frac{1}{2}} \frac{\sqrt{(\lambda_1-\lambda_2)^2+(\lambda_2-\lambda_3)^2+(\lambda_1-\lambda_3)^2}}{\sqrt{\lambda_1^2+\lambda_2^2+\lambda_3^2}} \quad (4)$$

Here λ_1, λ_2 and λ_3 represents each of the eigenvalues generated from the diffusion tensor. Two right and two left ROIs centered in the WM anterolateral region per spinal cord section were used for data analysis **(Figure 3 b)**. Although it is feasible to examine all segments of the spinal cord (cervical and thoracic and lumbar), results from previous MRI diffusion *ex vivo* studies have shown predominant alterations in MRI diffusion specifically at the SC lumbar segments[28]. Thus, this study is focused in ALS white matter anomalies in this particular SC region.

2.4. Histology and fluorescence analysis

Animals for histology were rendered unconscious by CO_2 inhalation and transcardially perfused with PBS and 4 % paraformaldehyde (PFA) solution. Spinal cords from each animal were dissected

and removed using microsurgical techniques and placed in PFA for 48hrs and then in Phosphate Buffer Solution (PBS) 1x. For cryo-protection, spinal cords were placed in progressive solutions of sucrose [5-30 %] for an additional 24 hrs. After embedding in ~~optical~~ optimum cutting temperature (OCT) polymer compound (Tissue Tek, Sakura, Finetek, cat #4583), 50 µm-thick spinal cord sections were obtained using a 20° Celsius microtome (Leica cryostat CM 1850 Cryostat, Buffalo Grove, IL). Sections were mounted on slides (Fisher brand Superfrost, cat# 12-550-15) and dried out for 15 minutes. Then, OCT was removed by washing three times with Tris base buffer (TBS). For immunohistochemistry (IHC) procedures sections were permeabilized with Triton-X100 0.25 % for 10 minutes and blocked with 5 % goat serum for an hour in TBS. Spinal cord sections (50 µm thickness) were mounted on slides. Each transversal section in the lumbar spinal cord (SC) was selected using similar points of reference using stereotaxic coordinates from previous DTI studies[29]. Specifically, our previous *ex vivo* studies centered in the anterolateral region of the spinal cord as has been extensively proved as an early region of axonal degeneration by previous histological and DTI studies [21,28,30].

To further elucidate the relationship between axonal connectivity and glia markers (oligodendrocytes cells), we stained for myelin basic protein (MBP) (Phospho-solutions, Aurora, CO) (Cat# 1120-MBP, 1:500). In order to evaluate the role of water permeability in ALS, we used anti Aquaporin-4 channels staining (AQP4) (StressMarq Bioscence Inc., Victoria, BC) (Cat #SPC-505D, 1:400). To study the structural relationship of the apparent diffusion coefficient with protein structural scaffolds, we used anti Collagen I (a1 telopeptide sequence) antibodies (Phospho-solutions, Cat#322 COLT, 1:200). To evaluate altered neurofilaments and active transport in degenerating axons, an additional non-phosphorylated neurofilament staining with SMI-32 markers was performed (Covance, Cat # SMI-32R, 1:1000).To avoid fluorescence bleeding through effects, we used secondary antibodies in the far red spectrum (Invitrogen, Fisher Scientific, Alexa647 IgG

nm anti-rabbit, and anti-chicken Cat# A-21244 and A-21449, 1:500) Finally, slides were washed with PBS 1X, dried and mounted in Vecta-Shield mounting media (Vector Laboratories, Burlingame, CA) and counterstained with Ethidium bromide or DAPI.

Images were acquired by confocal microscopy (Leica LMS-710 confocal microscope, Germany). Fluorescent imaging procedures were performed by background subtraction using samples without primary antibody as negative controls following standard procedures described elsewhere[31]. Specifically, imaging data was gathered in two independent channels: 534 nm channel for the YFP signal and 647 nm channel to detect fluorescent emission from other biochemical primary antibody markers previously described in this section. IHC quantitative measures were obtained by accounting mean pixel value per equal picture area using auto-threshold methods and the pixel aggregates of each figure compiled and tabulated for analysis. Briefly, the procedure divides the image into objects and background by taking an initial threshold. Averages of pixels at, below, or above the threshold were computed and subsequent averages of these two values were used. Multiple confocal z- stack planes and ImageJ plugins were used for 3D reconstructions. To measure the degree of association between MRI and histology, fluorescent signal in two ROIs from histological slices from animal from each group were quantitatively analyzed and compared with MRI diffusion values from similar groups.

2.5. Statistical analysis

Quantitative data were tabulated and analyzed using Graph Pad Prism 6 software (La Jolla, CA). For quantitative statistical analysis of MD, FA, RD and AD values, un-paired Student t-tests were used to determine statistical differences between experimental and control mice groups at each time point. Results were replicated by application of non-parametric statistical tools (Mann–Whitney test). To measure degree of association between MRI parameters and histology markers,

we used non-parametric Spearman's correlation analysis. A value of $p<0.05$ was used to demonstrate statistical significance. Error bars in all the figures represent standard error of the mean (S.E.M.).

3. Results

3.1. Measurements of spinal cord areas by MRI are not able to detect presymptomatic changes in the ALS mice.

Although previous clinical MRI studies in symptomatic ALS patients have demonstrated significant reduction in ALS in cross-sectional SC areas at symptomatic stages of the disease[18,32] its role as a preclinical bioimaging marker has been unclear. To investigate if these simple metrics constitute an early imaging biomarkers in ALS, we measured cross-sectional areas in lumbar regions of the G93A-SOD1 and control animal groups at earlier stages of the disease **(Figure 3a)**. Manual segmentations across all lumbar white matter (WM) and grey matter (GM) were obtained from the most distal scan from our SC series during presymptomatic (P80) and symptomatic stages (P120) **(Figure 1a)**. Averages from entire SC cross-sectional areas (WM + GM) have shown significant differences in SC areas in the symptomatic stage (P120) ALS groups compared to the control mice group (YFP,G93A-SOD1 = 4.09+/-0.25 mm^2 vs. YFP control mice group = 5.21 +/- 0.45 mm^2 ($p<0.01$). Nonetheless, when WM and GM segments from these symptomatic P120 group were analyzed independently, we observed a significant reduction in WM (YFP, G93A-SOD1 mice group = 2.18 +/- 0.07 mm^2 vs. YFP control mice group = 2.55 +/-0.07 mm^2) ($p<0.04$) with no significant changes in GM in the YFP, G93A-SOD1 mice group, reassuring a selective impairment of WM and axonal pathology in ALS. Nonetheless, no significant changes were observed between groups at presymptomatic stages (P80) of the disease **(Figure 1b)**, limiting the use of these parameters as presymptomatic biomarkers.

3.2. Isotropic Apparent Diffusion Coefficient (MD) in spinal cord of the ALS mice cannot detect structural changes in white matter at early stages of the disease.

Besides alterations in apparent diffusion coefficients (ADC) reported in symptomatic patients with ALS, the validity of the ADC signal as a presymptomatic marker in ALS remains to be determined. To address such question, we determined the MD, as a measure of the isotropic ADC in consecutive SC lumbar regions of the YFP, G93A-SOD1 mice and YFP control groups. Results from our combined group analysis showed a significant increase in the isotropic ADC average values in the YFP, G93A-SOD1 (7.9 +/- 1.3 10^{-4} mm^2/s) compared to YFP control groups (7.1 +/-1.2 10^{-4} mm^2/s) ($p<0.001$) at a symptomatic stage P120 **(Figure 2a)**. The isotropic ADC increases in the ALS group were homogenously distributed at each level of the SC. However, no significant differences were found at early stage of the disease (P80).

Recent bioengineering studies have focused on the role of cytoskeletal and scaffold proteins towards specific changes in ADC values [33]. To this end, we performed IHC staining targeting the proto- collagen scaffold in the SC of the YFP, G93A-SOD1 mice. Histological fluorescent imaging and confocal z- stack reconstructions of WM axons (yellow) and collagen fibers (red) indicated significant alterations in architectural organization produced by the YFP, G93A-SOD1 mutation compared to isotropic organization of the control mice at similar symptomatic stages of the disease (P120) **(Figure 2b)**. In the context of ALS[34], changes in ADC has also been related to changes in aquaporin 4 (AQP4) expression. To study this marker further and describe the role of membrane water permeability related this diffusion signal in our experimental model, we performed a histological analysis of AQP4 in spinal cord white matter from our ALS and control groups. Particularly, increased expression of AQP4 levels were not only observed the symptomatic stage (P120) changes but also at the presymptomatic stage (P80) as well. However, our current MRI studies

were not able to link the increase of ADC diffusion signals to an increased expression of AQP4 water membrane channels at early ALS stages *in vivo* **(Figure 2c)**.

3.3. Changes in Fractional Anisotropy can detect early microstructural alteration in ALS spinal cord white matter.

A growing number of clinical imaging MRI diffusion studies relied on changes in the evaluation of fractional anisotropy (FA) to quantify the axonal microstructural organization in ALS [35-37].Thus, we segmented white matter (WM) and grey matter (GM) from anisotropy maps values from the lower lumbar segments extracting and analyzing FA values **(Figure 1a)**. Results from this analysis pointed towards a significant increase in FA GM values (YFP, G93A-SOD1 mice group = 0.41 +/- 0.02 vs. YFP mice group = 0.29 +/- 0.02) at symptomatic age (P120) ($p<0.01$) with no differences between groups at presymptomatic stages (P80). However, a decrease in FA from WM lumbar regions was statistically significant the symptomatic stages (YFP, G93A-SOD1 mice group = 0.72 +/- 0.01 vs. YFP mice group = 0.65 +/- 0.01) ($p<0.01$) and also at the presymptomatic stage of the disease (YFP, G93A-SOD1 mice groups = 0.76 +/- 0.01 vs. YFP mice group = 0.69 +/- 0.01) ($p<0.01$). Thus, changes in FA are showing the sensitivity of this parameter to early WM microstructural changes in ALS (Figure 1b).

Previous studies by our group have shown that *ex vivo* changes in ALS mice spinal cord occurs in a distal to proximal progressive pattern. To increase the topographical sensitivity in our studies and elucidate the reproducibility of this finding *in vivo*, we scanned six consecutive SC lumbar segments from ALS mice and littermate's controls **(Figure 3a, b)**. Analysis from these SC regions pointed to a significant decrease in FA from lower lumbar segments (six to four) at presymptomatic stages (P80) to additional upper segments (six to two) at the symptomatic stage (P120) **(Figure 3c)**.

3.4. Spinal cord alterations in Axial and Radial diffusivities are associated to presymptomatic axonal structural changes.

Previous studies proposed axial (AD) and radial diffusivity (RD) as presymptomatic MRI markers for axonal damage and demyelination in ALS spinal cord (SC)[21,38-40]. Our studies have shown that changes in RD progressively increase from presymptomatic ages (P80) in the YFP, G93A-SOD1 mice group = 3.2 +/- 0.8 10^{-4} mm^2/s) compared to YFP control groups = 3.7 +/-0.7 10^{-4} mm^2/s) ($p<0.02$) to symptomatic stages of the disease (P120): YFP, G93A-SOD1 mice group = 3.3 +/- 0.7 10^{-4} mm^2/s compared to YFP control groups = 3.9 +/-0.6 10^{-4} mm^2/s ($p<0.004$) **(Figure 4a)**. Interestingly, alteration of RD showed a progression from SC lower levels (five to six) at P80 to a more progressive and extensive change involving higher planes as well (three to six) following a distal to proximal pattern of neurodegenerations. While radial diffusivity increase is mainly accepted and correlated with myelin content, we used myelin basic protein (MBP) to describe the progressive reduction of myelin levels in spinal cord of the YFP,G93A-SOD1 group **(Figure 4b)**.

Axial diffusivity (AD) has been implicated as one of the main markers in axonal integrity [41]. Presymptomatic evaluations (P80) in our ALS animal model have shown an overall decrease in AD values in the YFP, G93A-SOD1 groups (1.3 +/-0.2 10^{-3} mm^2/s compared to YFP control groups (1.6 +/- 0.2 10^{-3} mm^2/s) ($p<0.01$). Additional changes in AD were observed between animal groups at symptomatic stages (YFP = 1.6 +/- 0.3 10^{-3} mm^2/s vs. 1.4 +/-0.3 10^{-3} mm^2/s) ($p<0.05$). **(Figure 4c)**. Histological alterations reported by the YFP fluorescent markers across longitudinal sections of WM spinal cord (SC) centered in similar MRI ROIs have shown a decrease in axonal caliber and an increase in axonal tortuosity in the YFP, G93A-SOD1 mice group (P80) at the presymptomatic age and an increase in inter-axonal space at symptomatic stage (P120) **(Figure 4d)**.

4. Discussion

Amyotrophic Lateral Sclerosis (ALS), was first described in the literature more than 150 years ago by French neurologist Jean-Martin Charcot as a highly debilitating disease caused by progressive degeneration of upper and lower motor neurons[4]. To date, over 150 different mutations have been linked to the disease, some causing a long clinical course, while others trigger an exceptionally aggressive and short form of the disease[42]. Considering the short time frame between diagnosis and demise in ALS patients, the need for a non-invasive technique allowing for early detection of the disease is necessary. However, this approach cannot be used in patients with sporadic ALS (90% of the total ALS population), who are a high risk for developing ALS that cannot be identified until they develop symptoms[43]. Thus, the development of noninvasive methods to detect the disease before symptoms occur could be a better strategy to help in the development of new therapeutic strategies to preserve axonal connectivity in patients with ALS.

In previous *ex vivo* ALS mice studies (B6JL mice) from our group, we have shown DTI is able to detect microstructural changes in early stage of the disease[28]. Hence, this study is a continuation from our previous work using a new line of mice (C57 mice) with a fluorescent reporter *in vivo.* In addition, a growing number of published work have pointed differences between *ex vivo* models has been previously discussed in the literature, choosing the best model for the purposes of each study; *ex vivo* for precision, *in vivo* for time-course[44-46]. Moreover, recent studies have pointed that significant structural difference in nervous tissues can be seen between *ex vivo* and *in vivo* preparations with clear trade-off between these two imaging approaches[46]. Specifically, we analyzed SC microstructural changes in animal ALS using a different animal-model (YFP, G93A-SOD1 mice). Results from our MRI segmentation analysis have shown that macrostructural alterations in WM can be detected at the symptomatic stage of the disease, demonstrating the particular susceptibility of this CNS tissue to the

SOD1 mutation **(Figure 1)**. Nonetheless, considering the limitations of this approach to the early detection of this disease, we proceeded with the analysis of additional MRI diffusion techniques to interrogate further WM microstructural changes.

Diffusion MRI is one of the most relevant techniques to unveil the pathophysiological mechanisms of multiple diseases in the central nervous system. Among basic diffusion MRI techniques, ADC is sensitive to the integrity of cellular membranes and the impedance of water molecules diffusion[47]. In living organisms, measurements of ADC are sensitive to alterations or changes of different biological tissues, including the spinal cord[33,48]. Previous clinical and basic ALS studies using ADC markers in symptomatic cervical spinal cords showed a particular increase in ADC during the course of the disease[49,50]. In ALS patients, several studies have also proven ADC can be used as sensitive parameter to investigate anomalies in spinal cord tracts [34] and GM brain regions[50]. Although changes in ADC have been linked to different biological markers, the particular role of ADC as an ALS presymptomatic markers in the SC region has not been identified yet[51].

Recent tissue engineering studies have shown that ADC values can be attributed to a change in the magnetic interaction between the water molecules and collagen fibers[52]. Moreover, the relation between ADC and collagen density in cartilage engineered tissues has been proven where the increase in collagen polymerization and fiber packing lead to a decrease in ADC values[48]. Similarly, our data support a structural alteration in collagen fibers which may explain the changes in ADC at the symptomatic stage of ALS **(Figure 2a, b)**. This may indicate that structural scaffolds such as collagen are highly preserved in early stages of the disease and not suitable as early biomarkers. Overall, ADC is a collective measurement of how fast water diffuses in a complex diffusion environment. Consequently, in an environment mixed with free diffusion and restricted diffusion, ADC only reveals an overall measurement and is b-value dependent, including gradient strength and duration. In our data sample,

the isotropic ADC (MD) was not able to capture differences between control and ALS mice at P80. The specific relationship between each structural protein with the ADC signal is not well-known it is possible that specific alterations of many other biochemical markers of axonal structure and function can indeed modify ADC parameters during disease **(Supplementary figure 1)**.

As water constitutes approximately 60%-70% of the human body and its diffusion depends on random Brownian motion[12,53] extracellular environments experience relatively free diffusion of water while intracellular molecules show relatively restricted diffusion[54,55]. In biological tissues, water diffusion is regulated on its passageway across tissues by specialized mechanisms[56,57]. One of the key molecules regulating the passage of water in the nervous systems are transmembrane proteins called Aquaporin (AQP)[58]. Among all the isoforms, the Aquaporin 4 (AQP4) is one of the most abundant in neuronal tissue and glia (astrocytic) cell lines[59]. AQPs are also abnormally expressed in neurodegenerative conditions[60-62] pointing toward the possibility that both events are indeed related. As an example, recent studies demonstrated that the induced expression of AQPs in cellular constructs was associated to an increase in ADC values[63]. Similar to previous SC studies of rodent models of ALS[64], our experiments have visualized an increase in AQP4 expression **(Figure 2c).** However, our present MRI setup was not able to relate the increase of this biomarker to ADC changes at early stage of ALS (P80). Overall, our current *in vivo* experiments pointed ADC as a limited presymptomatic marker of disease. However, the dependence of ADC on gradients properties (diffusion time, gradient duration and strength) using higher magnetic field studies would determine the validity of this parameter as a useful presymptomatic bioimaging marker and subject of future experiments at higher gradients and high resolution MRI settings. Nonetheless, different studies have shown that DTI derived parameters had higher sensitivity and specificity than ADC values in patients with SC pathology[65,66].

Diffusion tensor imaging (DTI) exploits this property to produce micro-architectural detail of white matter tracts and provides information about white matter integrity[67]. Moreover, the use of anisotropic indices such as fractional anisotropy (FA) has been accepted as a common neuroimaging tool in basic and clinical practice, opening new insights into the understanding of the ALS pathology [16,19,32,37,67]. Particular to our study, FA was able to detect early white matter changes associated with axonal structural changes **(Figure 1b)**. The advantage of the spatial resolution of this imaging technique allowed us to study individual SC segments providing a glimpse into the real-time evolution of the disease **(Figure 3b)**. Hence, this work corroborate several neuropathological reports[8,68,69], where the WM deterioration in ALS followed a distal to proximal progression of the disease **(Figure 3c)**.

One of the major discoveries involving the G93A-SOD1 mice is that ALS is not cell specific, which means that glial cells surrounding motor neurons are probably involved in the mechanisms leading to the selective death of motor neurons[70] and specific differences have been described between axonal damage and reduction in myelin content using DTI[41,71,72]. Particularly, our study aimed to unveil the role of radial diffusivity (RD) and myelin degeneration in ALS, at earlier stages of the disease[70,73]. Consistent with previous reports of oligodendrocyte impairments in ALS[73,74], our investigations have determined a presymptomatic increase in RD associated with an early reduction of MBP in the YFP, G93A-SOD1 mice **(Figure 4 a, b)**. Remarkably, patterns of alterations in RD followed a similar arrangement of alterations previously described in axonal microstructure and organization (measured by FA). The similar distal to proximal alteration in AD and RD may indicated a multicellular degeneration produced by an early disruption of a trophic relationship between glial and neuronal cells[75,76]**.**

The study of axial diffusivities (AD) to measure axonal damage in ALS has been elusive due to numerous different and contradictory results reported between animal models (decreased AD)[21,22,77]

and human studies (no significant changes in AD)[19,36,49,78,79]. In that context, our data suggested AD followed decrease in values in the mutant group compared to controls, particularly at the presymptomatic stage **(Figure 4c)**. Moreover, our histological studies have shown that such diffusion changes (decrease in AD in the YFP, G93A-SOD1 mice group) could be explained by complex alterations in axonal organization during the development of ALS **(Figure 4d)**. However, presymptomatic (mild axonal) damages are mostly associated to biochemical and molecular alterations leading to impairment in axonal function **(Supplementary figure 1)** and leading to ultra-structural anomalies restricting the water diffusion in the axial plane **(Supplementary figure 2)**. Overall, our work has been able to describe an association between presymptomatic MRI, histological & molecular markers **(Supplementary figure 3)** and identified potential early biological and structural markers of ALS *in vivo* **(Table 1).**

Based on inadequate therapeutic results and the lack of optimistic outlook for new therapies, the utility of animal models in the preclinical phase for identifying therapeutic agents in ALS has been doubted [80]. One of the central explanation for such disparities in this apparent therapeutic inefficacy is based in common practice of early administration of experimental drugs in animal models before the onset of symptoms. Important limitations to this study and imaging techniques have to be addressed. From a technical standpoint, the application of MRI methods at the spinal level presents multiple challenges. Due to the small size of the cord relative to the brain, a higher spatial resolution is required. Physiological motions (respiration, heart beats) create ghosting artifacts and partial volume effects due to the surrounding cerebrospinal fluid, chemical shift artifacts arising from the epidural fat and other nearby structures, and geometric distortions arising from magnetic field inhomogeneity in nearby intervertebral disks and lungs[13,35]. Another major physical limitation in MRI resolution is its voxels size (>100 microns) compared to individual axonal diameter (1 to 20 microns). In addition, *in vivo* studies require a short scanning time (typically 0.5–2.0 hr.), which also limits the achievable

resolution. In addition, one limitation of our longitudinal MRI studies is the use of a different animal groups for histological preparations, particularly at early stages of the disease. This factor could increase the variance between MRI and histological markers presented in this *in vivo* study. Considering these limitations, our approach is dedicated to complement such shortcuts, including the examination of comparable histological regions by optical techniques with higher imaging spatial resolutions allowing us to acquire more information (including biochemical changes) during early stages of the neuropathological processes. Another limitation is the oversimplification of complex multicellular changes described by a unique diffusion value. Hence, the characterization of the anomalous diffusion in biological porous tissue requires a new approach to improve the detection of multicellular events at earlier stages of the disease in order to continue improving this MRI technique as an optimal imaging tool for early detection and monitoring in ALS patients.

5. Conclusion

Our results demonstrate that fractional anisotropy (FA), axial diffusivity (AD), and radial diffusivity (RD) are able to identify changes in nervous tissue at presymptomatic stages of ALS. The combination of MRI diffusion and fluorescent histological techniques confirmed the sensitivity of DTI biomarkers and revealed the ultrastructural origin of the diffusion signals to be axonal pathological process associated with ALS. Considering the initial complex cellular processes governing water diffusion in living tissues over the course of the disease, the development of more representative diffusion models is still needed. Overall, we believe this work can be adapted to assist in better understanding the biological nature of each MRI tensor biomarker and ultimately be used to test the effectiveness of therapeutic strategies in patients with ALS.

6. Conflict of interest statement

The authors do not have any conflict of interest to report.

7. Author contributions

RG conceived and designed the experiments. RG and RM wrote the manuscript. RG performed all the histology experiments and data analysis. JG and WL performed all the imaging experiments.

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Figure Legends:

Figure 1

Measurements of cross-sectional lumbar spinal cord areas are not amenable to detect presymptomatic changes in ALS mice. **Figure 1a -** Fractional anisotropy maps showing manual segmentation of regions of interest (ROI) from white matter (WM: thick white dashed line) and grey matter (GM: thin white dashed line) from YFP control and YFP, G93A-SOD1 mice lumbar spinal cord (SC) at presymptomatic (P80) and symptomatic (P120) disease stages. **Figure 1b -** Comparative changes in total spinal cord areas (GM and WM) between YFP control and ALS mice (upper right) a decrease in WM at symptomatic stage P120** ($p<0.01$) (upper right) without significant changes at earlier stages of the disease P80. ** ($p<0.01$), *($p<0.05$), ($n=5$). Scale bar = 1 millimeter.

Figure 2

Modifications in isotropic Apparent Diffusion Coefficients are connected to structural changes in molecular biomarkers in ALS. **Figure 2a -** DTI analysis have shown a significant increase in the mean diffusivity (MD) – or isotropic Apparent Diffusion Coefficient (ADC) – signal from the anterolateral region of the white matter (WM) in the YFP, G9A-SOD1 group. Increases in isotropic ADC were widespread across distal to proximal SC regions only in the YFP, G93A-SOD1 symptomatic (P120) animal group. **Figure 2b -** Coronal sections of WM shows YFP labeled axons (Yellow), an increased staining for proto-collagen (Red) can be seen at symptomatic stages (P120). Confocal microscopy 3D z- stacks reconstructions shows morphological alterations in collagen fibers the P120 YFP, G93A-SOD1 mice group. **Figure 2c -** Coronal sections of WM SC show YFP labeled axons (Yellow) an increased staining for Aquaporin 4 (AQP4) membrane channels (Cyanine) at presymptomatic and symptomatic stages (P80 and P120). Note changes in expression of

water channel membranes are ostensible in presymptomatic ALS. Dist., Distal; Prox., Proximal; SC, Spinal Cord; WM, white Matter, AQP4, Aquaporin 4; YFP, Yellow Fluorescence Protein; G93A-SOD1: Mutation of the super oxidase isoform 1 by substitution of alanine to glycine amino-acid residue. *** ($p<0.001$), ** ($p<0.01$), *($p<0.05$) (n=5). Nuclear Counterstaining with DAPI (Blue). Scale bar = 10 microns.

Figure 3

Alterations in Fractional Anisotropy can be detected in the presymptomatic stage of ALS. **Figure 3a –** Sagittal topographic T2 map showing levels of axial sections centered at lumbar spinal cord (SC) segments and ROIs selected for this study (Six axial slides). Scale bar =1cm. **Figure 3b** – Fractional Anisotropic (FA) maps from axial SC lumbar sections showing n YFP control and YFP, G93A-SOD1 mice. ROIS from grey matter (GM) (crosses) and white matter (WM) (asterisks) were used to calculate mean FA values at each segment. **Figure 3c** - FA analysis from GM and WM (lower right left) detected significant changes in WM axonal organization at P80 *** ($p<0.001$) indicating microstructural alterations in early stages of ALS (n=5 animals per group. Scale bar = 1 millimeter. FA plots from each spinal cord level showing significant reduction in distal sections of the spinal cord at the symptomatic stage (P120) and presymptomatic stage (P80). Note the distal to proximal alteration in the SC as the disease progresses (dying-back pattern of neurodegeneration).DTI; Diffusion tensor imaging, GM; Grey matter; WM; white matter; FA; Fractional anisotropy, YFP; Yellow fluorescent protein; G93A-SOD1, Mutation of the super oxidase isoform 1 by substitution of alanine to glycine amino-acid residue. Dist.; Distal, Prox.: Proximal, ** ($p<0.01$), *($p<0.05$), (n=5). Scale bar = 1 millimeter.

Figure 4

Presymptomatic structural changes are associated with Radial Diffusion alterations in ALS. **Figure 4a** - DTI analysis has shown a significant increase in Radial Diffusion (RD) in the anterolateral region of the white matter (WM) in the YFP, G9A-SOD1 group. Note that changes followed a progressive distal to proximal pattern in the symptomatic (P120) and presymptomatic (P80) animal groups. **Figure 4b -** Coronal sections of the WM spinal cord (SC) show YFP labeled axons (yellow), a decrease in MBP staining (Magenta) and an increase in inter-axonal space in the YFP, G93A-SOD1 groups (black) at presymptomatic and symptomatic stages (P80 and P120). Note the decrease in MBP content and increase in the inter-axonal space in presymptomatic WM which may contribute to the increase in RD in the ALS mice groups. **Figure 4c -**Axia**l** Diffusion alterations are associated to axonal structural changes in presymptomatic ALS mice**.** DTI analysis have shown an early reduction in Axial Diffusion (AD) in the anterolateral region of the white matter (WM) in the YFP, G9A-SOD1 group. **Figure 4d** - Longitudinal sections of the WM spinal cord (SC) centered in similar ROI WM show a decrease in axonal caliber and increase in axonal tortuosity in the YFP, G93A-SOD1 mice group at the presymptomatic Stage (P80). Dist.; Distal, Prox.; Proximal, MBP: Myelin Basic Protein, SC: Spinal Cord; WM, white Matter; YFP; Yellow Fluorescence Protein. *** ($p<0.001$), ** ($p<0.01$), *($p<0.05$), ($n=5$). Nuclear Counterstaining with DAPI (Blue). Scale bar = 10 microns.

Supplementary figure 1

Changes of axonal function can be detected in concurrence with MRI diffusion anomalies. **Supplementary figure 1a** -Diagram from a lumbar spinal cord (SC) axial section showing white matter (WM) and grey matter (GM) regions used for Immunohistochemistry (IHC) staining. An early increase of non-phosphorylated neurofilament staining (SMI-32) can be observed in the G93A-SOD1 mice, indicating an early impairment in axonal function in ALS. **Supplementary figure 1b -**

Higher magnification of anterolateral region of lumbar SC shows an increase of SMI-32 markers across axonal populations. Scale bar =10 microns. Ultrastructural.

Supplementary figure 2

Ultrastructural WM anomalies as a basis of changes in MRI diffusion. **Supplementary figure 2a -** Longitudinal spinal cord (SC) sections demonstrate early ultrastructural anomalies in WM organization (tortuosity) along with individual morphological axonal changes (beading and varicosities) (white arrows) seen in the fluorescent reporter SOD1 mice. The combination of these combined and individual structural anomalies could be responsible in the decrease in axial diffusion (AD). **Supplementary figure 2b** - Diagram representing a longitudinal section from SC control mice showing well-organized parallel axonal tracts group allowing unrestricted water diffusion across the WM tissue. Illustration of similar WM regions of the presymptomatic ALS mice where changes in axonal morphology could leads to a restriction (decrease) in axial water diffusion. Scale bar =10microns

Supplementary figure 3

Correlative plots showing the association between SC MRI diffusion changes and WM biological markers at early and symptomatic stages of the disease. **Supplementary figure 3a-** A negative association between radial diffusion (RD) and Myelin basic protein (MBP) levels can be observed in spinal cord (SC) tissues of the YFP control and YFP,G93A-SOD1 (ALS mice) (R^2= 0.60). Greater correlation between RD and MBP can be seen in the ALS mice group alone (right) (R^2= 0.67). **Supplementary figure 3b** - A positive correlation between fractional anisotropy (FA) and the axonal tissue content (measured by YFP levels) in control (YFP) and ALS mice (YFP, G93A-SOD1)

(R^2= 0.57). Greater correlation can be seen between FA and YFP levels (right) in the ALS mice group alone (R^2= 0.79). AU, Arbitrary Units; FA, Fractional Anisotropy; MBP, Myelin Basic Protein; RD, Radial Diffusion; YFP, Yellow Fluorescent Protein.