Title	Histological correlation of diffusional kurtosis and white matter modeling metrics in cuprizone-induced corpus callosum demyelination
Author(s)	Falangola, MF; Guilfoyle, DN; Tabesh, A; Hui, ESK; Nie, X; Jensen, JH; Gerum, SV; Hu, C; LaFrancois, J; Collins, HR; Helpern, JA
Citation	NMR in Biomedicine, 2014, v. 27 n. 8, p. 948-57
Issued Date	2014
URL	http://hdl.handle.net/10722/199057
Rights	NMR in Biomedicine. Copyright © John Wiley & Sons Ltd.

Histological Correlation of Diffusional Kurtosis and White Matter Modeling Metrics in the Cuprizone-Induced Corpus Callosum Demyelination

Maria F. Falangola[1,2,3,*], David N. Guilfoyle[4], Ali Tabesh,[1,2], Edward S. Hui[1,2], Xingju Nie[1,2], Jens H. Jensen[1,2], Scott V. Gerum[4], Caixia Hu[4], John LaFrancois[5], Heather R. Collins[2], Joseph A. Helpern[1,2,3]

[1]Department of Radiology and Radiological Science, Medical University of South Carolina, Charleston, SC 29425; [2]Center for Biomedical Imaging, Medical University of South Carolina, Charleston, SC 29425; [3]Department of Neurosciences, Medical University of South Carolina, Charleston, SC 29425. [4]Center for Advanced Brain Imaging, Nathan S. Kline Institute, Orangeburg, NY 10962. [5]Dementia Research, Nathan S. Kline Institute, Orangeburg, NY 10962.

*Corresponding Author:
Maria F. Falangola, M.D., Ph.D.
Department of Radiology and Radiological Science
Center for Biomedical Imaging, MSC 120
Medical University of South Carolina
68 President St, Bioengineering Building Rm 212
Charleston SC 29425-0120
Email: falangol@musc.edu
Tel: 843.876.2466
Fax: 843.876.2469

Word count: 7833

Short title: DK and WMM in the Cuprizone-Induced Mouse Brain Demyelination

Key words: DKI, cuprizone, corpus callosum, mouse, demyelination, MRI, diffusion

Abbreviations: diffusion MRI (dMRI); diffusion tensor imaging (DTI); diffusion tensor (DT); diffusional kurtosis imaging (DKI); axial kurtosis ($K_{//}$); radial kurtosis ($K_{\perp}$); white matter modeling (WMM); extra-axonal space (EAS); axonal water fraction (AWF); intrinsic diffusivity inside the axons(Da); axial diffusivity in the extra-axonal space ($D_{e||}$); radial diffusivity in the extra-axonal space ($D_{e\perp}$); tortuosity (α); cuprizone treated group (CPZ); control group (NC); corpus callosum (rostral (aCC), middle (bCC), and caudal (pCC)); immunohistochemistry (IHC); Glial fibrillary acidic protein (GFAP); ionized calcium binding adapter molecule 1 (Iba1).

Grant support: This work was supported by the NIH grants NIH 5R03EB009711-2 (MFF) and 1S10RR023534-01.

ABSTRACT:

The cuprizone mouse model is well-established for studying the processes of both demyelination and remyelination in the corpus callosum, and it has been utilized together with diffusion tensor imaging (DTI) to investigate myelin and axonal pathology. Although some underlying morphological mechanisms contributing to the changes in diffusion tensor (DT) metrics have been identified, the understanding of specific associations between histology and diffusion measures remains limited. Diffusional kurtosis imaging (DKI) is an extension of DTI that provides metrics of diffusional non-Gaussianity, for which an associated white matter modeling (WMM) method has been developed. The main goal of the present study was to quantitatively assess the relationships between diffusion measures and histological measures in the mouse model of cuprizone-induced corpus callosum demyelination. The diffusional kurtosis (DK) and WMM metrics were found to provide additional information that enhances the sensitivity to detect the morphological heterogeneity in the chronic phase of the disease process in the rostral segment of the corpus callosum. Specifically, in the rostral segment axonal water fraction ($d = 2.6$; $p<0.0001$), radial kurtosis ($d = 2.0$; $p=0.001$) and mean kurtosis ($d = 1.5$; $p=0.005$) showed the most sensitivity between groups with respect to yielding statistically significant p-values and high Cohen's d values. These results demonstrate the ability of DK and WMM metrics to detect white mater changes and inflammatory processes associated with cuprizone-induced demyelination. They also validate, in part, the application of these new WMM metrics for studying neurological diseases, as well as help to elucidate their biophysical meaning.

INTRODUCTION

The cuprizone mouse model (1-3) is well-established for studying the processes of both demyelination and remyelination in the corpus callosum. In this model, young adult mice are fed the copper chelator cuprizone (bis-cyclohexanone oxaldihydrazone), leading to reproducible central nervous system (CNS) demyelination within weeks. Removal of the toxin from their diet results in spontaneous remyelination (4,5). Cuprizone intoxication follows a consistent pattern with loss of myelin and oligodendrocytes during the first 3 to 4 weeks of cuprizone administration accompanied by recruitment of microglia, macrophages and astrocytes (acute demyelination); as a response to the demyelination process, oligodendrocyte progenitors begin to accumulate within the lesion and after 5 or 6 weeks of continuous cuprizone administration, demyelination is intense (subacute demyelination) and spontaneous remyelination starts to occur. With continued cuprizone administration and successive waves of demyelination, a chronically demyelinated state is reached. Considerable variation in the degree of myelination of the different segments of the corpus callosum is apparent at all stages of the process (5-8). This mouse model develops both motor and behavioral deficiencies, that correlate well with the extent of demyelination (9,10).

Both in-vivo and ex-vivo diffusion tensor imaging (DTI) have been used to quantify myelin and axonal pathology in the CC of the cuprizone mouse model (11-20). Broadly, these reports have shown a decrease in axial diffusivity ($D_{||}$) and an increase in radial diffusivity ($D_{\perp}$) associated with the process of demyelination. Significant correlations between such diffusion tensor (DT) metrics and histological measurements of tissue damage have also been reported (14,18,20). Although some underlying morphological mechanisms contributing to the changes in DT metrics have been proposed (14,16,18),

the understanding of specific associations between histology and diffusion measures is still limited.

Diffusional kurtosis imaging (DKI) is an extension of DTI that provides, in addition to the conventional DT metrics, diffusional non-Gaussianity metrics as quantified by the kurtosis of the diffusion displacement probability density function (21-23). These extra diffusional kurtosis (DK) metrics, including mean kurtosis (MK), axial ($K_{//}$) and radial kurtoses ($K_{\perp}$), are unavailable with DTI, which is based on a Gaussian diffusion signal model. The DK metrics are complementary to the DT metrics and serve to more completely characterize tissue microstructure.

Recently, DKI has been applied in humans to study several brain diseases (24-34) resulting in novel observations. Additionally, animal studies have shown that DK metrics can help differentiate brain tissues (35) and are sensitive to changes in brain microstructure associated with aging (36), Aβ deposition (37,38), stroke (39-40), axonal degeneration in the autoimmune encephalomyelitis rat model (41), traumatic brain injury (42) and developmental changes in a rat model of Huntington's disease (43).

In order to better understand the biophysical meaning of diffusion metrics in the brain, tissue modeling has frequently been employed to construct explicit links to various microstructural properties, such as cell densities and sizes, as well as to compartment-specific diffusion properties. One approach, referred to as white matter modeling (WMM), has been developed specifically for the analysis of DKI data in white matter (44). Briefly, this method relates DKI data to several microstructural parameters: the axonal water fraction (AWF); the intrinsic diffusivity inside the axons, D_a; the axial and radial diffusivities in the extra-axonal space (EAS), $D_{e,||}$ and $D_{e,\perp}$ respectively; and tortuosity (α) of the EAS defined as $D_{e,||}/D_{e,\perp}$. Although the relationship between these indices and pathological

tissue changes can be subtle, AWF is expected to be particularly sensitive to axonal loss, while $D_{e,\perp}$ and α are expected to be particularly sensitive to demyelination, but can also be related to other changes in extra-axonal diffusion, e.g., extracellular inflammation. In a recent application of this WMM model, our group was able to distinguish normal subjects from subjects with mild cognitive impairment (MCI), particularly in the corpus callosum (45), and to show that myelin in late-myelinating white matter tracts preferentially deteriorates in the course of Alzheimer's disease (46).

The goal of this study is to quantitative characterize the DK-WMM metrics in the chronic stage of demyelination in the cuprizone mouse model, and to investigate the correspondence between changes in diffusion metrics and morphological alterations associated with myelin pathology. These results may be of value for interpreting DKI-based studies of neurological disorders, such as multiple sclerosis and Alzheimer's disease, for which myelin changes are believed to play a prominent role.

MATERIALS AND METHODS

Animal Model

A total of 20 (8-10 week old) male C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were used in this study. The cuprizone treated group (CPZ; n=10) were fed a diet containing cuprizone (0.2%), (Bis(cyclohexanone) oxaldihydrazone, Sigma-Aldrich) mixed into milled chow pellets for a period of 10 weeks to induce a chronic state of CNS demyelination. The control group (NC; n=10) was maintained on a normal diet for 10 weeks. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Nathan Kline Institute and Medical University of South Carolina, and in accordance with the National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals. All animals were housed in temperature- and humidity-controlled

rooms on a 12-h light/dark cycle (lights on at 6:00 AM) in an accredited animal care facility.

MRI Protocol

Mice were anesthetized using an isoflurane vaporizer set at the following percentages: 3% for induction, 2% during pilot scanning, and 1.5% during data acquisition. An animal monitoring unit (SA instruments, Inc., model 1025, Stony Brook, NY) was used to record respiration and rectal temperature. Respiration was measured with a pressure transducer placed under the abdomen just below the ribcage. Body temperature was maintained using forced warm air, controlled by a feedback circuit between the heater and thermistor. After induction, mice were placed on a holder and restrained using a bite bar and ear bars placed half way into the auditory canal. Oxygen was used as the carrier gas and delivered at a low flow rate (≤ 0.5 L/min.) to a cone positioned before the bite bar, where gases mixed with air and passed over the rodent's nose. All animals were maintained at 37.0 ± 0.2 °C and respiration ranged between 50 to 70 breaths per minute with a median heart rate of 500 beats per minute during scanning. All data were obtained on a 7.0 T Agilent (Santa Clara, CA) 40 cm bore system. The gradient coil insert had an internal diameter of 12 cm with a maximum gradient strength of 600 mT/m and minimum rise time of 200 μs with customized second and third order shim coils. A Rapid (Rimpar, Germany) volume transmit coil (72 mm ID) and a 2 channel receive-only surface coil was used for RF transmission and reception, respectively. A respiration-gated 4-shot SE-EPI sequence was used for DKI acquisition. The sequence parameters were: TR/TE=3000/30ms, δ/Δ=5/17ms, slices=7, slice thickness = 1mm, data matrix = 128 × 128, image resolution = 234 × 234 μm^2, number of averages = 4, 30

gradient directions (47) and 5 b-values for each gradient direction (0.5, 1.0, 1.5, 2.0 and 2.5 ms/μm^2).

DKI post-processing was performed using in-house software (48) (http://nitrc.org/projects/dke). The software generates parametric maps by fitting the DKI signal model (21,22) to the diffusion MRI (dMRI) signal measurements for each voxel using a linearly constrained weighted linear least squares fitting algorithm (Tabesh et al., 2011), resulting in parametric maps of the conventional DT metrics of mean diffusivity (MD), $D_{||}$, $D_{\perp}$, and fractional anisotropy (FA), as well as the additional kurtosis metrics of MK, $K_{||}$, and $K_{\perp}$. All of these metrics are estimated from the DT and DK tensors (22). The MD corresponds to the diffusivity averaged over all possible diffusion directions, $D_{||}$ corresponds to the diffusivity in the direction of the principal diffusion tensor eigenvector, and $D_{\perp}$ corresponds to the diffusivity averaged over all diffusion directions perpendicular to the principal diffusion tensor eigenvector. The additional metrics of MK, $K_{||}$ and $K_{\perp}$, are kurtosis analogs of MD, $D_{||}$ and $D_{\perp}$ that quantify the diffusional non-Gaussianity (21). It is worth noting that, due to the inclusion of non-Gaussian effects, the DKI-derived estimates of diffusivities will generally be more accurate than those obtained with DTI (49). In addition, parametric maps were estimated for WMM metrics of: AWF, D_a, $D_{e,||}$, $D_{e,\perp}$ and α as described previously (44).

Brain regions of interest (ROIs) at the level of corpus callosum (rostral (aCC), middle (bCC), and caudal (pCC)) were manually drawn on the FA map using ImageJ software (version 1.42q; http://rsb.info.nih.gov/ij/) (50) (Fig.1). To meet the WMM assumption (44) of fiber bundles that are highly oriented in a uniform parallel pattern, we limited the ROIs to the center of the CC at each level. We sampled two MRI slices for aCC and bCC and one MRI slice at the level of pCC. All ROIs contained at least 9 voxels. The

average regional value for each metric was obtained from the voxels within each ROI. To minimize the effect of cerebrospinal fluid (CSF) contamination, all voxels with MD>1.5 μm^2/ms were excluded from the ROIs prior to parameter quantification.

Histological Protocol

Following the MRI scan, while still under anesthesia, mice were perfused with ice-cold phosphate buffered saline (PBS) followed by paraformaldehyde (4%) in PBS. Mice brains were then removed and post-fixed overnight in the same fixative, and then transferred to a storage buffer (Na Cacodylate). All mice brain samples were processed by NeuroScience Associates (Knoxville, TN) using MultiBrain® Technology, where 25 mouse brains are embedded together per block, and freeze-sectioned at 30 μm in the coronal plane through the CC area of the mouse brain (~3 mm in length). Sections were stained free-floating at room temperature for: A) Solochrome to reveal myelin (51); B) Amino cupric silver to reveal axonal degeneration (52); C) Glial fibrillary acidic protein (GFAP) to reveal astrocyte activity (53), and D) ionized calcium binding adapter molecule 1 (Iba1) to reveal microglia (54). It is important to note that all immunohistochemistry (IHC), for each antibody, was performed at the same time for all sections used in the quantitative analysis.

All slices were then digitized using a National Optical model DC5-163 SD Series digital microscope with a built-in 2.0 mega pixel camera and version 2.0 of Motic® Images Plus software. All slices for each histological marker were imaged in one session to avoid possible image intensity variation. Images (4x magnification) were acquired and split into multicolor channels. The blue channel was chosen for the solochrome stain images and the red channel for the amino cupric silver and GFAP and Iba1 IHC images. The channels were then converted and saved as 8-bit grayscale (1600 x 1200 pixels) images.

Approximately 18 consecutive 30 μm corpus callosum histological digital images were analyzed for each mouse using ImageJ. The immuno-stain intensity for each of the digitized images was then quantified. Histological ROIs at the level of corpus callosum were manually drawn, centered at bregma 1.10 for rostral (aCC), -1.46 for middle (bCC) and -2.54 for caudal (pCC) segments (Figure 1) (55). For each immuno-stain, the mean intensity values from all pixels in each ROI were averaged. In this analysis, we used the mean intensity value to represent the degree of histological staining.

Statistical Analysis:

One-way ANOVA, corrected for multiple comparisons using the Sidak method, was performed to compare the means of the two groups for all the metrics. All error bars correspond to the standard error of the mean (SEM).

Spearman's rank correlation was used to examine the association between diffusion metrics and immunohistochemistry metrics. The correlation analysis was performed for the entire CC, including all three segments for each group (control and cuprizone) separately (intra-group correlations), based on z-scores from values obtained at each CC segment. The z-score for each mouse and each metric was calculated as the departure from the mean of the control group for each segment of the CC, divided by the standard deviation of the control group (z-score = (metric – NC mean)/NC stdev).

To investigate which morphological, diffusion and WMM metrics would yield the strongest differentiation between the NC and CPZ an effect size (Cohen's d) was calculated for each metric at each CC level. All analyses were performed using SPSS version 20.0 (IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp). All reported p values were considered statistically significant at $p \leq 0.05$.

RESULTS:

Morphological and Diffusion MRI Assessment for the Control Group

Here we present an overview of the control group, based on a two-tailed t-test for two samples of equal variance, uncorrected; the mean values for all morphological and diffusion metrics are presented in Tables 1-4.

In agreement with the morphological heterogeneity previously described between the segments of the CC [56-61], our histological results for the control group demonstrated a lesser degree of myelination in the aCC compared with bCC and pCC. Interestingly, our results also showed more microglia cells in the aCC and bCC when compared with pCC, but no differences in the number of astrocytes. Finally, the amino cupric silver stain revealed some degree of axonal damage in the aCC, when compared with bCC and pCC.

The diffusion MRI results for the control group associated well with the morphological aspects in each CC segment. Briefly, FA had a caudal-rostral pattern, where higher FA was present in the pCC, accompanied by higher $D_{||}$, $D_{e,||}$ and α values, and with lower $D_{\perp}$ and $D_{e,\perp}$ values compared with aCC and bCC. This is probably due to the high density/packing of highly myelinated axons at this level. FA was also higher in the bCC when compared with aCC, with lower $D_{\perp}$, $K_{\perp}$, $D_{e,\perp}$, perhaps due to the presence of highly myelinated axons and a broader distribution in axonal caliber and more extra-cellular space than aCC. No MD, MK, AWF or D_a differences were detected between the three CC segments in the control group.

Morphological and Diffusion MRI Assessment between Groups

Histological Assessment

As expected, after 10 weeks of cuprizone treatment, which is considered a chronic phase, the Solochrome stain revealed intense, complete demyelination in the CC of the CPZ mice, as indicated by higher pixel intensity values relative to NC mice. The aCC was characterized by being the least affected segment and the one with the greatest morphological heterogeneity. Group differences were statistically significant in all three regions of the CC. The demyelination process was accompanied by mild neurodegeneration and intense inflammation represented by the statistically significant increase in the amino cupric silver stain and in the immunoreactivity for Iba1 (macrophage/microglia) and GFAP (astrocyte activity) markers in the CPZ group. For the entire CC, and in each of the three regions of the CC, the inflammatory markers GFAP and Iba1 were the best differentiators between the two groups. Specifically, GFAP ($d = 15.19$; $p<0.0001$) in the bCC and Iba1 ($d = 10.23$; $p<0.0001$) in the aCC were the best differentiators between the two groups. All histological measures for each segment of the corpus callosum are presented in Table 1.

Diffusion MRI Assessment

For the DT metrics (FA, MD and $D_{\perp}$), results consistent with previous reports throughout the entire CC of the CPZ mice were observed (i.e. reduced FA and increased MD and $D_{\perp}$ in comparison to NC mice, except for the FA in the aCC). $D_{||}$ was significantly increased in the aCC and bCC, but did not change in the pCC. All DT metrics for each segment of the corpus callosum are presented in Table 2.

All kurtosis metrics in the CPZ mice showed significant decreases, except for $K_{||}$ which did not change in the aCC. All DK metrics for each segment of the corpus callosum are presented in Table 3.

For the WMM metrics, CPZ mice had a significant AWF decrease in the entire CC, more intensely in the bCC and pCC. Significant D_a increase was only seen in the bCC, with no significant change in the aCC or pCC. For the extra axonal metrics, $D_{e,\parallel}$ showed a slight increase in the bCC and a decrease in pCC, with no change in the aCC; $D_{e,\perp}$ increased in both bCC and pCC, with no change in the aCC. A significant decrease in tortuosity was seen in the bCC, and more intensely in the pCC, with no change in the aCC. All WMM metrics for each segment of the corpus callosum are presented in Table 4.

In the aCC, AWF (d = 2.6; p<0.0001), $K_\perp$ (d = 2.0; p=0.001) and MK (d = 1.5; p=0.005) showed the most sensitivity between groups with respect to yielding statistically significant p-values and high Cohen's d values. In the bCC, where demyelination was especially pronounced, $D_\perp$ (d = 4.1; p<0.0001), MD (d = 3.8; p<0.0001), AWF (d = 3.6; p<0.0001) and $K_\perp$, (d = 3.1; p<0.0001) yielded the best differentiation. In the pCC, $D_\perp$ (d = 3.4; p<0.0001), FA (d = 2.4; p<0.0001) and AWF (d = 2.3; p=0.0001) best differentiated the two groups.

Correlation between Diffusion and Histological Metrics

A series of Spearman rank-order correlations were conducted in order to determine if any relationships existed between the diffusion metrics and the morphological metrics for the entire CC. This correlation analysis was performed for the entire CC for each group (control and cuprizone) separately (intra-group correlations). No significant correlations were detected in the control group, except for a negative correlation between FA and Iba1 (r(28) = -0.449; p=0.01) and a positive correlation between MK and Iba1 (r (28) = 0.393; p= 0.03).

There was a statistically significant relationship between diffusion and morphological metrics for the CPZ group. Solochrome, a myelin marker, correlated with all

diffusion metrics, except $D_{e,||}$ and α. The strongest correlation was found between Solochrome and MD ($r(28) = 0.837$; $p<0.0001$), followed by FA ($r(28) = -0.637$; $p=0.0001$), $D_{\perp}$ ($r(28) = -0.672$; $p<0.0001$), and $D_{e,\perp}$ ($r(28) = 0.623$; $p=0.0002$). The inflammatory marker for microglia, Iba1, also correlated with several diffusion metrics, but the GFAP marker for astrocyte reactivity correlated only with $D_{||}$ ($r(28) = -0.441$; $p=0.01$). No correlation was observed for the marker of neurodegeneration (amino cupric silver) with any diffusion metric (Table 5).

DISCUSSION:

This study is the first to use DK and associated WMM metrics to investigate the non-Gaussian diffusion patterns of chronic demyelination observed in the cuprizone mouse model. We demonstrated the ability of DK and WMM metrics to detect the CC white mater changes and inflammatory response associated with cuprizone-induced demyelination. Our results also replicate previous studies utilizing DTI (11-20).

The morphological assessment revealed demyelination with a rostro-caudal gradient (i.e., more intense in the body and posterior segment of the corpus callosum), accompanied by a mild degree of axonal damage and intense inflammatory response. These results are consistent with several previous studies (4-8) that reported activation of microglia and astrocytosis, predominantly in the caudal CC, associated with the breakdown of the myelin. Differently from the normal morphological aspect that shows less myelinated axons in the aCC compared with bCC and pCC (56), in the chronic stage of demyelination the rostral segment of the CC is less affected in the process (14,16) and maintain more myelinated axons compared with both bCC and pCC (Table 1).

DT, DK and WMM metrics estimated well the extent of the demyelination process in the bCC and pCC, but DT metrics underestimated the disease process at the aCC

level. In the aCC, FA was not able to distinguish the two groups, and the diffusivity metrics showed marginal statistical significance, probably due to the greatest morphological heterogeneity being present at this level. Indeed, it is well-described (14,16) that the anterior part of the CC is less damaged during the cuprizone toxicity-induced demyelination process, presenting with a heterogeneous pattern of demyelination, as our morphological results also demonstrated (Figure 3). Similar to DT metrics, changes in the DK and WMM metrics were more evident in the bCC and pCC, with statistical significance differentiating the two animal groups. However, DK and WMM metrics were also able to capture the heterogeneity of the process in the aCC, and despite the variability in the degree of myelin loss at this level, we observed significant decrease in MK, $K_{\perp}$ and AWF, likely reflecting myelin breakdown and decrease in axonal packing which, albeit less, is still morphologically evident at this level.

Based on the morphological changes represented by myelin breakdown and loss, one would expect fewer diffusion barriers and less structural complexity in the CC microenvironment, thereby causing a decrease in the diffusion metrics. Indeed, associated with an increase in the diffusivities (MD, $D_{||}$ and $D_{\perp}$) we observed decreases in MK, $K_{||}$, $K_{\perp}$ and AWF.

However, since the aCC, bCC and pCC differ in the degree of myelination, axonal density, distribution and alignment, which lead to differences in extra cellular space, not all the dMRI changes are straightforward to interpret. Additionally, the morphological changes due to the toxic process is complex, not only with demyelination and presence of myelin debris, but with damaged axons, decrease in axonal diameter and changes in cellularity (apoptotic oligodendrocytes, reactive microglia and astrocytes) (5-8). Therefore, the individual components of this process and the combination of

morphological components have different effects on the behavior of water diffusion as reflected in the dMRI metrics. This is evident in the WMM metrics for example, where D_a increased in bCC, but did not significantly change in the aCC and pCC. We can speculate that because bCC has a broader distribution containing axons with large and mid-size (mixed) caliber, the decrease in axonal diameter that occurs during the toxic process in this chronic stage (62) narrows the axonal distribution, leading to a better alignment of the axons, and subsequently increase in D_a. Additionally, reactive astrocytes known for high diffusion rates, and intimately associated with small damaged axons, may also contribute to the increase in D_a. In this segment, the demyelination process leads to an increase in $D_{e,\perp}$ and $D_{e,||}$, but a decrease in tortuosity due to the stronger effect of the $D_{e,\perp}$ increase (Table 4).

On the other hand, in the pCC, which has a high density of small packed axons, the dMRI patterns behaved slightly differently. As a result of the demyelination process $D_{e,\perp}$ increased; however, at this level, the uneven demyelination probably creates imperfections in the original dense fiber alignment resulting in less tension of the fibers and changing the geometry of the extracellular space, which may be the reason we see decrease in $D_{e,||}$. In this segment, the effect size of the $D_{e,\perp}$ increase is higher than the decrease in $D_{e,||}$, explaining the decrease in tortuosity.

One interesting observation that may appear to be contradictory to previous results (12-14,19,63) is the increase in $D_{||}$ in the aCC and bCC. However, previous studies showed a decrease in $D_{||}$ in the acute phase of the demyelinating process, not in the chronic phase. Indeed, both in vivo and ex vivo previous studies reported no significant difference or slightly increased $D_{||}$ after 6 weeks of toxin exposure (11,12,16,20). Another possible explanation for this difference is that DKI-derived estimates of diffusivities are

assumed to provide more accurate estimates of diffusion metrics (49), and kurtosis metrics are less sensitive to CSF partial volume (64).

The strong correlation between dMRI metrics and Solocrome, particularly MD, FA, $D_{\perp}$ and $D_{e,\perp}$ confirms that these metrics are sensitive to myelin abnormalities. The inflammatory marker for microglia, Iba1, also correlated with several diffusion metrics, particularly with $D_{\perp}$, which is in agreement with the fact that microglia infiltration is correlated with intense myelin breakdown (4-8). The lack of correlation between the amino cupric silver and the diffusion metrics is an unexpected observation and needs to be investigated in a future study. Similarly, the presence of a correlation between Iba1 with FA (negative) and MK (positive) in the CC of normal mice is also interesting, but at this time the reason is still unknown and needs to be further investigated. Likewise, the presence of a correlation between Iba1 with FA (negative) and MK (positive) in the CC of normal mice is interesting, but at this time the reason is still unknown and needs to be further investigated.

Confirming the WMM assumptions for regions such as the CC, which is formed predominantly with WM fiber bundles aligned in a consistent parallel orientation (57,65), the DKI-WMM metrics provided unique information regarding the underlying morphological alterations associated with the demyelination process, particularly in the rostral segment of the CC, where DKI-WMM metrics such MK, $K_{\perp}$ and AWF were more sensitive to the heterogeneity of the toxic process.

One limitation of this study is the fact that we did not investigate the temporal process of demyelination and/or the recovery phase. We acknowledge the importance of investigating water diffusion at those stages of the pathological process, but since we were investigating new diffusion metrics we decided to focus only on the phase where the demyelination is intense and complete. Therefore, these results should be carefully

interpreted since they represent only the chronic phase and would probably be different in the acute and/or recovery stage. Additionally, technical limitations for both dMRI and histology techniques, with different spatial resolutions, should be considered when interpreting the results. Finally, partial volume effects due to the larger voxel size, particularly in the body of the CC, may have had an effect in the results; however it is known that DKI metrics are less sensitive to partial volume effects (64), and masking for CSF using MD > 1.5um^2/ms, reduced the possibility of the results being determined by CSF contamination.

In conclusion, we have demonstrated that kurtosis and WMM metrics can be used as markers of the morphological changes associated with chronic demyelination in the cuprizone model. We have found that DK and WMM metrics provide complementary information enhancing the sensitivity to the morphological heterogeneity of the disease processes seen in the rostral segment of the corpus callosum. However, further studies are needed to delineate the underlying mechanisms associated with the temporal changes in the dMRI parameters, particularly with the WMM metrics. In part, these results also help validate these new WMM metrics, which should assist in the interpretation of results from future DKI studies using these metrics to investigate WM abnormalities in neurological diseases.

Acknowledgements

We thank Dr. Hiroko Hama, from Medical University of South Carolina, for her assistance with laboratory space and with the experimental cuprizone treatment.

REFERENCES

1. Matsushima GK, Morell P. The neurotoxicant, cuprizone, as a model to study demyelination and remyelination in the central nervous system. Brain Pathol. 2001; 11(1):107-16.

2. Torkildsen O, Brunborg LA, Myhr KM, Bø L. The cuprizone model for demyelination. Acta Neurol Scand. 2008; 188:72-6.

3. Kipp M, Clarner T, Dang J, Copray S, Beyer C. The cuprizone animal model: new insights into an old story. Acta Neuropathol. 2009; 118(6):723-36.

4. Stidworthy MF, Genoud S, Suter U, Mantei N, Franklin RJ. Quantifying the early stages of remyelination following cuprizone-induced demyelination. Brain Pathol. 2003;13(3):329-39.

5. Skripuletz T, Gudi V, Hackstette D, Stangel M. De- and remyelination in the CNS white and grey matter induced by cuprizone: the old, the new, and the unexpected. Histol Histopathol. 2011 Dec;26(12):1585-97. Review.

6. Hiremath MM, Saito Y, Knapp GW, Ting JP, Suzuki K, Matsushima GK. Microglial/macrophage accumulation during cuprizone-induced demyelination in C57BL/6 mice. J Neuroimmunol. 1998;92(1-2):38-49.

7. Remington LT, Babcock AA, Zehntner SP, Owens T. Microglial recruitment, activation, and proliferation in response to primary demyelination. Am J Pathol. 2007;170(5):1713-24.

8. Hibbits N, Yoshino J, Le TQ, Armstrong RC. Astrogliosis during acute and chronic cuprizone demyelination and implications for remyelination. ASN Neuro. 2012; 4(6):393-408.

9. Franco-Pons N, Torrente M, Colomina MT, Vilella E. Behavioral deficits in the cuprizone-induced murine model of demyelination/remyelination. Toxicol Lett. 2007;169(3):205-13.

10. Hibbits N, Pannu R, Wu TJ, Armstrong RC. Cuprizone demyelination of the corpus callosum in mice correlates with altered social interaction and impaired bilateral sensorimotor coordination. ASN Neuro. 2009;1(3).

11. Song SK, Yoshino J, Le TQ, Lin SJ, Sun SW, Cross AH, Armstrong RC. Demyelination increases radial diffusivity in corpus callosum of mouse brain. Neuroimage. 2005; 26(1):132-40.

12. Sun SW, Liang HF, Trinkaus K, Cross AH, Armstrong RC, Song SK. Noninvasive detection of cuprizone induced axonal damage and demyelination in the mouse corpus callosum. Magn Reson Med. 2006; 55(2):302-8.

13. Harsan LA, Poulet P, Guignard B, Steibel J, Parizel N, de Sousa PL, Boehm N, Grucker D, Ghandour MS. Brain dysmyelination and recovery assessment by noninvasive in vivo diffusion tensor magnetic resonance imaging. J Neurosci Res. 2006; 83(3):392-402.

14. Wu QZ, Yang Q, Cate HS, Kemper D, Binder M, Wang HX, Fang K, Quick MJ, Marriott M, Kilpatrick TJ, Egan GF. MRI identification of the rostral-caudal pattern of pathology within the corpus callosum in the cuprizone mouse model. J Magn Reson Imaging. 2008; 27(3):446-53.

15. Yang HJ, Wang H, Zhang Y, Xiao L, Clough RW, Browning R, Li XM, Xu H. Region specific susceptibilities to cuprizone-induced lesions in the mouse forebrain: Implications for the pathophysiology of schizophrenia. Brain Res. 2009; 1270:121-30.

16. Xie M, Tobin JE, Budde MD, Chen CI, Trinkaus K, Cross AH, McDaniel DP, Song SK, Armstrong RC. Rostrocaudal analysis of corpus callosum demyelination and axon damage across disease stages refines diffusion tensor imaging correlations with pathological features. J Neuropathol Exp Neurol. 2010; 69(7):704-16.

17. Boretius S, Escher A, Dallenga T, Wrzos C, Tammer R, Brück W, Nessler S, Frahm J, Stadelmann C. Assessment of lesion pathology in a new animal model of MS by multiparametric MRI and DTI. Neuroimage. 2012; 59(3):2678-88

18. Chandran P, Upadhyay J, Markosyan S, Lisowski A, Buck W, Chin CL, Fox G, Luo F, Day M. Magnetic resonance imaging and histological evidence for the blockade of cuprizone-induced demyelination in C57BL/6 mice. Neuroscience. 2012; 202:446-53.

19. Zhang J, Jones MV, McMahon MT, Mori S, Calabresi PA. In vivo and ex vivo diffusion tensor imaging of cuprizone-induced demyelination in the mouse corpus callosum. Magn Reson Med. 2012; 67(3):750-9.

20. Thiessen JD, Zhang Y, Zhang H, Wang L, Buist R, Del Bigio MR, Kong J, Li XM, Martin M. Quantitative MRI and ultrastructural examination of the cuprizone mouse model of demyelination. NMR Biomed. 2013;26(11):1562-81.

21. Jensen JH, Helpern JA, Ramani A, Lu H, Kaczynski K. Diffusional Kurtosis Imaging: The Quantification of Non-Gaussian Water Diffusion by Means of MRI. Magn. Reson. Med. 2005; 53:1432-1440.

22. Lu H, Jensen JH, Ramani A, Helpern JA. Three-dimensional characterization of non-gaussian water diffusion in humans using diffusion kurtosis imaging. NMR Biomed. 2006; 19(2):236-247.

23. Jensen JH, Helpern JA. MRI quantification of non-Gaussian water diffusion by kurtosis analysis. NMR Biomed. 2010; 23(7):698-710.

24. Falangola MF, Jensen JH, Babb JS, Hu C, Castellanos FX, Di Martino A, et al. Age-related non-Gaussian diffusion patterns in the prefrontal brain. J MagnReson Imaging 2008; 28(6):1345-50.

25. Jensen JH, Falangola MF, Hu C, Tabesh A, Rapalino O, Lo C, et al. Preliminary observations of increased diffusional kurtosis in human brain following recent cerebral infarction. NMR Biomed. 2011; 24(5):452-7.

26. Fung SH, Roccatagliata L, Gonzalez RG, Schaefer PW. MR diffusion imaging in ischemic stroke. Neuroimaging Clin N Am. 2011; 21(2):345-77.

27. Hui ES, Fieremans E, Jensen JH, Tabesh A, Feng W, Bonilha L, Spampinato MV, Adams R, Helpern JA. Stroke assessment with diffusional kurtosis imaging. Stroke. 2012; 43(11):2968-73.

28. Raab P, Hattingen E, Franz K, Zanella FE, Lanfermann H. Cerebral gliomas: diffusional kurtosis imaging analysis of microstructural differences. Radiology. 2010; 254(3):876-81.

29. Van Cauter S, Veraart J, Sijbers J, Peeters RR, Himmelreich U, De Keyzer F, Van Gool SW, Van Calenbergh F, De Vleeschouwer S, Van Hecke W, Sunaert S. Gliomas: Diffusion Kurtosis MR Imaging in Grading. Radiology. 2012; 263(2):492-501.

30. Helpern JA, Adisetiyo V, Falangola MF, Hu C, Di Martino A, Williams K, et al. Preliminary evidence of altered gray and white matter microstructural development in the frontal lobe of adolescents with attention-deficit hyperactivity disorder: a diffusional kurtosis imaging study. J Magn Reson Imaging. 2011; 33(1):17-23.

31. Grossman EJ, Ge Y, Jensen JH, Babb JS, Miles L, Reaume J, et al. Thalamus and Cognitive Impairment in Mild Traumatic Brain Injury: A Diffusional Kurtosis Imaging Study. Journal of Neurotrauma. 2011; 29(13):2318-27.

32. Gao Y, Zhang Y, Wong CS, Wu PM, Zhang Z, Gao J, Qiu D, Huang B. Diffusion abnormalities in temporal lobes of children with temporal lobe epilepsy: a preliminary diffusional kurtosis imaging study and comparison with diffusion tensor imaging. NMR Biomed. 2012; 25(12):1369-77.

33. Falangola MF, Jensen JH, Tabesh A, Hu C, Deardorff RL, Babb JS, Ferris S, Helpern JA. Non-Gaussian diffusion MRI assessment of brain microstructure in mild cognitive impairment and Alzheimer's disease. Magn Reson Imaging. 2013; 31(6):840-6.

34. Coutu JP, Chen JJ, Rosas HD, Salat DH. Non-Gaussian water diffusion in aging white matter. Neurobiol Aging. pii: S0197-4580(13)00619-2. doi:10.1016 / j.neurobiolaging. 2013. [Epub ahead of print] PubMed PMID: 24378085.

35. Cheung MM, Hui ES, Chan KC, Helpern JA, Qi L, Wu EX. Does diffusion kurtosis imaging lead to better neural tissue characterization? A rodent brain maturation study. Neuroimage. 2009; 45(2):386-92. PMID: 19150655.

36. Falangola MF, Guilfoyle D, Hui ES, Hu C, Gerum S, LaFrancois J, Nie X, Jensen JH, Tabesh A, Helpern JA. Diffusional Kurtosis Imaging Detects Age-related Grey matter Changes in the Normal Mouse Brain. Proc Intl Soc Mag Reson Med. 2012; 20:1241.

37. Falangola MF, Branch C, Jensen JH, Hu C, Xuan L, Duff K, Nixon R, Helpern JA. Assessment of Brain Microstructure in a Transgenic Mouse Model of AB-Amyloid Deposition. Proc Intl Soc Mag Reson Med. 2007; 15:310.

38. Vanhoutte G, Pereson S, Delgado Y Palacios R, Guns PJ, Asselbergh B, Veraart J, Sijbers J, Verhoye M, Van Broeckhoven C, Van der Linden A. Diffusion kurtosis imaging to detect amyloidosis in an APP/PS1 mouse model for Alzheimer's disease. Magn Reson Med. 2013;69(4):1115-21.

39. Hui ES, Du F, Huang S, Shen Q, Duong TQ. Spatiotemporal dynamics of diffusional kurtosis, mean diffusivity and perfusion changes in experimental stroke. Brain Res. 2012; 1451:100-9.

40. F. Grinberg, et al., Diffusion Kurtosis Imaging and Lognormal Distribution Function Imaging Enhance Visualization of Lesions in Animal Stroke Models; NMR Biomed. 2012; 25(11):1295-304.

41. Cheung MM, Hui ES, Wu EX. Comparison of directional diffusion kurtoses and diffusivities in EAE-induced spinal cord. Proc Intl Soc Mag Reson Med. 2008; 16:3328.

42. Zhuo J, Xu S, Proctor JL, Mullins RJ, Simon JZ, Fiskum G, Gullapalli RP. Diffusion kurtosis as an in vivo imaging marker for reactive astrogliosis in traumatic brain injury. Neuroimage. 2012;59(1):467-77.

43. Blockx I, De Groof G, Verhoye M, Van Audekerke J, Raber K, Poot D, Sijbers J, Osmand AP, Von Hörsten S, Van der Linden A. Microstructural changes observed with DKI in a transgenic Huntington rat model: evidence for abnormal neurodevelopment. Neuroimage. 2012;59(2):957-967.

44. Fieremans E, Jensen JH, Helpern JA. White matter characterization with diffusional kurtosis imaging. Neuroimage. 2011; 58(1):177–188.

45. Fieremans E, Benitez A, Jensen JH, Falangola MF, Tabesh A, Deardorff RL, Spampinato MV, Babb JS, Novikov DS, Ferris SH, Helpern JA. Novel white matter tract integrity metrics sensitive to Alzheimer disease progression. AJNR Am J Neuroradiol. 2013 [Epub ahead of print] PubMed PMID: 23764722.

46. Benitez A, Fieremans E, Jensen JH, Falangola MF, Tabesh A, Ferris SH, Helpern JA. (2013) White matter tract integrity metrics reflect the vulnerability of late-myelinating tracts in Alzheimer's disease. Neuroimage Clin; 4:64-71. doi: 10.1016/j.nicl.2013.11.001.

47. Jones DK, Horsfield MA, Simmons A. Optimal strategies for measuring diffusion in anisotropic systems by magnetic resonance imaging. Magn Reson Med 1999; 42:515–525.

48. Tabesh A, Jensen JH, Ardekani BA, Helpern JA. Estimation of tensors and tensor-derived measures in diffusional kurtosis imaging.Magn Reson Med. 2011; 65(3):823-36.

49. Veraart J, Poot DHJ, Van Hecke W, et al. More accurate estimation of diffusion tensor parameters using diffusion Kurtosis imaging. Magn Reson Med. 2011;65(1):138–145

50. Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2012.

51. Page KM. A stain for myelin using solochrome cyanin. J Med Lab Technol. 1965; 22(4):224-5.

52. Switzer RC 3rd. Application of silver degeneration stains for neurotoxicity testing. Toxicol Pathol. 2000; 28(1):70-83.

53. Jacque CM, Vinner C, Kujas M, Raoul M, Racadot J, Baumann NA (January 1978). "Determination of glial fibrillary acidic protein (GFAP) in human brain tumors". J. Neurol. Sci. 1978; 35 (1): 147–55.

54. Gómez-Nicola D, Fransen NL, Suzzi S, Perry VH. Regulation of microglial proliferation during chronic neurodegeneration. J Neurosci. 2013; 33(6):2481-93.

55. Paxinos G, Franklin K. The Mouse Brain in Stereotaxic Coordinates: Compact (Academic,San Diego), 2003; 2nd Ed.

56. Sargon MF, Mas N, Senan S, Ozdemir B, Celik HH, Cumhur M. Quantitative analysis of myelinated axons of commissural fibers in the rat brain. Anat Histol Embryol. 2003; 32(3):141-4.

57. Innocenti, Giorgio M.: General Organization of Callosal Connections in the Cerebral Cortex. Cerebral Cortex, 1986, Vol. 5, Jones, E. G., and A. Peters, eds. New York: Plenum, pp. 291–353.

58. Schmidt T, Awad H, Slowik A, Beyer C, Kipp M, Clarner T. Regional heterogeneity of cuprizone-induced demyelination: topographical aspects of the midline of the corpus callosum. J Mol Neurosci. 201349(1):80-8.

59. Barazany D, Basser PJ, Assaf Y. In vivo measurement of axon diameter distribution in the corpus callosum of rat brain. Brain. 2009; 132(Pt 5):1210-20.

60. Reyes-Haro D, Mora-Loyola E, Soria-Ortiz B, García-Colunga J. Regional density of glial cells in the rat corpus callosum. Biol Res. 2013; 46(1):27-32.

61. Olivares R, Michalland S, Aboitiz F. Cross-species and intraspecies morphometric analysis of the corpus callosum. Brain Behav Evol. 2000; 55(1):37-43.

62. Mason JL, Langaman C, Morell P, Suzuki K, Matsushima GK. Episodic demyelination and subsequent remyelination within the murine central nervous system: changes in axonal calibre. Neuropathol Appl Neurobiol. 2001; 27(1):50-8.

63. Wang Y, Wang Q, Haldar JP, Yeh FC, Xie M, Sun P, Tu TW, Trinkaus K, Klein RS, Cross AH, Song SK. Quantification of increased cellularity during inflammatory demyelination. Brain. 2011;134(Pt 12):3590-601.

64. Yang AW, Jensen JH, Hu CC, Tabesh A, Falangola MF, Helpern JA. Effect of cerebral spinal fluid suppression for diffusional kurtosis imaging. J Magn Reson Imaging. 2013; 37(2):365-71.

65. Leergaard TB, White NS, de Crespigny A, Bolstad I, D'Arceuil H, Bjaalie JG, Dale AM. Quantitative histological validation of diffusion MRI fiber orientation distributions in the rat brain. PLoS One. 2010; 5(1):e8595.

Table 1: Histological estimates (mean ± standard error, p-values, percentage differences and Cohen's d) for each segment of the corpus callosum.

		Solochrome	**Amino**	**GFAP**	**Iba1**
		Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
aCC	NC	171.37 ± 0.73	211.50 ± 0.57	169.79 ± 1.15	216.44 ± 0.34
	CPZ	177.15 ± 1.42	204.66 ± 1.75	86.53 ± 2.49	196.03 ± 0.88
	p-values	p<0.002	p<0.002	p<0.0001	p<0.0001
	Cohen's d	1.70	1.75	14.32	10.23
	% Difference	3.37	-3.23	-49.03	-9.43
bCC	NC	168.82 ± 0.54	201.22 ± 0.62	168.64 ± 1.19	217.08 ± 0.53
	CPZ	183.50 ± 0.68	193.38 ± 2.16	84.36 ± 2.33	196.38 ± 0.97
	p-values	p<0.0001	p<0.003	p<0.0001	p<0.0001
	Cohen's d	7.99	1.65	15.19	8.84
	% Difference	8.69	-3.89	-49.98	-9.54
pCC	NC	168.92 ± 0.76	200.76 ± 1.08	167.23 ± 1.24	222.06 ± 1.21
	CPZ	179.89 ± 1.21	191.62 ± 1.29	93.95 ± 2.79	203.95 ± 1.74
	p-values	p<0.0001	p<0.0001	p<0.0001	p<0.0001
	Cohen's d	3.62	2.56	11.31	4.03
	% Difference	6.49	-4.55	-43.82	-8.15

Histological quantification (grey mean intensity) showing significant decrease in myelin stain (Solochrome) and increase in neurorodegeneration (Amino) and inflammatory markers ((GFAP) and IBa1) in the cuprizone group. Note that higher mean intensity (MI) values correspond to less staining. Percentage difference calculated as (MI cuprizone – MI control) x 100)/MI control. Corpus callosum rostral (aCC), middle (bCC) and caudal (pCC) levels; control group (NC) and cuprizone group (CPZ); p values for group differences.

Table 2: DT metrics estimates (mean ± standard error, p-values, percentage differences and Cohen's d) for each segment of the corpus callosum.

		FA	**MD**	**$D_{//}$**	**$D_{\perp}$**
		Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
			μm2/ms	μm2/ms	μm2/ms
aCC	NC	0.26 ± 0.01	1.00 ± 0.02	1.24 ± 0.02	0.87 ± 0.02
	CPZ	0.24 ± 0.01	1.06 ± 0.02	1.30 ± 0.02	0.94 ± 0.02
	p-values	n/s	p=0.02	p=0.04	p=0.03
	Cohen's d	0.80	1.18	1.03	1.14
	% Difference	-8.53	6.53	4.51	7.80
bCC	NC	0.32 ± 0.00	0.94 ± 0.01	1.20 ± 0.01	0.80 ± 0.01
	CPZ	0.27 ± 0.01	1.07 ± 0.01	1.32 ± 0.02	0.94 ± 0.01
	p-values	p<0.0001	p<0.0001	p<0.0001	p<0.0001
	Cohen's d	2.84	3.79	2.48	4.12
	% Difference	-16.88	13.66	9.98	16.56
pCC	NC	0.46 ± 0.01	0.95 ± 0.01	1.51 ± 0.03	0.68 ± 0.01
	CPZ	0.36 ± 0.01	1.00 ± 0.01	1.43 ± 0.03	0.78 ± 0.01
	p-values	p<0.0001	p=0.01	n/s	p<0.0001
	Cohen's d	2.45	1.36	0.83	3.41
	% Difference	-21.65	4.51	-4.85	15.49

DT metrics showing changes between the control group (NC) and cuprizone group (CPZ); fractional anisotropy (FA); mean diffusivity (MD); axial diffusivity ($D_{//}$); radial diffusivity ($D_{\perp}$); p values are for group differences.

Table 3: DK metrics estimates (mean ± standard error, p-values, percentage differences and Cohen's d) for each segment of the corpus callosum.

		MK	**$K_{//}$**	**$K_{\perp}$**
		Mean ± SEM	Mean ± SEM	Mean ± SEM
	NC	0.69 ± 0.02	0.77 ± 0.02	0.78 ± 0.02
aCC	CPZ	0.62 ± 0.01	0.75 ± 0.02	0.67 ± 0.02
	p-values	p=0.005	n/s	p=0.001
	Cohen's d	1.46	0.33	1.97
	% Difference	-9.62	-2.59	-13.85
bCC	NC	0.64 ± 0.02	0.72 ± 0.02	0.68 ± 0.04
	CPZ	0.49 ± 0.02	0.58 ± 0.02	0.49 ± 0.02
	p-values	p=0.0001	p=0.0001	p<0.0001
	Cohen's d	2.79	2.27	3.12
	% Difference	-23.56	-20.19	-28.42
pCC	NC	0.65 ± 0.01	0.70 ± 0.01	0.68 ± 0.04
	CPZ	0.54 ± 0.02	0.63 ± 0.01	0.48 ± 0.05
	p-values	p=0.001	p=0.001	p=0.005
	Cohen's d	1.93	1.92	1.52
	% Difference	-16.80	-10.14	-28.40

DK metrics showing changes between the control group (NC) and cuprizone group (CPZ); mean kurtosis (MK); axial kurtosis ($K_{//}$); radial kurtosis ($K_{\perp}$); p values are for group differences.

Table 4: WMM metrics estimates (mean ± standard error, p-values, percentage differences and Cohen's d) for each segment of the corpus callosum.

		AWF	**Da**	**$D_{e,\parallel}$**	**$D_{e,\perp}$**	**α**
		Mean ± SEM	Mean ± SEM µm2/ms	Mean ± SEM µm2/ms	Mean ± SEM µm2/ms	Mean ± SEM
aCC	NC	0.26 ± 0.0	0.57 ± 0.02	1.59 ± 0.03	1.11 ± 0.03	1.43 ± 0.02
	CPZ	0.24 ± 0.0	0.58 ± 0.01	1.64 ± 0.02	1.17 ± 0.02	1.40 ± 0.02
	p-values	p<0.0001	n/s	n/s	n/s	n/s
	Cohen's d	2.57	0.17	0.58	0.75	0.56
	% Difference	-8.56	1.76	2.83	5.21	-2.27
bCC	NC	0.25 ± 0.0	0.56 ± 0.02	1.53 ± 0.02	1.01 ± 0.02	1.51 ± 0.02
	CPZ	0.21 ± 0.0	0.67 ± 0.02	1.61 ± 0.03	1.12 ± 0.01	1.44 ± 0.01
	p-values	p<0.0001	p=0.001	p=0.04	p=0.0001	p=0.01
	Cohen's d	3.61	1.79	1.01	2.34	1.30
	% Difference	-16.87	19.18	5.22	10.76	-5.16
pCC	NC	0.26 ± 0.0	0.60 ± 0.02	1.92 ± 0.04	0.86 ± 0.02	2.24 ± 0.07
	CPZ	0.21 ± 0.01	0.55 ± 0.02	1.78 ± 0.04	0.94 ± 0.01	1.88 ± 0.05
	p-values	p=0.0001	n/s	p=0.03	p=0.001	p=0.001
	Cohen's d	2.26	0.87	1.12	1.85	1.90
	% Difference	-17.83	-8.82	-7.60	9.77	-16.06

WMM metrics showing changes between the control group (NC) and cuprizone group (CPZ); axonal water fraction (AWF); the intrinsic diffusivity inside the axons, (D_a); the axial and radial diffusivities in the extra-axonal space, ($D_{e,\parallel}$) and ($D_{e,\perp}$) respectively; and the tortuosity (α); p values are for group differences.

Table 5: Spearman's correlation between diffusion metrics and histological quantitative measures for the cuprizone group (CPZ); Correlation Coefficient significant (bold) at: * p≤0.05, ** p≤0.01, and *** p≤0.001 (2-tailed).

	FA	MD	$D_{\|\|}$	$D_{\perp}$	MK	$K_{\|\|}$	$K_{\perp}$	AWF	D_a	$D_{e\|\|}$	$D_{e\perp}$	α
Solochrome	**- 0.637*** **	**0.837*** **	**0.518** **	**0.672*** **	**- 0.510** **	**- 0.495** **	**- 0.498** **	**- 0.365***	**0.431***	0.271	**0.623*** **	-0.276
Amino	0.035	-0.080	-0.085	0.033	-0.003	-0.258	0.279	-0.239	-0.347	-0.072	0.043	-0.157
GFAP	0.042	-0.161	**-0.441** **	0.184	0.019	-0.111	0.225	0.175	-0.207	-0.321	0.072	0.018
Iba1	-0.340	0.175	**- 0.376***	**0.649*** **	**- 0.423***	**-0.483** **	-0.024	-0.326	-0.316	**- 0.435***	**0.434***	**- 0.365***

LEGENDS

Figure 1:
First Row: Coronal diagram of the mouse brain with slices (Paxinos' mouse Brain Atlas) centered at anatomical positions corresponding to the bregma location for corpus callosum (CC) rostral (aCC), middle (bCC) and caudal (pCC) levels; **Second Row:** Representative fraction anisotropy (FA) maps (NC mice) with CC ROIs at each level. **Third Row:** Representative of the Solochrome stain (NC mice) with CC ROIs at each level.

Figure 2:
Histological examples (4x) of the solochrome (A) and amino cupric silver (B) stains, and GFAP (C) and Iba1 (D) immunohistochemistry with detailed insert (100x) at the body from of the CC. Control group (NC) and cuprizone group (CPZ). Scale bar = 100 µm.

Figure 3:
Morphological heterogeneity of the demyelination process in the cuprizone group; the figure illustrates histological sections from 3 cuprizone mice, showing the different degree of demyelination in the aCC (177.15±4.50) and similar, complete demyelination at the bCC (183.50±2.14) and pCC(179.89±3.83) for all three mice. Note the higher SD at the level of the aCC when compared is bCC and pCC, reflecting the morphological demyelination heterogeneity in the aCC. Intensity values as mean ± SD (arbitrary units).