Conduction Velocity, G-ratio, and Extracellular Water as Microstructural Characteristics of Autism Spectrum Disorder

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Abstract:

The neuronal differences contributing to the etiology of autism spectrum disorder (ASD) are still not well defined. Previous studies have suggested that myelin and axons are disrupted during development in ASD. By combining structural and diffusion MRI techniques, myelin and axons can be assessed using extracellular water, aggregate g-ratio, and a novel metric termed aggregate conduction velocity, which is related to the capacity of the axon to carry information. In this study, several innovative cellular microstructural methods, as measured from magnetic resonance imaging (MRI), are combined to characterize differences between ASD and typically developing adolescent participants in a large cohort. We first examine the relationship between each metric, including microstructural measurements of axonal and intracellular diffusion and the T1/T2 ratio. We then demonstrate the sensitivity of these metrics by characterizing differences between ASD and neurotypical participants, finding widespread increases in extracellular water in the cortex and decreases in aggregate g-ratio and aggregate conduction velocity throughout the cortex, subcortex, and white matter skeleton. We finally provide evidence that these microstructural differences are associated with higher scores on the Social Communication Questionnaire (SCQ) a commonly used diagnostic tool to assess ASD. This study is the first to reveal that ASD involves differences of myelin and axonal development with implications for neuronal and behavioral function. We also introduce a novel neuroimaging metric, aggregate conduction velocity, that is highly sensitive to these changes. We conclude that ASD may be characterized by otherwise intact structural connectivity but that functional connectivity may be attenuated by network properties affecting neural transmission speed. This effect may explain the putative reliance on local connectivity in contrast to more distal connectivity observed in ASD.

Keywords: Conduction velocity; diffusion MRI; autism spectrum disorder; sex differences

Introduction:

Autism spectrum disorder (ASD) represents a complex, multifaceted condition having a significant sex bias in both diagnosis rate and behavioral symptom profile[1]. This sex difference has driven considerable research interest in determining diagnostic and sex-specific associations with brain structure and function as measurable using magnetic resonance imaging (MRI)-based techniques.

ASD Neuroimaging: MRI techniques have long been a focus in ASD for potential diagnosis and increased understanding of brain patterns that might explain symptom severity and clinical variation. Reviews of neuroimaging literature going back as far as 2003 report consistently detectable mean increases in total brain, parietal, temporal, and cerebellar volumes compared to typically developing (TD) comparison subjects, though there were apparent differences in findings depending on the age cohort being studied and an almost total lack of female subjects[2,3]. Using a large subset of male subjects from the Autism Brain Imaging Data Exchange (ABIDE), a more recent study has described widespread increased cortical thickness that diverges from TD during childhood but rapidly decreases to levels equivalent to TD participants upon entering adulthood[4]. Another study supported this finding, used a distance-based feature selection paradigm to find differences within paired ASD/TD subjects to see differences in cortical thickness that may map to areas key to functional networks. However, these areas of significant difference were relatively sparse throughout the brain[5]. Meanwhile, longitudinal studies examining volume changes during adolescence in key subareas such as the hippocampus and amygdala have shown no difference between TD and ASD[6]. While there is ample evidence that differential neuroimaging features are present to differentiate ASD from TD, machine learning techniques have only achieved 80% ROC classification success from combined fMRI and structural data across the brain[7]. It has also been questioned if there are reliable sex differences in ASD or if observed sex-by-diagnosis interactions map to areas typically observed to show sex differences during development[8,9]. Studies on sex differences have suggested that ASD males and females may not exhibit sex-specific differences seen in typically developing individuals[10]; however, experimental parameters such as small sample sizes, lack of consistent experimental control factors, and a relative lack of female subjects have generally impacted many studies, making it difficult to gain insight on the neurobiological basis for ASD from an imaging perspective.

Connectomic Differences in ASD: Altered functional connectivity in the brain is a widely noted feature of ASD. However, functional connectivity alterations are not uniform across the brain and are associated with a brain-wide pattern of hypo- and hyperconnectivity depending on the active region being examined[11–14]. A mega-analysis of nearly 2,000 individuals between 5-58 years old found that hypoconnectivity of the sensory

and attentional network and hyperconnectivity between cortical and subcortical systems were associated with clinically relevant autistic features such as social impairments, repetitive behavior, and sensory processing[11]. This pattern is potentially present in early childhood, as a recent study by Yoon et al. (2022) found lower network efficiencies and reduced longer-range anterior-posterior resting-state connectivity in 2-6 year-olds with ASD[13]. Results from functional connectivity studies led to the development of a cortical underconnectivity theory, which postulated that the observed differences resulted from a deficiency in the presence or function of longer-range distal connections and an overreliance on shorter-range or local connections[15–17]. While initially controversial[18], several studies have supplied a variety of functional evidence in agreement with this theory[12,19–22]. Structural data has also provided evidence supporting reduced long-distance connectivity, with the corpus callosum (key to long-distance inter-hemispheric connectivity), in autistic individuals having lower fiber counts and volumes than comparison TD participants[23]. While the functional evidence describes the consequences of altered brain connections, it is essential to develop microstructural evidence that may highlight neuronal processes that lead to altered function to evaluate the underconnectivity theory and provide insight into the neurobiological basis for ASD.

Neuronal Microstructure: If the underconnectivity theory of ASD is accurate, it is likely that observed functional connectivity changes have a basis in the cellular microstructure of the neurons. Diffusion models of cellular microstructure can provide detailed information on architectural changes in brain structure at the sub-voxel level and describe specific cellular milieu rather than gross anatomy. In this framework, lower directional diffusion, referred to as *anisotropic*, and higher adirectional diffusion, referred to as *isotropic*, may indicate deficits in axonal morphology that would cause reduced action potential speed or efficiency and drive reduced connectivity deficits. Depending on which axons and axonal areas are affected, this might impact particular systems or longer/shorter range connections, particularly since longer-range connections rely on larger and higher diameter axons to efficiently transmit information across the brain[24–26]. Previous diffusion microstructure experiments have demonstrated that ASD participants display different axonal anatomy compared to TD participants, such as structural differences in neuronal white matter (WM), with ASD being associated with lower fractional anisotropy (FA) and higher diffusivity[27]. Using NODDI, one study has found increased extracellular water and decreased neurite density in ASD throughout major tractography bundles in the brain[28,29]. Other work has applied constrained spherical deconvolution to examine differences in axonal volume between ASD and TD participants using fixel-based analysis. In this context, a 'fixel' refers to an individual fiber population within a voxel, derived from the WM-FODs calculated using constrained spherical

deconvolution, and allows for fiber-specific metric calculation relating to intra-axonal volume[30–33]. This analysis found that participants with ASD had significantly lower fiber density cross-section, a volume and fiber bundle diameter modified measure of intra-axonal volume, compared to TD participants across several major WM bundles of interest throughout the brain[27].

Neuroimaging-based Assessment of Neuronal Conduction Velocity: Beyond axonal fiber density, it is also possible to describe the relationship between axons and the myelin sheath surrounding them, providing the hallmark of the structural and diffusion microenvironment. The g-ratio is a quantitative proportion between axon diameter and myelin thickness first described by Rushton[34]. Increased myelin improves the resistivity of the axonal cellular membrane between the Nodes of Ranvier and provides for increased speed and energetic efficiency conducting action potentials[35,36]. Both axonal diameter and myelin thickness affect neuronal conduction velocity and spatial, energetic, and conductive properties within the human nervous system, leading to an optimal g-ratio of around 0.6-0.7[34,37,38]. The g-ratio thus describes a fundamental linkage between neurons' structure and function (via conduction velocity). Genetic[39,40], electrical[41–45], and functional[8,46–49] evidence suggests that neural activity is disrupted in ASD. Post-mortem histological studies also demonstrate that myelin and axons display abnormal morphology in ASD[50]. MRI-based measures of g-ratio, conduction velocity, and microstructural axonal properties are needed to establish *in vivo* measurements to give insights into the underpinnings of ASD.

Developing more sensitive models of microstructure and axonal properties may provide insight into structural and cellular processes in ASD. The g-ratio is an important measure of axonal integrity, and that accounts for axonal diameter and the diameter of the myelin sheath surrounding the axon[51,52]. Conduction velocity is an important metric that addresses the capacity of the axonal fiber to carry information, and this can be derived, in part, from the g-ratio[53]. The present study aims to establish novel means of calculating both these metrics, in aggregate form across all axonal fibers in a voxel, using a combination of diffusion and structural MRI and applying these metrics to an ASD cohort.

Methods:

Participants: The neuroimaging and neuropsychological data analyzed were drawn from Wave 1 of the Autism Centers of Excellence (ACE) GENDAAR project (KAP, PI), based at the University of Virginia (UVA), which were collected across four participating institutions (Yale University, Harvard University/Boston Children's Hospital, University of California Los Angeles, and University of Washington/Seattle Children's

Research Institute). Male and female youth (8–17 years old) were recruited for inclusion in the ASD or typically developing cohorts. Unaffected siblings of ASD participants were also recruited but were not included as part of this analysis. Subject recruitment was approved to begin on 10/16/2013 and was closed on 09/18/2017. The parents of the subjects provided their written informed consent, whereas, the participating children provided written assent. Procedures for data collection were conducted in compliance with requirements from Institutional Review Board at the University of Virginia (UVA), each institution's local IRB, and the Declaration of Helsinki. All data were archived, managed, securely stored, deidentified, etc. at UVA, where the analyses reported here were conducted.

Exclusionary criteria for all groups included: full-scale IQ (FSIQ) ≤ 70 as estimated via the Differential Ability Scales, Second Edition General Conceptual Ability Standard Score[54]; twin status; active tic disorder that would interfere with imaging; pregnancy; metal in the body; active seizures within the past year; or current use of any benzodiazepine, barbiturate, or anti-epileptic medications. Other medication use was required to have been stable for over six weeks. Specifically, group-specific exclusionary criteria were considered as follows:

Typically Developing (TD). Subjects were excluded if diagnosed, referred, or suspected of ASD, schizophrenia, intellectual disability, learning disability, or other developmental or psychiatric disorder; had a first- or second-degree relative with ASD; recorded a total t-score > 60 on the Social Responsiveness Scale, Second Edition[55]; a raw score > 11 on the Lifetime version of the Social Communication Questionnaire[56]; a clinical impression suggesting ASD, other developmental delay or disorder, broader autism phenotype, or any significant psychiatric disorder.

Autism Spectrum Disorder (ASD): Exclusionary criteria specific to the ASD group were having a known single gene disorder related to ASD or syndromic form of ASD (e.g., Fragile X); medical conditions likely to be etiological (e.g. focal epilepsy or infantile spasms); any particular neurological disorder involving pathology dorsal to the brainstem, other than uncomplicated non-focal epilepsy; history of significant pre- or perinatal injury, *i.e.* birth at < 36 weeks and weight < 2000 grams, or neonatal intensive care unit hospital stay > 3 days; history of neonatal brain damage; any known environmental circumstances that might account for the presence of ASD characteristics in the proband (e.g., evidence of severe nutritional or psychological deprivation); clinically significant visual or auditory impairment following correction; or any other sensorimotor difficulties which might generally preclude the valid use of diagnostic assessments.

Diagnostic confirmation: Children and adolescents who received the Autism Diagnostic Observation Schedule, Second Edition (ADOS-2) Module 3 (for which revised algorithms were available from the outset of the study) were required to achieve a Calibrated Severity Score (CSS) of ≥ 4; adolescents who received Module 4 (for which a revised algorithm became available during the course of data collection[57]) were required to meet ASD criteria according to either the updated algorithm (CSS ≥ 4) or the version of the algorithm published with the ADOS-2 (Communication + Social Interaction Total ≥ 7). Scores on the Autism Diagnostic Interview-Revised (ADI-R), a standardized parent interview designed to obtain ASD symptom information, were obligatory to meet the diagnostic algorithm to within one point.

Resulting Sample: Two-hundred seventy-three (mean age = 154.3 months ±35.21 S.D., 133 female [49%]) subjects met these study inclusion/exclusion criteria. The study cohort included 148 individuals diagnosed with ASD (mean age = 150.8 months ±34.31 S.D., 70 female [47%]) and 124 neurotypical participants (mean age = 154.3 months ±35.21 S.D., 62 female [50%]). All subject data were de-identified in accordance with the Health Insurance Portability and Accountability Act of 1996 (HIPAA; Public Law 104-191, 104th U.S. Congress). Neuroimaging and other study data are available from the National Institute of Mental Health Data Archive (NDA; Collection: #2021).

Social Communication Questionnaire (SCQ): All participants were assessed using the SCQ, a parent-report questionnaire comparing behaviors characteristic of ASD between ages 4-5 and present[58]. Scores on the SCQ range from 0-40 with higher scores indicating the presence of more behaviors characteristic of ASD. The SCQ is based on the Autism Diagnostic Interview – Revised (ADI-R)[59] and has been validated with the ADI-R and with ASD diagnosis[58]. A cutoff score of 15 has been reported to indicate ASD[60,61].

Image Acquisition: Diffusion, T1-weighted, and T2-weighted images were acquired from each subject. Diffusion images were acquired with an isotropic voxel size of 2x2x2mm, 64 non-colinear gradient directions at b=1000 s/mm2, and 1 b=0. T1-weighted MPRAGE images with a FOV of 176x256x256mm and an isotropic voxel size of 1x1x1mm, TE=3.3; T2-weighted images were acquired with a FOV of 128x128x34 with a voxel size of 1.5x1.5x4mm, TE=35.

Image Data Processing: A simple overview of the image processing pipeline, showing which imaging metrics contributed to calculation of other imaging metrics, is presented in Fig. 1. As described in Newman et al.[62], diffusion images were denoised[63], corrected for Gibbs ringing artifacts[64], and corrected for inhomogeneity fields using FSL's *topup* and *eddy* commands utilizing outlier detection and replacemnet[65–67], and the final preprocessed diffusion images were up-sampled to an isotropic voxel size of 1.3x1.3x1.3mm[68].

WM, GM, and CSF tissue response functions were generated using the *Dhollander* algorithm[69], and single-shell 3-tissue constrained spherical deconvolution was used to generate the WM fiber orientation distribution (FODs) and GM and CSF representations. 3-Tissue Constrained Spherical Deconvolution[70–73] was used to calculate the voxel-wise maps of the fraction of signal arising from each of 3 compartments: an intracellular anisotropic, intracellular isotropic, and extracellular isotropic freely diffusing water compartment by setting the sum of all FOD coefficients equal to unity. WM-FODs were then used to create a cohort-specific template with a subset of 40 individuals counterbalanced between sex and diagnosis[74]. All subject's WM-FODs were registered to this template using an affine non-linear transform warp, and then the template was registered to a b-value matched template in stereotaxic MNI space[75,76].

Estimating Axonal Fiber Diameter: A fixel-based morphometry (FBM)[30,31] approach was used to estimate the intra-axonal cross-sectional area within each voxel to be used as an apparent axonal volume fraction (AVF). FBM combines fiber density, computed from the integral of each WM-FOD spherical harmonic lobe, and cross-sectional area, computed using the Jacobian matrix determinant, which reflects the change in cross-sectional area when normalized to the template image. The cross-sectional change is used as a weight to the fiber density, and the two metrics are multiplied on a voxel-wise basis to obtain the fiber density cross-section related to the total intra-axonal volume (AVF)[30,33]. Each subject's AVF maps were then registered to MNI space using the ANTs SyN nonlinear registration technique by aligning each to the 11-year-old adolescent template developed by Richards et al. Note a template approximately 1 standard deviation below the mean age of this study was used to better register the comparatively much smaller younger subjects[77,78].

Myelination Density: T1w and T2w images were processed as described in the MICA-MNI pipeline[79]; this involved performing N4 bias correction from ANTs[80,81] on both T1w and T2w images, rescaling both images from 0-100 based on their maximum values, co-registering the T2w to the T1w image using ANTs[81] rigid registration and then calculating the T1/T2 ratio on a voxel-wise basis[82]. T1/T2 ratio images were then registered to MNI space using the ANTs SyN nonlinear registration technique by aligning each to the same Richards et al. templates used to register the AVF maps.

Conduction Velocity Determination: The aggregate g-ratio was calculated on a voxel-wise basis according to Stikov et al. and was used according to Mohammadi & Callaghan as displayed in Equation 1[51,52,83,84]. As a measure of intra-axonal volume, the fiber density cross section was used as the AVF[31], and as a metric of myelin density, the T1/T2 ratio was used as the myelin volume fraction (MVF). Both of these metrics

represent the total sums of each respective compartment across the volume of the voxel and are a volume-based equivalent to the original formulation of g as the ratio of axon diameter (d) to fiber diameter (D).

$$g = \frac{d}{D} = \sqrt{1 - \frac{MVF}{MVF+AVF}} \quad (1)$$

Aggregate conduction velocity was calculated based on the calculations of Rushton[34] and Berman et al.[53]; reiterating Rushton's calculation that conduction velocity (θ) is proportional to the length of each fiber segment (l), and that this is roughly proportional to *D,* which in turn can be defined as the ratio between *d* and the g-ratio (g). Furthering the considerations of Rushton, Berman et al. show that a value proportional to conduction velocity can be calculated using axon diameter and the g-ratio as in equation 2[53]:

$$\theta \propto l \propto Dg\sqrt{-\ln(g)} \propto d\sqrt{-\ln(g)} \quad (2)$$

Herein, aggregate conduction velocity is calculated at the whole-voxel level, similar to Stikov et al.'s original conceptualization of an aggregate g-ratio, with each voxel representing an ensemble of g-ratios within each individual axonal segment with arbitrary orientation. Thus, instead of the direct measurement of axonal or myelin diameters, per se, the present study utilized a function of the arbitrarily oriented ensemble of intra-axonal volumes, returning to the AVF. It follows that increased or decreased diameter *in aggregate throughout a voxel* is proportional to intra-axonal volume.

All imaging metrics, 3T-CSD signal fraction compartments, T1/T2 ratio, aggregate g-ratio, and aggregate conduction velocity (Fig. 2) were averaged across each of 214 ROIs taken from the JHU-ICBM WM atlas (48 ROIs)[85] and the Destrieux Cortical Atlas (164 ROIs)[86]. Additionally, two composite ROIs were included, one of all 48 JHU ROIs and one of 150 neocortical regions from the Destrieux Atlas.

Statistical Approach: General linear models (GLMs) were used to test the relationship between each microstructural metric (intracellular anisotropic, intracellular isotropic, extracellular free water, T1/T2 ratio, aggregate g-ratio, and aggregate conduction velocity) on diagnosis and sex differences as well as on SCQ total score with sex as a control. All GLMs featured subject age, IQ, scanner/site, and intracranial volume as control terms. All sets of microstructural results were corrected for multiple comparisons using the Benjamini & Hochberg[87] method across all 214 ROIs.

Results:

Given how the aggregate g-ratio and aggregate conduction velocity metrics were derived from diffusion and T1/T2 ratio information, these metrics were first compared using linear models across all 214 ROIs with subsequent Benjamini and Hochberg correction for multiple comparisons. These results can be viewed in Fig. 3, and as expected, the methods derived from T1/T2 ratio tended to be largely in agreement in both axonal ROIs from the JHU-ICBM atlas and in cortical ROIs from the Destrieux atlas. There were also many ROIs in the axonal areas where the ICA signal fraction was significantly associated with T1/T2 ratio. This is especially notable as the WM-FODs that contribute to the ICA signal fraction are used to derive the fixel-based estimate of AVF. However, far less significant associations existed between ICA and either aggregate g-ratio or aggregate conduction velocity. In the cortex, there was a widespread negative association between ECI (extracellular water) signal fraction and aggregate g-ratio and aggregate conduction velocity, suggesting that in this adolescent cohort, the intra-axonal volume mediates the volume of extracellular space in the cortex.

These metrics were then assessed using the full model for relationships to variables of interest. For full summarized results of all adjusted p-values, see Table 1, for raw means from each metric in every ROI, see Supplementary Table 1, for all p-values before and after adjustment, see Supplementary Tables 2-7 for ECI, ICI, ICA, T1/T2 ratio, aggregate g-ratio, and aggregate conduction velocity respectively. Following multiple comparison corrections, 93 ROIs were found to have significantly greater extracellular water signal fraction in individuals with ASD compared to TD participants (Fig. 4), including in both composite ROIs covering the WM and neocortex. The significant ROIs were almost entirely located in the cortex, and no individual JHU-ICBM WM atlas ROIs, other than the entire composite ROI, survived multiple comparison corrections (Fig. 5). 7 ROIs located in the subcortical gray matter also displayed significantly greater extracellular water signal in ASD compared to participants. These included the nucleus accumbens, hippocampus, and thalamus on the left exclusively, and bilateral caudate and putamen. Areas with the strongest relationship between extracellular water signal and ASD were the bilateral caudate nuclei and a bilateral series of pre- and post-central gyri in the parietal, superior temporal, and superior frontal lobes (Fig. 4).

Sixty-one ROIs were found to have significantly reduced aggregate g-ratio in ASD compared to TD participants (Fig. 4), including the composite WM ROI but not the composite cortical ROI (Fig. 5). Significant ROIs were located throughout the neocortex and axonal areas but were especially prominent in the superior

parietal lobe. Only three subcortical areas showed significant differences, the right and left thalamus and the right nucleus accumbens.

One-hundred and fifty-five ROIs were found to have significantly reduced aggregate conduction velocity in ASD compared to TD participants (Fig. 4), including both composite ROIs in the neocortex and WM (Fig. 5). Significant ROIs included the majority of both atlases, with widespread associations between diagnosis and aggregate conduction velocity. Subcortically, there were significant associations with the bilateral amygdala, caudate, putamen, thalamus, and the right hippocampus. There was also a strong positive association between aggregate conduction velocity and IQ, with 206 ROIs surviving multiple comparison corrections, including every WM ROI from the ICBM-JHU-WM atlas. Four of the non-significant ROIs were the bilateral temporal and occipital poles, and the remaining three were the left inferior occipital gyrus and sulcus, the right lunate sulcus, and the right lateral orbital sulcus. Receiver operator characteristic (ROC) analysis was performed using the 'pROC' package in RStudio on the linear model for each composite ROI to determine which microstructural metric with significant ROIs was most sensitive and specific in regards to subject diagnosis (Fig. 6)[88]. The ECI signal fraction within the composite WM ROI had the highest area under the ROC curve (AUC), indicating the best sensitivity and specificity to diagnosis.

In regard to diagnosis, no other microstructure metric survived multiple comparison corrections in any ROI, including T1/T2 ratio. There was no significant effect of sex or of sex-by-diagnosis interaction in any ROI after multiple comparison corrections (Fig. 7). Though there were no significant associations with sex in any microstructural metric in any ROI, diagnosis groups show varying relationships with age that are not consistent across sexes, with far higher T1/T2 ratio values in TD males compared to TD females, while in ASD, females tend to have higher T1/T2 ratio, and ECI signal fraction values compared to males. ASD males, meanwhile, tend to have higher ICI (GM-like) signal fraction values, while in TD participants, age-associated ROIs tend to have higher ICI (GM-like) values in females. Aggregate conduction velocity and aggregate g-ratio do not seem to differ between ASD and TD in their age-by-sex interaction, despite having many significantly different ROIs between ASD and TD participants.

When microstructural metrics were compared to SCQ total score a similar pattern to diagnosis was observed (Fig. 8). There was a significant positive relationship between ECI signal fraction and SCQ total score in 55 ROIs, indicating that greater extracellular water was associated with a more ASD-like behavioral profile. All but one (the left putamen) were cortical ROIs including the composite cortical ROI. Aggregate g-ratio had a significantly negative association with SCQ total score in 12 ROIs, though this included all 3 ROIs covering the

entire corpus callosum. Aggregate conduction velocity however, had a significant negative relationship with SCQ total score in 139 ROIs including both composite cortical and white matter ROIs, indicating that reduced conduction velocity was associated with a more ASD-like behavioral profile (Fig. 9). No other microstructural metric in any ROI was significantly associated with SCQ total score after multiple comparison correction.

Discussion:

This study's results support the underconnectivity theory in ASD by describing for the first time subcellular findings that could be the neurobiological basis for reduced long-range connectivity[15–17]. This study reveals widespread significant mean differences between ASD and TD participants while also highlighting significant associations between microstructural metrics and behavioral measures of ASD. These findings were primarily located in the neocortex in the ECI signal fraction, in the axonal WM areas for aggregate g-ratio, and in both neocortex and axonal WM areas for aggregate conduction velocity. ROC analysis showed that aggregate microstructural metrics are highly discriminatory between ASD and TD individuals and are strongly correlated with behavioral metrics.

Multiple studies have previously described consistent increases in cortical thickness[2,4,89] and mean diffusivity with corresponding decreases in FA[90–92] in ASD compared to TD participants. Against this background, the widespread increases of extracellular water in cortical ROIs and decreased aggregate conduction velocity and aggregate g-ratio observed in this study can potentially be interpreted as widespread problems with myelination or as the microstructural result of increased cortical thickness. However, there was no significant difference in T1/T2 ratio (used as the MVF in this study) between ASD and TD, implying that there is a more complex relationship between myelination and ASD than simply a deficit compared to TD. By weighting intra-axonal volume (measured via the total sum of FDC) into both aggregate g-ratio and aggregate conduction velocity, it is possible that differences are driven by changes to axonal morphology at the sub-cellular level. This may be an architectural difference in cellular microstructure (i.e., axons themselves may be thinner or more dispersed in ASD), or, combined with the increased extracellular water exclusively in the cortex, there is increased active or previous neuroinflammation that stunted axonal and myelin development, leaving a sparser and higher volume cortex with a large portion of that increase simply being extracellular space. This finding is supported by the finding of increased extracellular water and decreased neuronal dispersion by other diffusion models such as NODDI[29].

Histological work performed on small samples of WM from both TD and ASD adults found that superficial and deep WM had significantly fewer large diameter axonal fibers, more small diameter axonal fibers, and significantly more branching axonal fibers in ASD compared to TD participants[50,93]. Individuals with ASD also had significantly thinner myelin sheaths in all sizes of axon[50]. Though this histological experiment was performed in adults instead of the adolescents in this study, given these two factors, a bias toward smaller diameter axons and thinner myelin, it is likely that the results reported here confirm this prior finding, as the aggregate g-ratio in this adolescent cohort is overall lower than expected in adults due to insufficiently developed myelin, and thus the TD group can be interpreted as nearer to developed myelin and thus higher g-ratio closer to optimum values[52,84,94,95]. It has also been reported that children with ASD have lower conduction velocity using magnetoencephalography[96,97] and in peripheral neurons[98], potentially due to reduced axonal diameter and the prevalence of small fibers. This study reinforces this finding by showing, for the first time noninvasively, widespread reduced aggregate conduction velocity in ASD throughout the brain. The observed reductions in aggregate conduction velocity, in particular, may indicate deficits, particularly in longer-range axonal connections dependent on higher-diameter fibers and myelination. This effect was observed in both aggregate g-ratio and aggregate conduction velocity throughout a wide range of the axonal skeleton, indicating that these functional deficits are systemic.

It should also be noted that extensive work has shown that extracellular water increases in the axonal WM skeleton with age and cerebrovascular injury[72,99–101], but that none of the axonal ROIs tested was significant for ECI signal fraction differences, suggesting that there is no active degeneration of brain cells or microstructure. The findings of extracellular water differences exclusively in cortex pairs with the lack of findings in the axonal signal compartment and in the T1/T2 ratio measurement of myelin in any ROIs examined. This is also an important reminder that T2-weighted signal and extracellular water are not interchangeable, especially in development. The water in the extracellular space is still close to the bound volume fraction pool water, and the T2-weighted signal was increased enough compared to the T1-weighted signal to serve as a marker of ASD in the T1/T2 ratio metric. Note, however, that the ECI signal fraction had the highest number of negative correlations with g-ratio and conduction velocity, but, as expected, those metrics were closely related to T1/T2 ratio in nearly every ROI, despite T1/T2 ratio subsequently having no ROIs that were different between ASD and TD participants. An additional possibility is that changes in synaptic formation as suggested by several genetic associations between ASD and growth-cone-related genes such as CRMP1 and CRMP2[102]. Recent work using FDC in other model systems, such as the optic nerve, has suggested

that fiber cross-section may be a key component of information transfer and downstream neuronal development[103]. Validation studies in animal models or via further histopathological study are required to further investigate the cellular basis of these observed effects.

These alterations in axonal morphology may be responsible for functional changes and subsequent behavioral outcomes. In this study, we observe that reduced aggregate conduction velocity is strongly associated with increased ASD behaviors, as measured by the SCQ. This association is present across a large number of cortical and WM regions, and is very similar to the association between aggregate conduction velocity and ASD diagnosis, suggesting that slowed axonal conduction velocity within and between brain regions could contribute to ASD etiology. Interestingly, this association is present between ASD behavioral metrics and extracellular water in a wide variety of cortical regions but only present between aggregate g-ratio and ASD behavioral metrics in the corpus callosum and a small number of other regions. This matches reports from histological studies where differences in axonal area are much larger than differences in myelination between ASD and TD individuals[93]. This is particularly relevant to our calculation of aggregate conduction velocity which relies on aggregate axonal volume per voxel. Combined with the lack of significant T1/T2 ratio associations, this suggests that behavioral components of ASD are more likely associated with reduced axonal area and the subsequently slower conduction velocity, than due to a difference in myelination.

Conclusion:

Advanced diffusion and structural microstructure models can characterize a combination of increased extracellular water and decreased aggregate g-ratio and aggregate conduction velocity between ASD and TD participants. Microstructural metrics are similarly associated with a validated measure of ASD behavior, suggesting that particularly slower aggregate conduction velocity relate to higher scores on ASD behavioral evaluations. This study introduces a novel means to calculate aggregate g-ratio using diffusion MRI techniques and a novel calculation of aggregate conduction velocity as well as applying both to the study of ASD for the first time. These findings suggest that differences in axon morphology and subsequently slowed conduction velocity, are widespread throughout the brain and may contribute to the clinical picture of ASD. Altered signal transduction along affected fiber pathways also provides an explanation for recent observations of local versus distal connection dependencies more specifically in those diagnosed with ASD.

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Conflicts of Interest:

James C. McPartland consults with Customer Value Partners, Bridgebio, Determined Health, and BlackThorn Therapeutics, has received research funding from Janssen Research and Development, serves on the Scientific Advisory Boards of Pastorus and Modern Clinics, and receives royalties from Guilford Press, Lambert, Oxford, and Springer. Other authors declare no conflicts of interest.

Authorship Contributions:

BTN conducted the analyses and wrote the manuscript; ZJ curated the data sets used in the analyses; SV contributed to the theoretical concepts; TJD contributed expertise in clinical radiology; JDVH conceived of the overall study, contributed to the theoretical and conceptual considerations, and helped to write the manuscript; KAP contributed expertise in ASD research and provided ACE program leadership (NIH R01 MH100028); SJW, NAK, and JCM provided expertise in ASD clinical assessment and neurophysiological research.

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Figures:

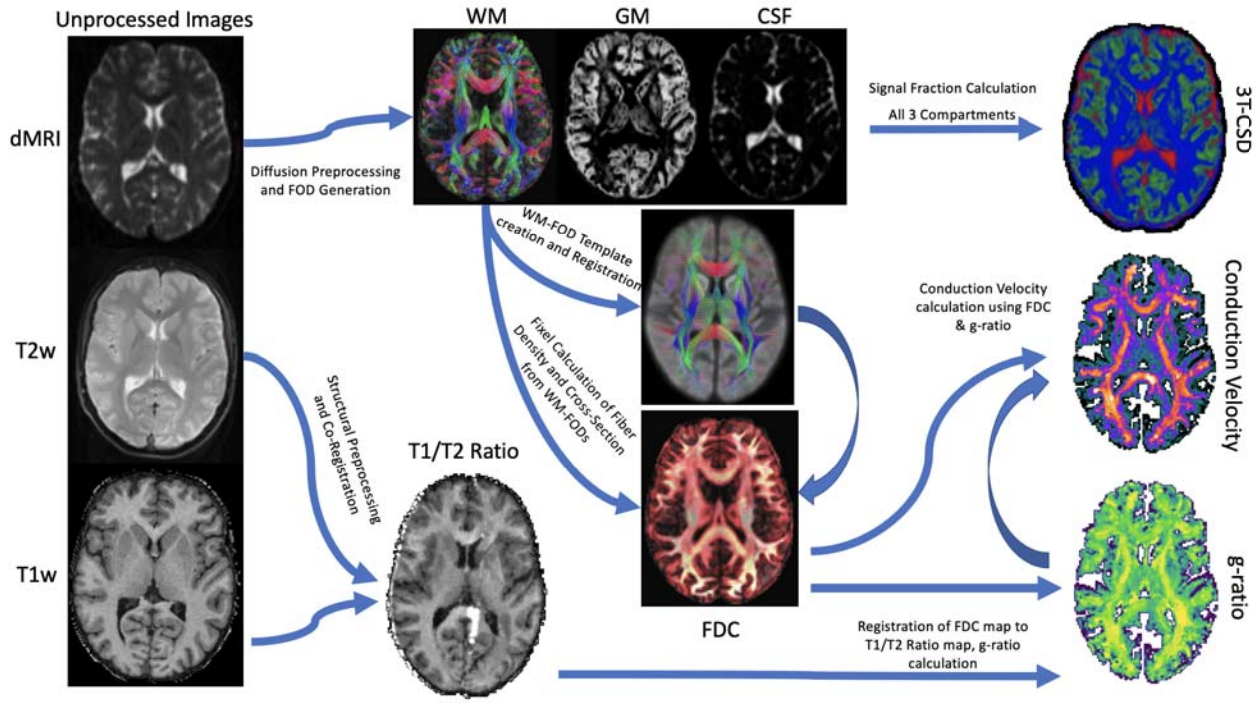


Figure 1: Simple flow diagram displaying which imaging metrics contributed to which output metrics. Fiber density cross-section (FDC) was derived from WM-FODs as part of a fixel analysis pipeline, then summed voxel-wise as an intra-axonal volume fraction (AVF) estimate while T1/T2 ratio was used as a myelin volume fraction (MVF) for the calculation of g-ratio and conduction velocity. It should also be noted that 3T-CSD metrics were separately registered to the MNI space atlases via a different procedure than the metrics derived from T1/T2 ratio.

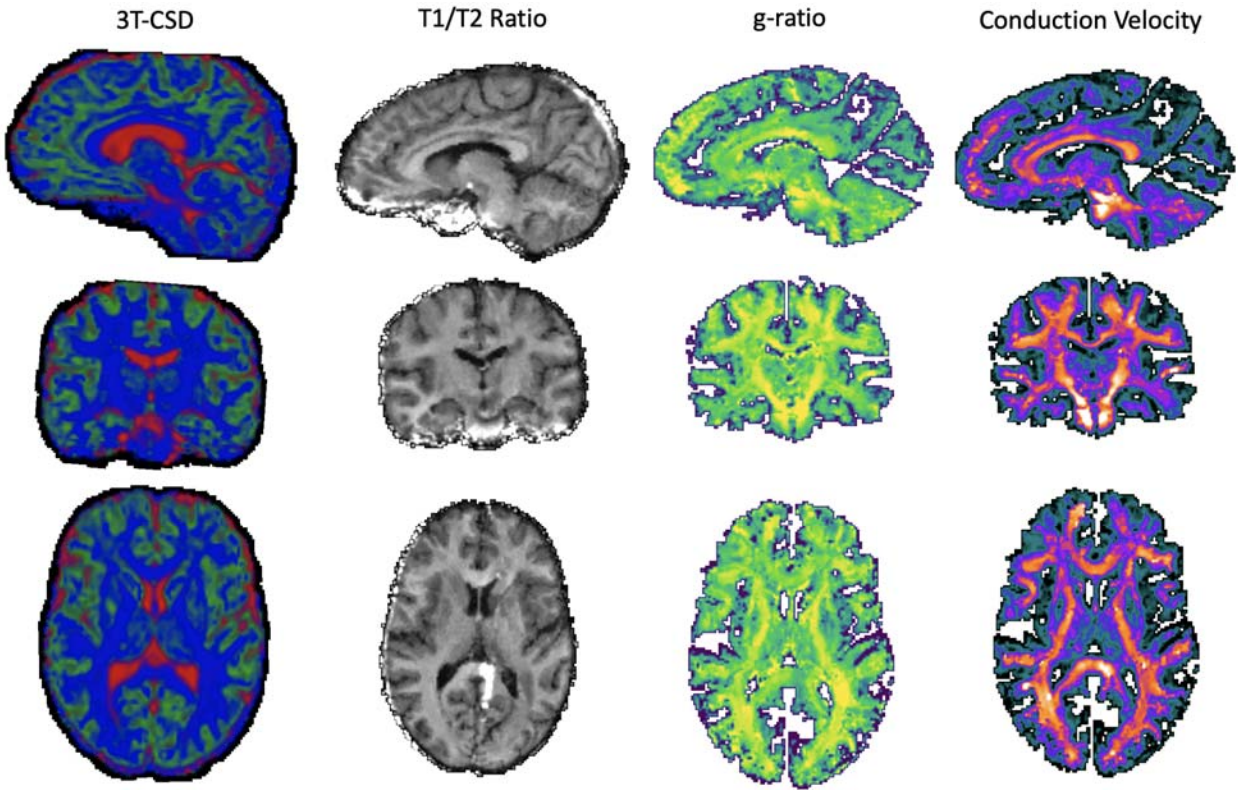


Figure 2: Illustration of each brain cellular microstructure metric from a single individual in this study. 3T-CSD is displayed as a composite of each of the 3 constituent tissue compartments having a value for each voxel, with blue voxels representing majority ICA (WM-like) signal, green voxels representing majority ICI (GM-like) signal, and red representing majority ECI (extracellular free water) signal. T1/T2 ratio, aggregate g-ratio, and aggregate conduction velocity are colored with arbitrary scales to emphasize differences between brain areas.

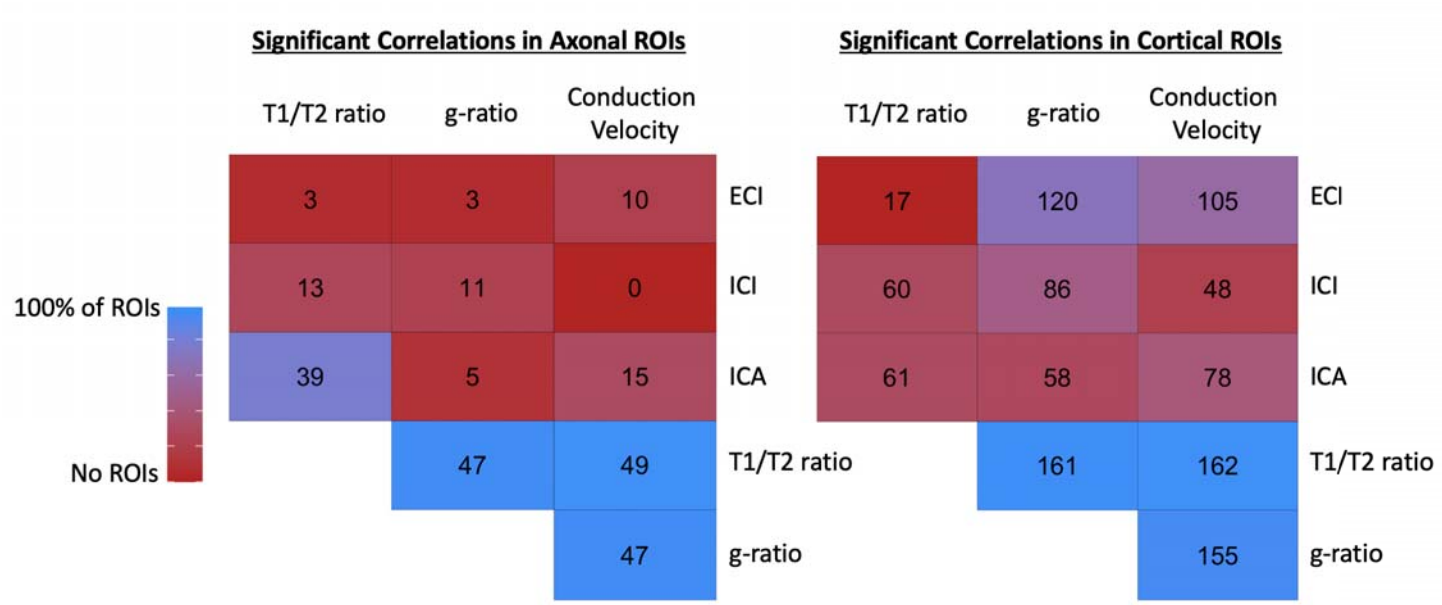


Figure 3: Matrix displaying number of ROIs across axonal areas and cortex that remained significantly associated within the same ROI with each of the different metrics used in this study. Metrics derived from T1/T2 ratio tended to have near universal significant associations between themselves (T1/T2 ratio, aggregate g-ratio, and aggregate conduction velocity). There was not very widespread associations in the axonal ROIs between metrics derived only from diffusion and metrics derived from T1/T2 ratio except for the ICA signal fraction (which is intended to measure intra-axonal signal fraction). ECI (extracellular water) signal fraction had negative associations while ICI and ICA had positive associations with T1/T2 ratio derived metrics. This was particularly true in the cortical ROIs where ECI signal fraction was significantly associated with aggregate g-ratio and aggregate conduction velocity in a majority of ROIs.

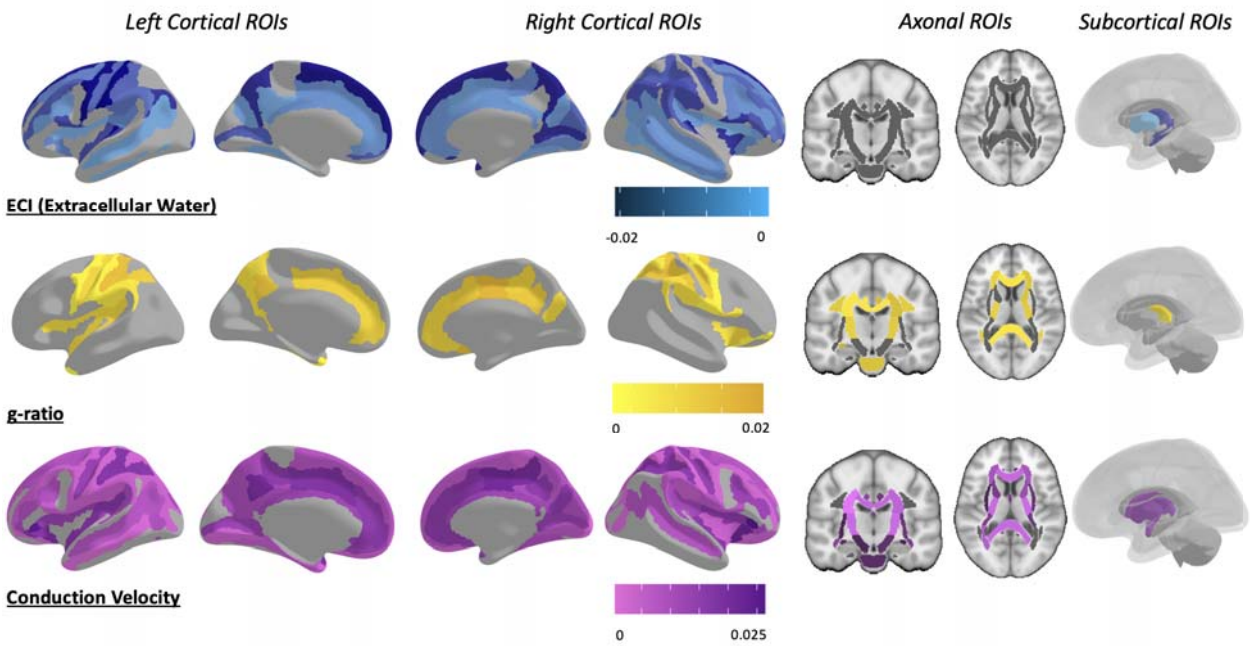
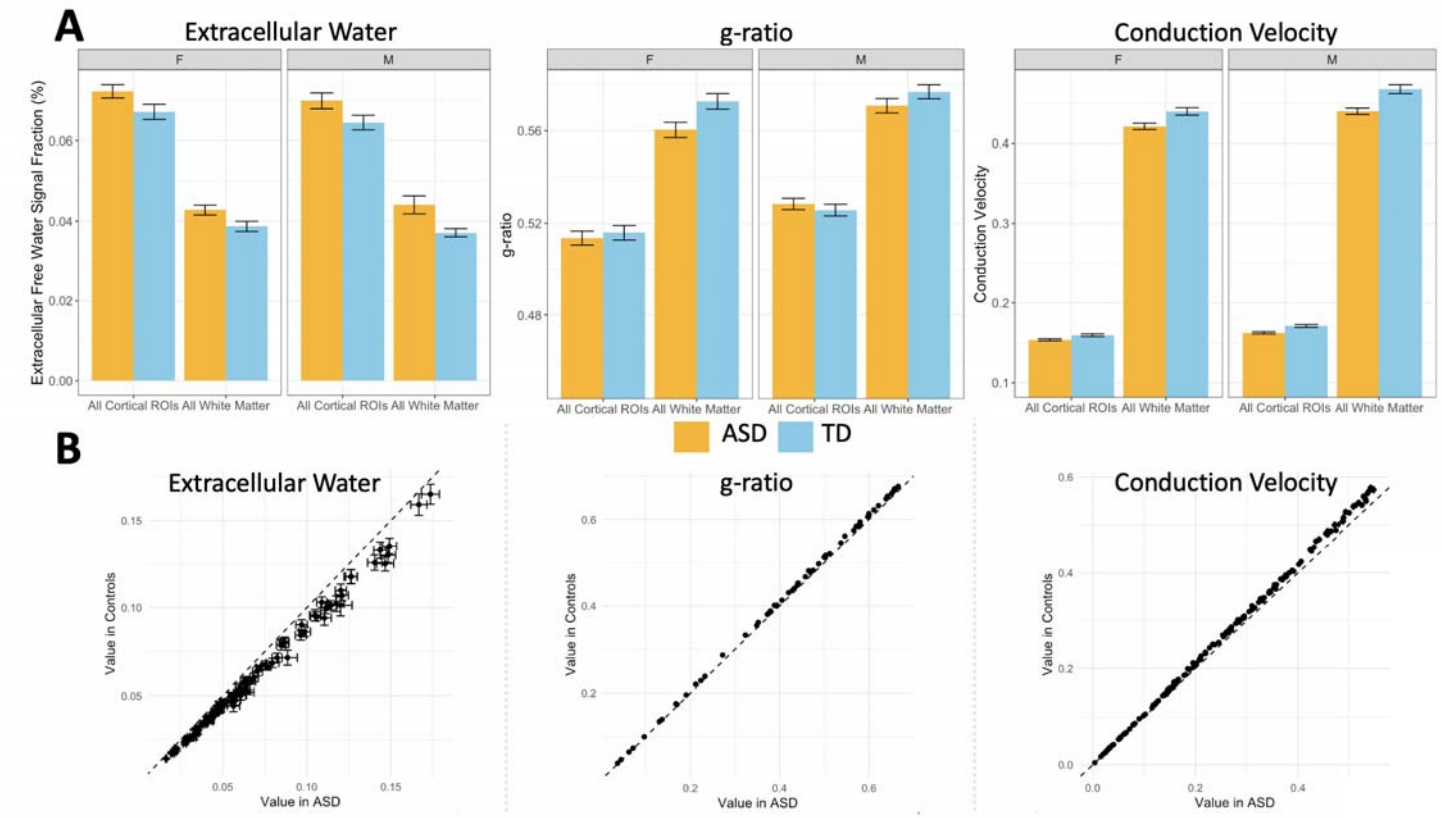


Figure 4: Map of ROIs that were significant at the p<0.05 level between ASD and TD after multiple comparison corrections. Colored by the slope of the relationship, with ASD individuals having greater extracellular water signal fraction, reduced aggregate g-ratio, and reduced aggregate conduction velocity in each of the significant ROIs. Aggregate conduction velocity was by far significantly different in more ROIs than any other metric, followed by the extracellular water compartment. However, there was a high degree of spatial specificity between the metrics, with aggregate g-ratio and aggregate conduction velocity involving extensive axonal areas and extracellular water differences primarily arising in the cortex and subcortex.



<u>Figure 5</u>: Chart displaying differences between extracellular free water, aggregate g-ratio, and aggregate conduction velocity levels in bilateral composite ROIs compared to TD participants, separated by sex. (A) Both boys and girls with ASD had significantly increased extracellular water, significantly decreased aggregate g-ratio, and significantly decreased conduction velocity. (B) Mean value in each significant ROI, after multiple comparison testing, with error bars representing standard error in each group. Dotted line represents x=y, showing that each individual ROI followed the directionality seen in the whole brain ROIs. Additionally, the effect appears to become more pronounced with higher mean value in extracellular value and aggregate conduction velocity.

Microstructural Metric	Anatomical Region	AUC
ECI (Extracellular Water)	All WM ROIs	0.8971
	All Cortical ROIs	0.7189
G-Ratio	All WM ROIs	0.7063
	All Cortical ROIs	0.4909
Conduction Velocity	All WM ROIs	0.7282
	All Cortical ROIs	0.7007

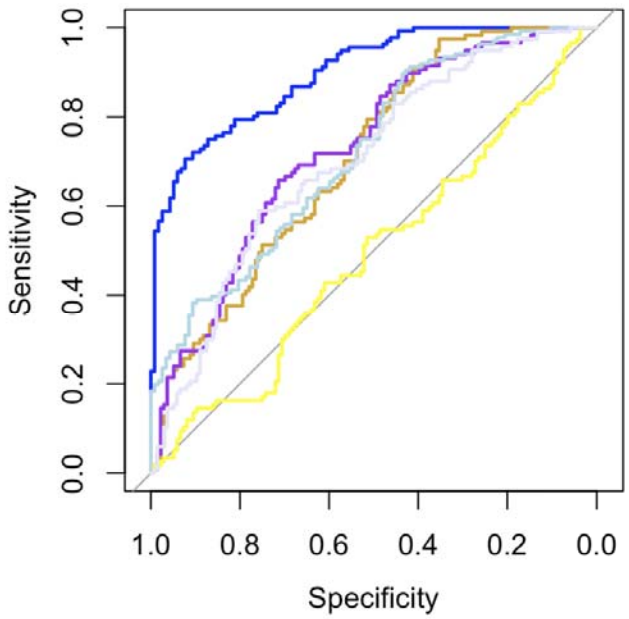


Figure 6: ROC analysis of the 3 microstructural metrics measured within the composite ROIs (axonal WM ROIs and Cortical ROIs, from the JHU WM atlas and Destrieux atlas) including AUC calculation. The same linear model as the ROI analysis was used to predict classification of subject diagnosis. Analysis indicated that ECI signal fraction within the composite WM ROI had the highest sensitivity and specificity to subject diagnosis, followed by aggregate conduction velocity within the composite WM ROI and ECI within the composite cortical ROI. Note that aggregate g-ratio had a sensitivity and specificity of approximately chance within the composite cortical ROI however this ROI was not statistically significant in the primary regional analysis.

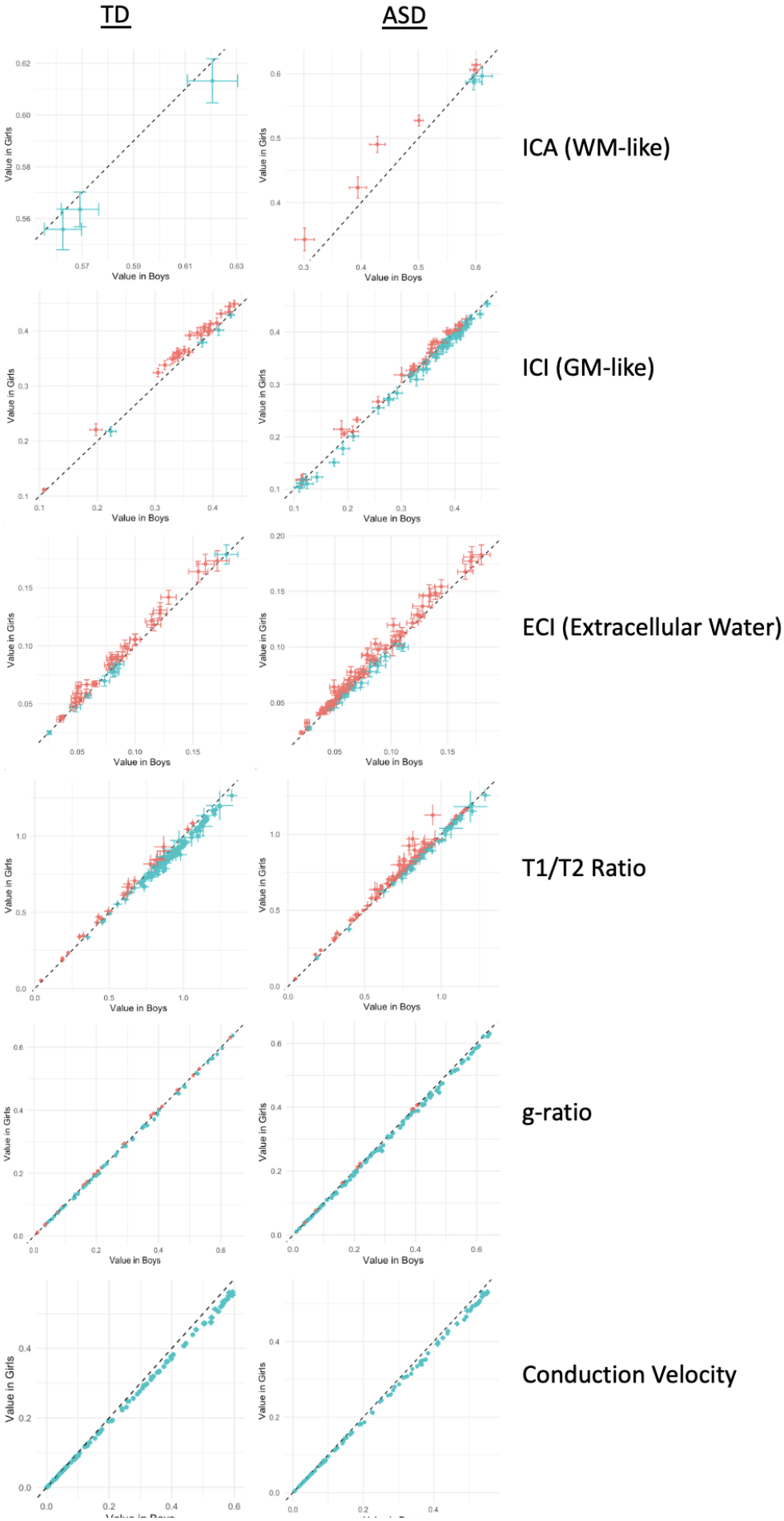

TD
ASD
ICA (WM-like)
ICI (GM-like)
ECI (Extracellular Water)
T1/T2 Ratio
g-ratio
Conduction Velocity
Value in Girls
Value in Boys

*Figure 7: Series of charts displaying mean microstructural values of ROIs significantly associated with age, colored by sex and separated between ASD and TD participants. Diagnosis groups show varying relationships with aging that are not consistent across sexes, with far higher T1/T2 ratio values in TD boys compared TD girls, while in ASD, girls tend to have higher T1/T2 ratio and ECI signal fraction values compared to boys. ASD boys meanwhile tend to have higher ICI (GM-like) signal fraction values, while in TD participants age-associated ROIs tend to have higher ICI (GM-like) values in girls. Aggregate conduction velocity and aggregate g-ratio do not seem to differ between ASD and TD in their age*sex interaction.*

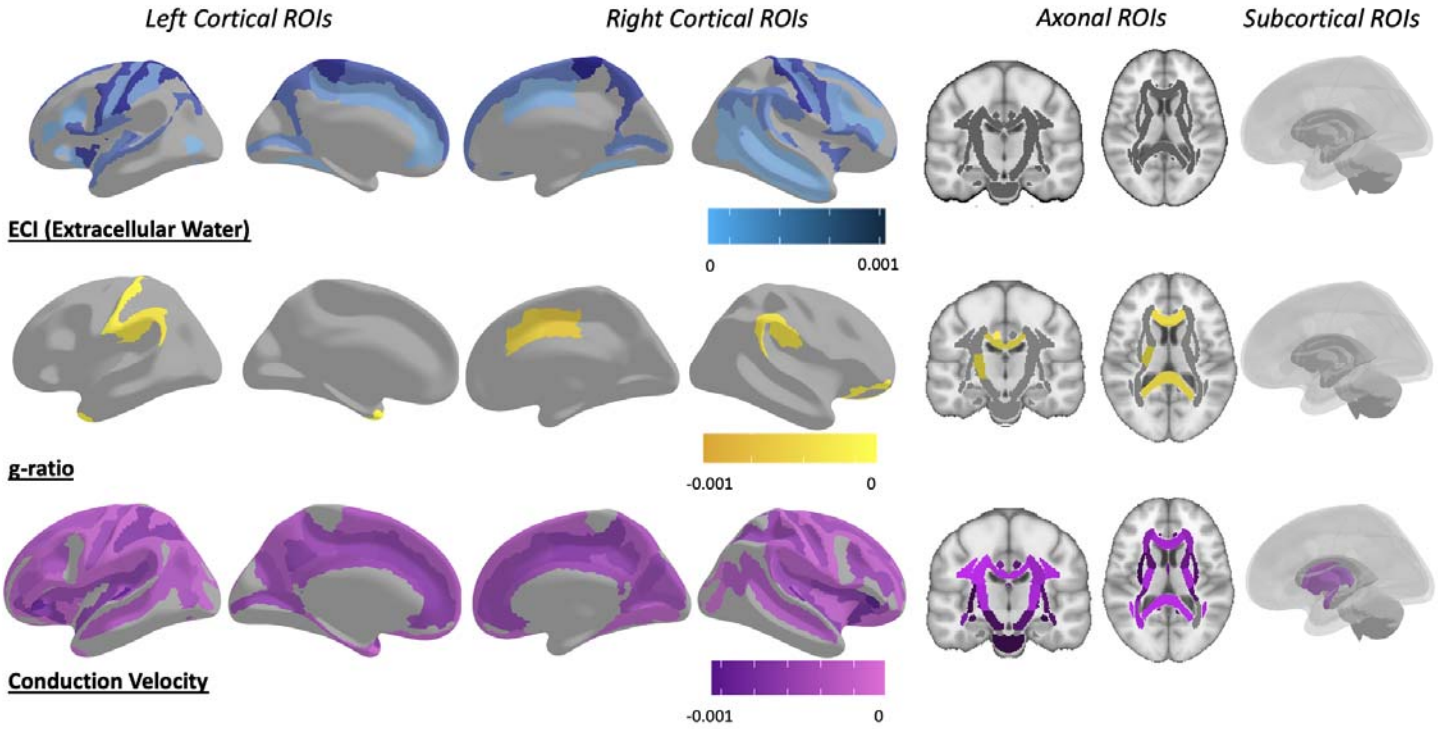
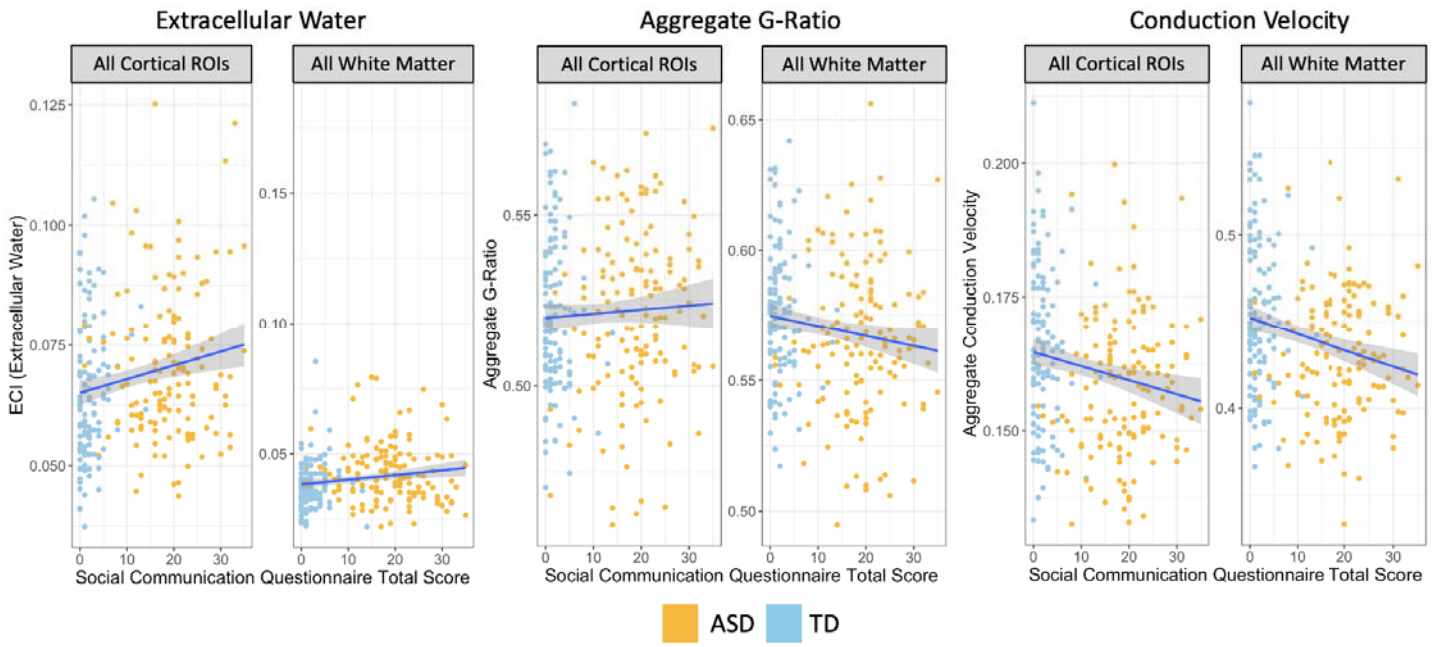


Figure 8: Map of ROIs that were significant at the p<0.05 level between each mean microstructural metric and SCQ total score after multiple comparison corrections. Colored by the slope of the relationship, with extracellular water positively associated, aggregate g-ratio negatively associated, and aggregate conduction velocity negatively associated, in each significant ROI. There was an even more pronounced special specificity in results, with ECI signal fraction ROIs located almost exclusively in cortex, aggregate g-ratio ROIs located across the entire corpus callosum, and aggregate conduction velocity ROIs located throughout the cortex, white matter, and subcortical ROIs.



<u>Figure 9:</u> Charts displaying relationship between microstructural metrics with significant ROIs in composite ROIs, colored by ASD diagnosis. The directions of the associations were consistent across all significant ROIs in each microstructural metric. The strongest associations were found in extracellular water metrics in cortical ROIs and aggregate conduction velocity metrics.

Tables:

Microstructure Metric	Region	Diagnosis	Sex	Age	Intracranial Volume	IQ
ECI (Extracellular Water)	Total	93	0	94	2	0
	Subcortical GM	7	0	2	2	0
	Neocortex	84	0	91	0	0
	Axonal WM	0	0	0	0	0
ICI	Total	0	0	93	17	0
	Subcortical GM	0	0	2	3	0
	Neocortex	0	0	89	14	0
	Axonal WM	0	0	0	0	0
ICA	Total	0	0	36	12	0
	Subcortical GM	0	0	2	1	0
	Neocortex	0	0	33	11	0
	Axonal WM	0	0	0	0	0
T1/T2 Ratio	Total	0	0	200	128	0
	Subcortical GM	0	0	14	9	0
	Neocortex	0	0	138	86	0
	Axonal WM	0	0	46	31	0
Aggregate g-ratio	Total	61	0	144	17	1
	Subcortical GM	3	0	12	3	0
	Neocortex	38	0	111	7	0
	Axonal WM	19	0	20	7	1
Aggregate Conduction Velocity	Total	155	0	124	206	0
	Subcortical GM	11	0	3	14	0
	Neocortex	104	0	85	142	0
	Axonal WM	38	0	34	48	0

Table 1: Table summarizing the number of ROIs from each microstructural metric and each anatomical subgroup that were significant at the $p<0.05$ level for each variable after multiple comparison corrections. The total value for each includes the 2 additional composite ROIs. The right cerebral peduncle was the only aggregate g-ratio significantly related to IQ.