




Longitudinal Metabolite Changes in Progressive Multiple Sclerosis: A Study of 3 Potential Neuroprotective Treatments

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Background: ¹H-magnetic resonance spectroscopy (¹H-MRS) may provide a direct index for the testing of medicines for neuroprotection and drug mechanisms in multiple sclerosis (MS) through measures of total *N*-acetyl-aspartate (tNAA), total creatine (tCr), myo-inositol (mlns), total-choline (tCho), and glutamate + glutamine (Glx). Neurometabolites may be associated with clinical disability with evidence that baseline neuroaxonal integrity is associated with upper limb function and processing speed in secondary progressive MS (SPMS).

Purpose: To assess the effect on neurometabolites from three candidate drugs after 96-weeks as seen by ¹H-MRS and their association with clinical disability in SPMS.

Study-Type: Longitudinal.

Population: 108 participants with SPMS randomized to receive neuroprotective drugs amiloride [mean age 55.4 (SD 7.4), 61% female], fluoxetine [55.6 (6.6), 71%], riluzole [54.6 (6.3), 68%], or placebo [54.8 (7.9), 67%].

Field Strength/Sequence: 3-Tesla. Chemical-shift-imaging 2D-point-resolved-spectroscopy (PRESS), 3DT1.

Assessment: Brain metabolites in normal appearing white matter (NAWM) and gray matter (GM), brain volume, lesion load, nine-hole peg test (9HPT), and paced auditory serial addition test were measured at baseline and at 96-weeks.

Statistical Tests: Paired *t*-test was used to analyze metabolite changes in the placebo arm over 96-weeks. Metabolite differences between treatment arms and placebo; and associations between baseline metabolites and upper limb function/information processing speed at 96-weeks assessed using multiple linear regression models. *P*-value<0.05 was considered statistically significant.

View this article online at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/doi/10.1002/jmri.29017). DOI: 10.1002/jmri.29017

Received May 29, 2023, Accepted for publication Sep 1, 2023.

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Results: In the placebo arm, tCho increased in GM (mean difference = -0.32 IU) but decreased in NAWM (mean difference = 0.13 IU). Compared to placebo, in the *fluoxetine arm*, mIns/tCr was lower ($\beta = -0.21$); in the *riluzole arm*, GM Glx ($\beta = -0.25$) and Glx/tCr ($\beta = -0.29$) were reduced. Baseline tNAA ($\beta = 0.22$) and tNAA/tCr ($\beta = 0.23$) in NAWM were associated with 9HPT scores at 96-weeks.

Data Conclusion: ^1H -MRS demonstrated altered membrane turnover over 96-weeks in the placebo group. It also distinguished changes in neuro-metabolites related to gliosis and glutamatergic transmission, due to fluoxetine and riluzole, respectively. Data show tNAA is a potential marker for upper limb function.

Level of Evidence: 1

Technical Efficacy: Stage 4

J. MAGN. RESON. IMAGING 2023.

Proton magnetic resonance spectroscopic imaging (^1H -magnetic resonance spectroscopy [^1H -MRSI]) provides an in vivo opportunity to analyze the metabolic composition of brain tissue in multiple sclerosis (MS). Metabolites of interest in MS include total N-acetyl aspartate and N-acetyl-aspartylglutamate (tNAA)—a marker of neuroaxonal integrity and mitochondrial function, myo-inositol (mIns)—a marker of glial cell activity, most likely astrogliosis, glutamate, and its precursor glutamine (Glx); and total-choline (tCho)—a marker of membrane turnover.¹ While the metabolite profile has been comparatively well studied in the relapsing–remitting form of MS, less is known about alterations in the progressive forms of MS (PMS).¹

Previous natural history studies examining longitudinal metabolite changes in PMS have shown mixed results: over 24–30 months, several studies showed no change in tNAA, mIns, and tCho ($n = 17$ – 47)^{2–4}; while one study showed a decrease in tNAA and an increase in tCho in normal appearing white matter (NAWM; $n = 15$).⁵

^1H -MRSI also presents a method through which the biochemical target engagement of candidate drugs to treat PMS can be investigated. Several candidate drugs (fluoxetine, riluzole, and amiloride) had been proposed as they target pathways that result in neuroaxonal degeneration in PMS.⁶ Through MRS, fluoxetine has previously been shown to increase tNAA levels in cerebral white matter in MS.^{7–10} In a mixed MS cohort, it decreased tCho levels in cortical gray matter (GM).¹¹ Furthermore, fluoxetine may have an anti-inflammatory effect that could be confirmed by a decrease in mIns levels.¹²

Riluzole was proposed as a candidate as it decreases glutamatergic transmission in the central nervous system.¹³ Evidence for glutamatergic excitotoxicity has been demonstrated in experimental MS models.^{14,15} In animal studies, glutamatergic receptor antagonism ameliorates disease activity but translational studies have been mixed.^{15,16} Using ^1H -MRSI, we can track the effectiveness of riluzole in reducing glutamatergic transmission by a concomitant reduction in Glx concentration compared to placebo.¹³

Amiloride has evidence for a potential neuroprotective effect in MS from previous animal work suggesting that the acid sensing ion channel-1 (ASIC-1) receptor is involved in sodium and calcium influx that lead to axonal and

oligodendrocyte injury. Furthermore, blockage of this receptor may result in a neuroprotective effect, an effect that could be assessed by measuring NAA levels using ^1H -MRSI.¹⁷

The purpose of our study is therefore to explore the changes in neuro-metabolites in white and GM in an secondary progressive MS (SPMS) cohort over 96 weeks; explore how metabolites are affected by fluoxetine, riluzole, and amiloride to provide evidence of neuro-protection beyond atrophy for these candidate drugs; and determine if baseline metabolites effect measures of upper limb function and processing speed in SPMS at 96 weeks for those on and off treatment.

Methods

Participants and Clinical Assessments

Consent was obtained for all participants according to the Declaration of Helsinki and ethical approval for the study was provided by the Scotland A Research Ethics Committee [13/SS/0007].

A full description of the methods has been reported previously.²⁰ In brief, participants were recruited from the MS-SMART trial, aged 25–65 with an Expanded Disability Status Scale (EDSS) score of 4.0–6.5 who showed evidence of progression of SPMS over the last 2 years.^{18,19} A full breakdown of this is given in Table 1. Participants were randomized 1:1:1:1 to placebo, amiloride, fluoxetine, or riluzole and the primary outcome measure was the percentage brain volume change (whole brain atrophy) over 2 years. Participants were not on disease modifying treatments. Further details of the study protocol and its primary and secondary outcome measures (nine-hole peg test [9HPT] and Paced Auditory Serial Addition Test [PASAT3]) have been published previously.^{18,19}

Participants at the Queen Square MS Centre, University College London were invited to be involved in an optional “Advanced MRI sub-study” and thus consented to being scanned for ^1H -MRSI at baseline and 96-weeks. They underwent recruitment and follow-up between December 2014 and July 2018.

MRI Acquisition

Imaging was acquired using a Philips Achieva 3T MRI scanner (Philips Healthcare, Best) using a 16ch neurovascular coil.

TABLE 1. Baseline Characteristics of Study Participants

	Amiloride (N = 31)	Fluoxetine (N = 24)	Riluzole (N = 25)	Placebo (N = 28)	Total (N = 108)
Age (years)	55.4 (7.4)	55.6 (6.6)	54.6 (6.3)	54.8 (7.9)	55.1 (7.1)
Female	19 (61%)	17 (71%)	17 (68%)	19 (68%)	72 (67%)
EDSS	6.0 (5.5–6.5)	6.0 (5.5–6.1)	6.0 (5.5–6.0)	6.0 (5.2–6.0)	6.0 (5.5–6.5)
Time since onset of MS (years)	24.7 (10.7)	23.0 (8.9)	21.6 (8.7)	19.4 (9.6)	22.2 (9.7)
PASAT3 (out of 60)	44.8 (10.0)	42.0 (12.3)	38.6 (15.8)	46.2 (9.8)	43.1 (12.2)
Timed 25 foot walk (sec)	9.2 (6.9–14.3)	12.0 (7.2–16.3)	9.9 (7.9–14.8)	8.3 (6.8–11.3)	9.3 (7.0–15.0)
9-Hole peg test (sec ⁻¹)	0.04 (0.01)	0.03 (0.01)	0.03 (0.01)	0.04 (0.01)	0.04 (0.01)
T2 lesion volume (mL)	7.5 (4.2–15.9)	11.0 (5.3–17.4)	8.9 (2.7–22.5)	7.0 (3.0–13.8)	8.5 (3.5–17.1)
Baseline NBV (mL)	1405.5 (88.0)	1421.9 (73.9)	1419.1 (74.7)	1443.7 (91.737)	1422.2 (83.2)

Data presented are *n* (%), mean (SD), or median (IQR). Nine-hole peg test calculated by taking the reciprocal of each trial per hand, taking the mean of two trials and then taking the average.
EDSS = expanded disability status scale; NBV = normalized brain volume; PASAT3 = paced auditory serial addition test.

Acquisition parameters for the ¹H-MRSI and structural MR sequences and details of MR analysis including brain volume, lesion segmentation, spectra post-processing, and calculation of tissue specific metabolite levels have been described previously. Briefly for ¹H-MRSI, the inferior margin of the slice was positioned at the superior margin of the corpus callosum, angulated to the anterior commissure/posterior commissure line.²⁰ A 2D point-resolved spectroscopic sequence (TE/TR = 35/2000 msec), 210 × 160 mm² field of view (volume of interest subdivided into a 21 × 16 grid) was acquired with 10 × 10mm² voxels and 15 mm thickness (Fig. 1), spectral width = 2000 Hz.²⁰ Outer volume suppression using fat saturation was applied to limit artefacts, and the volume of interest was shimmed on the water peak using the pencil beam-auto technique, acceptable shims were determined on the shape of the peak, focusing on single Lorentzian line shapes, while linewidth criteria was assigned as a cut off all had a full width half maximum <15 Hz for the water peak.²¹ Chemical shift selective saturation pulses were used for water suppression. A reference scan with no water suppression was also collected for quantification and total scan time for MRS was 10 minutes 54 sec. The number of averages was one (with and without water suppression), and each spectra had 1024 samples. No frequency correction or motion correction was applied during acquisition.

T2 lesion volume was measured by outlining lesions on the T2 weighted image using the FLAIR as a reference. This was completed by trained personnel blinded to clinical data (FDA 5 years experience, AC 1 year experience, and DM 20 years experience) using a semi-automated method (Jim7, Xinapse, UK).^{18,20} From lesion filled 3D-T1 weighted images, Geodesic Information Flows algorithm was used to segment the brain.²² Normalized brain volume was then calculated using the FSL SIENAX method.^{23,24}

MRI Data Processing

Total NAA, mIns, glutamate and its precursor glutamine (Glx), total choline (tCho) and their ratios to total creatine (tCr) were measured using Linear Combination of Model Spectra (LCModel© version 6.3-1A) using a simulated basis spectrum and default values for water concentration and attenuation. The tCr levels were also estimated.

Basis sets were simulated in GAMMA²⁵ included 17 metabolites (L-alanine, aspartate, Creatine, phosphocreatine, gamma-aminobutyric acid, glucose, Glx, glutamate, glycerophosphocholine, phosphocholine, myo-Inositol, lactate, NAA, NAAG, scyllo-inositol, taurine, and guanidinoacetate).²⁵ Macromolecules at 0.9, 2.0, 1.2, 1.4, and 1.7 ppm and lipids at 0.9, 1.3, and 2.0 ppm were also

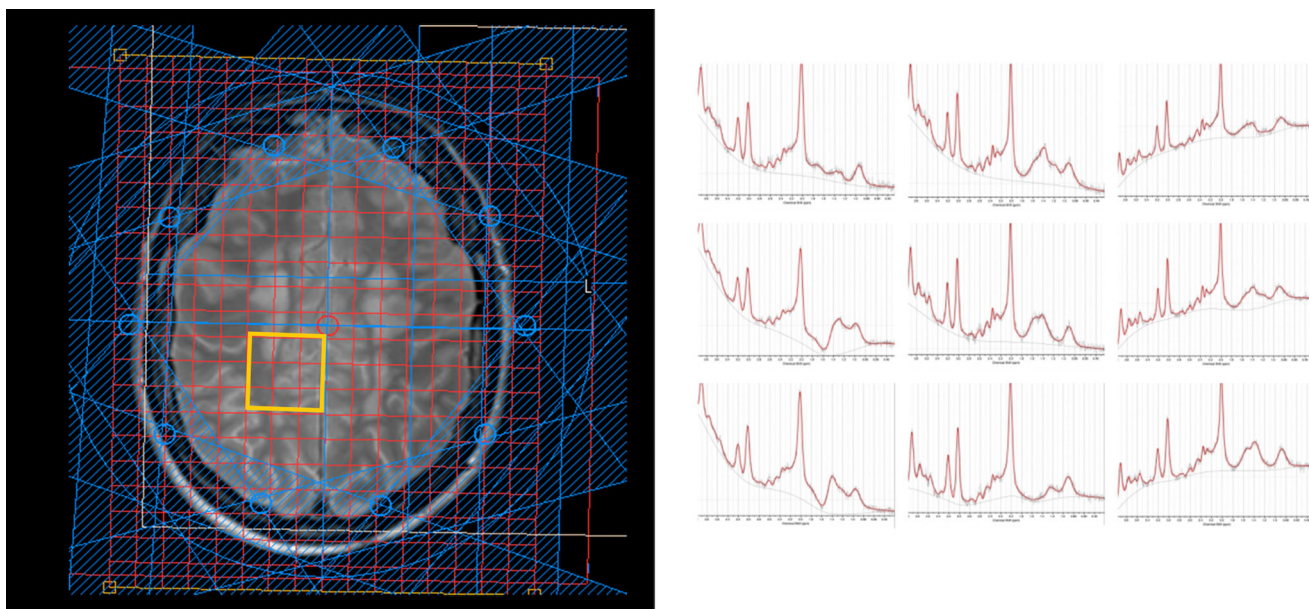


FIGURE 1: Example chemical shift imaging grid. An axial T2 slice containing the blue outer volume suppression bands. The yellow box highlights a sample chemical shift imaging grid from a participant in the study with the associated spectra shown on the right. Following the quality check, all spectra in this grid were accepted and included in the analysis.

included in the simulated basis set for macromolecular baseline correction.

Metabolites were scaled to water and expressed in institutional units (IU).²⁰ Spectra from individual voxels were automatically rejected if any neurometabolite Cramer-Rao lower bounds were $>20\%$, full width half-maximum of the tNAA spectral peak was >15 Hz or SNR <9 . In calculating metabolite levels in NAWM and GM, voxels containing $>15\%$ CSF and $>1\%$ WML were excluded before employing a regression method that has been described previously.²⁰ As previously described, the small lesion load in the acquired slice (average white matter lesion [WML] fraction in the SPMS cohort was 0.01) meant that the calculation of metabolite values in WML tissue could not be completed.²⁰ Metabolite outliers (defined as major outliers that fell outside three times the interquartile range below quartile one or three times interquartile range above quartile three) were also excluded.²⁰

Statistical Methodology

Statistical analysis was completed using R statistical software version 3.5.1.²⁶ All statistical tests and confidence intervals were two-sided; 95% confidence intervals were calculated with the significance of raw P -values assessed based on a 5% significance level. A complete case analysis following removal of metabolite outlying observations based on the intention-to-treat population. During the analysis, 48 comparisons were undertaken and each comparison was based on a specific hypothesis that was pre-specified a priori (except for choline as a post-hoc analysis) and therefore no adjustment for

multiplicity was made. The reporting of multiple analyses was guided by the considerations outlined by Patel et al.: multiplicity adjustment is not required when a list of hypotheses of primary importance are pre-specified; there is a focus on being explicit and transparent about the extent of multiplicity; and the magnitude of observed associations are interpreted in the context of the background literature.^{27,28}

A paired t -test was used to analyze metabolite changes in the placebo arm over 96-weeks. Metabolite differences between each treatment group and placebo were analyzed using a multiple linear regression method to calculate adjusted mean differences and 95% confidence intervals. Each metabolite was assessed in a separate model, where the week-96 metabolite concentration was the dependent variable. Independent variables included the trial arm (with placebo as the reference category), the baseline metabolite concentration (corresponding to the outcome variable), and minimization variables: age, sex, and EDSS score at randomization.

When analyzing the longitudinal association between baseline metabolites and week-96 clinical disability measures (9HPT and PASAT3 scores), separate linear regression models were initially fitted with 9HPT and PASAT3 performance at 96-weeks as the dependent variable. Trial arm was again included as an independent variable in each model. Where metabolites were associated with 96-week PASAT3 or 9HPT in this preliminary model (refer table 6), this relationship was further analyzed in a multiple linear regression model including the following covariates: age, sex, occurrence of a relapse in 2 years preceding randomization, baseline T2 lesion volume, baseline normalized brain volume, and trial

arm. Age and sex were chosen as key demographic variables, while the occurrence of relapses in the 2 years preceding study entry was added to assess the impact of potential inflammatory activity. Results are reported as standardized coefficients (β) with standardized 95% confidence intervals.

Distributional assumptions underlying the regression analyses were assessed by visual inspection of residual plots. Normality was examined by normal probability plots. Highly leveraged data observations were identified using Cook's distance.

Results

H-MRSI data were obtained at 96-weeks from 122/148 participants (82%) where 26 participants were lost to follow up or failed quality assurance (see Fig. 2). The 122 participants provided 7402 ^1H -MRSI voxels and after removing voxels containing >15% CSF, 3639 voxels remained. Removing voxels containing >1% WML left 2655 voxels from the 96-week timepoint for analysis [median 20 (range 1–53) per patient]. Fourteen participants were excluded as metabolite outliers and these were spread across each trial arm (three in amiloride, three in fluoxetine, seven in riluzole, and one in placebo arm); and this cohort had an overall median EDSS 6.5 (IQR 6.0–6.5), median T2 lesion volume (mL) 22.0 (IQR 15.6–30.5) and mean normalized brain volume (mL) of 1364 (SD 98.4). After calculating metabolite values and removing metabolite outliers (as previously described),²⁰ 108 participants were left for analysis and the baseline characteristics of this cohort are shown in Table 1.

The spectral signal to noise ratio and linewidth were of sufficient quality, consistent at each timepoint and across trial arms for this analyzed cohort ($n = 108$; Supplementary Table S3).

Metabolite Changes in SPMS over 96 Weeks

There was no significant decrease in tNAA or mIns in either the NAWM ($P = 0.34$; $P = 0.37$, respectively) or GM ($P = 0.32$; $P = 0.65$, respectively). There was a significant increase in GM tCho (and tCho/Cr) with mean differences of -0.32 IU, and -0.04 IU, respectively, while NAWM tCho decreased (mean difference = 0.13 IU) (Supplementary Table S1).

Effect of Candidate Drugs on Metabolites

These are shown in supplementary Tables S2 and S3. Compared to placebo, the only statistically significant changes were that mIns/tCr levels in NAWM was lower in the fluoxetine arm over 96-weeks ($\beta = -0.21$, 95% CI $[-0.40$ to $-0.02]$). In the riluzole arm, there was no change in NAWM Glx ($P = 0.24$); however, riluzole did significantly decrease GM Glx ($\beta = -0.25$, 95% CI $[-0.47$ to $-0.04]$) and Glx/tCr ($\beta = -0.29$, 95% CI $[-0.50$ to $-0.08]$) compared to placebo. There was no significant change in tNAA or

tNAA/tCr levels compared to placebo in the amiloride arm in NAWM ($P = 0.76$ and $P = 0.61$, respectively) or GM ($P = 0.07$ and $P = 0.51$, respectively).

Associations between Metabolites and Clinical Measures

In NAWM, baseline tNAA ($\beta = 0.22$, 95% CI $[0.02$ – $0.41]$) and tNAA/tCr ($\beta = 0.23$, 95% CI $[0.5$ – $0.42]$) were associated with week-96 9HPT scores after adjusting for model covariates (Table 2 and Supplementary Table S4).

Discussion

^1H -MRSI in SPMS has allowed us to interrogate at a metabolic level: natural history, effect of candidate drugs, and longitudinal disability measures. An increase in GM tCho and a reduction in NAWM tCho in SPMS not on treatment is demonstrated. Fluoxetine was shown to only reduce mIns/tCr levels, riluzole reduced the Glx level, while amiloride had no effect on tNAA. Baseline tNAA and tNAA/tCr levels in NAWM are associated with decreased performance in upper limb function at 96-weeks.

Metabolite Changes in SPMS over 96 Weeks

Previous longitudinal studies of metabolite changes in progressive MS have shown mixed results.^{2–5} However, based on mechanisms of SPMS progression, increases in tCho and mIns, and a reduction in tNAA was hypothesized. Two previous longitudinal studies of NAWM tCho in PMS have shown mixed results with one study showing no change ($n = 47$, median EDSS = 6.0) and another being inconclusive (SPMS = 15, median EDSS = 4.0).^{4,5} The tCho peak comprises free choline, phosphocholine, phosphatidylcholine (a major constituent of cell membranes), and glycerophosphocholine.²⁹ Studies in animal MS models and those correlating in vivo ^1H -MRS with histopathology have demonstrated increased tCho (and tCho/tCr) in acute WML,³⁰ with levels greatest in lesions with fibrillary gliosis.^{1,30,31} It is therefore postulated that the increased tCho level in GM could be due to a combination of increased membrane turnover, fibrillary gliosis, increased cell membranes from glial cell proliferation, and remyelination. This may also reflect the role of cortical GM damage mediated by meningeal inflammation in SPMS.³² The reduction in NAWM tCho may reflect a decrease in membrane turnover or cell membranes/myelin. The difficulty in interpretation stems from the fact that the tCho peak contains differing proportions of choline-containing compounds. These compounds are involved in membrane formation, membrane breakdown but also reflect membrane levels and therefore disentangling these contributions to underlying pathology remains an ongoing challenge when using ^1H -MRSI in MS.

No significant changes were seen in tNAA or mIns over 96-weeks in either tissue compartment. These findings are in keeping with the majority of previous studies.^{2–4} A single

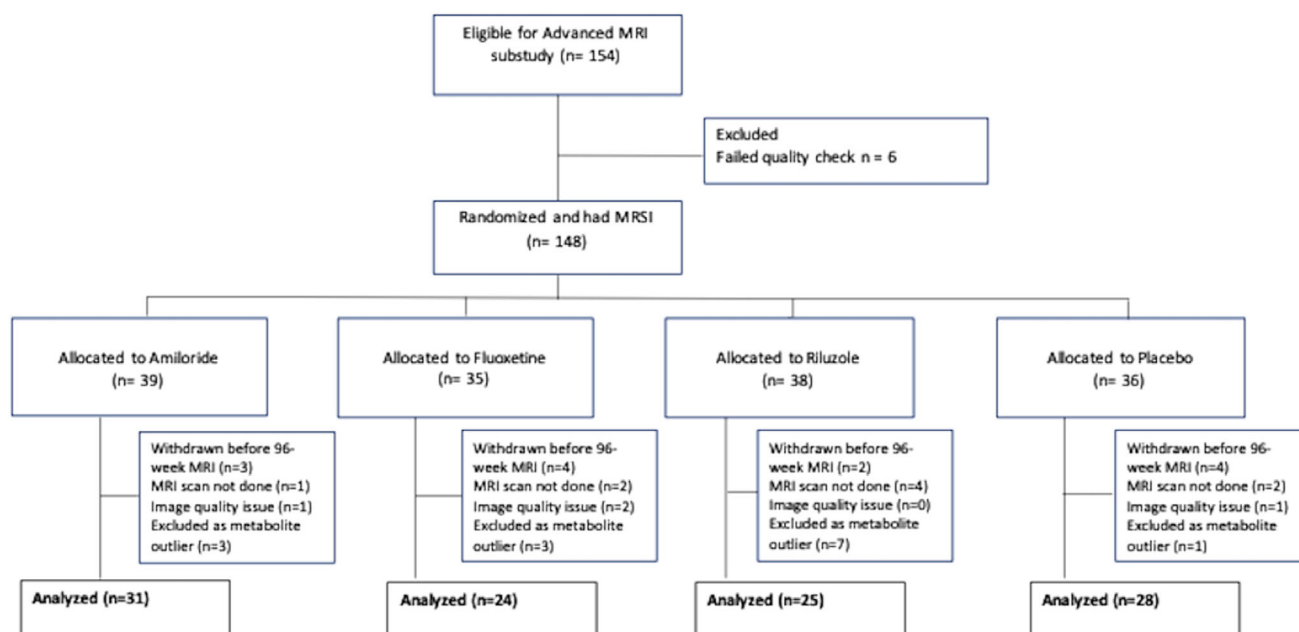


FIGURE 2: Study profile. At week 96, 26 participants were either lost to follow up or failed MR quality assurance. Of the remaining 122 participants a further 14 were removed as metabolite outliers: 3 from the amiloride arm, 3 from the fluoxetine arm, 7 from the riluzole arm, and 1 from the placebo arm leaving 108 for study analysis.

study demonstrated a reduction in tNAA/tCr over 24-weeks but this cohort had differing characteristics (median EDSS = 4.0) and used single voxel spectroscopy.⁵ The lack of change in tNAA and mIns (and their respective ratios to tCr) over this time frame could be due to several reasons: First, the rate of change within the 96-week study period could be better characterized by acquiring metabolite levels at additional timepoints. Second, acute axonal damage may decrease with disease duration of 10 years or more.³³ Therefore, changes in neuroaxonal integrity in nonactive SPMS may occur over a longer duration than was measured in this study. Last, longitudinal changes in tNAA in SPMS may encompass reductions in axonal density but also compensatory mechanisms to preserve neuro-axonal function. This can lead to partially reversible reductions in tNAA (tNAA/tCr) that may explain why tNAA did not decrease in our cohort.³⁴

Effect of Individual Drugs on Metabolite Levels

FLUOXETINE. There was no significant change in tNAA or tNAA/tCr in the fluoxetine treatment arm compared to placebo. The 95% confidence intervals for effect of fluoxetine on tNAA (and tNAA/tCr) exclude clinically important treatment effects. This analyzed cohort contained a larger sample with SPMS on fluoxetine (24/108; 3–6 times) compared to two previous smaller studies that demonstrated an increase in NAA/Cr in cerebral white matter (RRMS 7/11, SPMS 4/11)¹⁰; and an increase in WML after 2-weeks (RRMS 7/15, PMS 8/15).⁸ It also underscores the results of the MS-SMART and FLUOX-PMS trials that fluoxetine has no

significant therapeutic effect as a neuroprotective agent in non-inflammatory PMS.^{19,35}

NAWM mIns/tCr levels were reduced by fluoxetine, and in MS-SMART a reduction in new/enlarging lesions was seen with fluoxetine, which cautiously might infer an anti-inflammatory effect.¹⁹ There may therefore be further value in examining NAWM mIns/tCr as a marker of decreased astrogliosis as sequelae of inflammatory activity.

There was no reduction in NAWM or GM tCho (or tCho/tCr) in the fluoxetine arm compared to placebo. The overall treatment effect was relatively small with confidence intervals sufficiently narrow to exclude any potential treatment effect undetected by insufficient sample size. Our cohort contained only SPMS while the previously suggestive study cohort contained eight RRMS and seven PMS treated with fluoxetine for 2-weeks, while our cohort was treated for 96-weeks.¹¹

RILUZOLE. GM Glx in the riluzole treatment arm was significantly lower when compared to placebo but not in NAWM, confirming the action of riluzole on glutamate in this compartment. Our results suggest that riluzole may decrease Glx levels in GM but does not translate into a reduction in brain atrophy as evidenced by the main trial results from MS-SMART.¹⁹

The identification of a significant effect of riluzole on Glx in GM, but not NAWM indirectly supports experimental studies that predominantly examined effects of riluzole on cortical neuron ion channels.¹³ From animal studies, it remains unclear as to whether riluzole exerts similar mechanisms of action on NAWM. It may also reflect the differing causes of glutamatergic excitotoxicity in GM and NAWM—

TABLE 2. Regression Analysis Examining Association Between Baseline tNAA and tNAA/tCr in NAWM and Week 96 Nine-Hole Peg Test Performance

Predictors	Standardized beta	95% CI	P	R ²
Baseline tNAA in NAWM and week 96 nine-hole peg test performance (<i>n</i> = 107 ^a)				
Baseline tNAA	0.21	0.02–0.41	0.04	0.17
Age	0.14	–0.06 – 0.34	0.16	
Gender	0.15	–0.32 – 0.62	0.52	
Previous relapses	–0.28	–0.90 – 0.34	0.37	
Duration from MS onset	–0.05	–0.26 – 0.16	0.62	
Baseline T2LV	–0.02	–0.22 – 0.18	0.84	
Baseline NBV	0.24	0.00 – 0.47	0.05	
Baseline tNAA/tCr in NAWM and week 96 nine-hole peg test performance (<i>n</i> = 107 ^a)				
Baseline tNAA/tCr	0.23	0.05 – 0.42	0.02	0.18
Age	0.15	–0.05 – 0.35	0.14	
Gender	0.16	–0.30 – 0.62	0.49	
Previous relapses	–0.27	–0.88 – 0.35	0.39	
Duration from MS onset	–0.04	–0.25 – 0.17	0.70	
Baseline T2LV	–0.02	–0.22 – 0.18	0.86	
Baseline NBV	0.24	0.01 – 0.47	0.04	

Model covariates: Age, gender, T2 lesion volume, trial arm, occurrence of relapse in 2 years preceding randomization. Trial arm was not found to be a significant variable in this multiple regression model and so is not shown. The p-values of significant predictors highlighted in bold.

CI = confidence interval; NAWM = normal appearing white matter; NBV = normalized brain volume; tCr = creatine and phospho-creatine; tNAA = *N*-acetyl aspartate and *N*-acetyl aspartyl glutamate; T2LV = T2 lesion volume.

^aOne participant missing as they did not complete the nine-hole peg test at 96 weeks.

where riluzole is unlikely to impact white matter predominant mechanisms such as excess release by macrophages/microglia and dendritic cells but more likely to have an effect in GM where excess presynaptic release is the purported mechanism.¹⁵ There are several considerations when interpreting these changes in glutamate metabolism. First, ¹H-MRSI cannot differentiate between signal arising from intracellular or extracellular Glx. Furthermore, in a healthy adult brain, the majority of glutamate signal arises from the intracellular compartment compared to the extracellular compartment where the concentration is in micromoles (vs. millimoles) and therefore less likely to be detected by ¹H-MRS techniques. Therefore, one could argue that changes in intracellular glutamate are being measured. Though in MS the extracellular concentration may be higher due to mechanisms that result in excitotoxicity including excess release by immune cells, altered reuptake from decreased expression of EAAT-1 and EAAT-2 receptors, which may not have been captured.¹⁵ Second, the metabolism of glutamate is complex—it is

synthesized in astrocytes and neurons, involved in the Krebs cycle, is a precursor to the inhibitory neurotransmitter GABA and is taken up from extracellular areas by astrocytes.³⁶ Disentangling the various contributions to the Glx signal is therefore difficult using ¹H-MRSI.

AMILORIDE. There was no significant difference seen in tNAA or tNAA/tCr between the amiloride and placebo groups. Furthermore, the estimated treatment effects of amiloride on tNAA levels (in NAWM and GM) were small and with reasonably narrow confidence intervals that crossed zero, it is highly unlikely that there was a treatment effect that was not detected. There are several explanations for why target engagement between amiloride and a marker of neuroaxonal integrity was not identified. In animal studies, amiloride exerted its neuroprotective effect on axons when given at disease onset or at the onset of relapse, but this is different from our cohort of non-active progressing SPMS in whom disease progression was driven by various mechanisms

leading to neurodegeneration, rather than focal inflammation alone.¹⁷ ASIC-1 expression and co-localization with damaged axons was greatest at the borders of active lesions.^{17,37} It is therefore possible that an amiloride effect on tNAA may have been detected in lesions or peri-lesional areas but was not captured in this study as metabolite values in WML were unable to be measured. Given this, future work should aim to study tNAA in WML, using single voxel spectroscopy, higher field strengths or longer acquisitions to allow for the spatial resolution needed to reduce partial volume in lesional voxels.

Association with Upper Limb Function and Information Processing Speed

Baseline NAWM tNAA/tCr and tNAA is confirmed to be associated with decreased performance in upper limb function 2-years later.²⁰ When holding all other independent variables constant, for every 1-unit decrease in tNAA and tNAA/tCr levels, the 9HPT decreases. This would potentially underscore that reductions in neuroaxonal integrity and mitochondrial function are associated with decreased upper limb performance (9HPT is taken as the reciprocal and therefore higher values reflect increased upper limb function). There is only one previous small ($n = 15$) study in PMS that examined the relationship between baseline tNAA and longitudinal upper limb function, which did not find an association.⁵

None of the previously observed cross-sectional associations with PASAT3/information processing speed remained significant. This could be for several reasons: first, in the cross-sectional analysis, T2LV demonstrated the strongest association with information processing speed and the longitudinal results further emphasized this. Second, there may be region-specific metabolic relationships that were not analyzed in this study. Our findings are however, in keeping with the only previous study ($n = 31$, median EDSS = 4.5) that examined the relationship between metabolites in NAWM, lesion load and long-term performance on information processing speed in PMS, showing T2LV to be the only predictor of 5-year cognitive performance.³⁸

Limitations

First, when calculating metabolite levels in NAWM and GM, the partial volume effect was addressed by placing the slice above the lateral ventricles, avoiding CSF and using a nominal voxel size of $10 \times 10 \times 15 \text{ mm}^3$. Contamination by WML and CSF was limited by excluding voxels that contained >1% WML and >15% CSF before using a regression method to calculate metabolite levels in NAWM and GM.²⁰

While the spectral fit and linewidth of individual voxels were within acceptable limits, the next step to use a regression method to estimate per participant metabolite levels in NAWM and GM can lead to outliers, which were then excluded as outlined above and previously described.^{20,39} Second, resolving glutamate from Glx at 3T using standard

sequences is difficult and while a reduction in Glx was shown, the magnitude of reduction in glutamate levels could be more accurately identified by other MRSI techniques that were not feasible, due to scan time limitations, in this study.⁴⁰ Third, metabolite levels were measured using a multivoxel technique and then the mean of these voxels in NAWM and GM was used to obtain a single per patient metabolite value for each tissue type, respectively. Fourth, water was the internal reference for quantification but there are assumptions made about the water concentration of brain tissue in MS. One important assumption was taking the water concentration to be that of healthy white matter (35,880 mM), in both GM and NAWM. This may not be accurate for either tissue type, as the water volume fraction may change and differ due to the inclusion of even a small lesion fraction. Lesions in GM were not delineated at all and so using another more standard “GM” value here would also be inaccurate. Fifth, as a short TR = 2 sec was used to minimize scan time and measures of metabolite T1 were beyond the scope of this study, there will be a resulting confounding T1 variability in the metabolite values, but this was considered acceptable for the goals of this study. A longer TR to minimize T1 variation would have led to longer scan times, increasing the chance for motion and decreasing patient comfort and tolerability. No adjustment for T2 relaxation was made beyond that applied by LCModel. For these reasons, results are reported as estimated, not absolute concentrations. Last, healthy volunteers were not recruited as this was not part of this study and therefore any associations are confined to the SPMS population only.

Conclusion

In a large SPMS cohort undergoing longitudinal ¹H-MRSI over 2 years, there are changes in a marker of membrane turnover in both GM (increased) and NAWM (decreased). The known mechanism of action of riluzole (reducing Glx) is supported, but this is insufficient to reduce brain atrophy or have an effect on clinician or patient reported outcome measures. Baseline NAWM tNAA (and tNAA/tCr) are durable predictive markers of upper limb deterioration over this time frame, but not information processing speed. We therefore ultimately feel ¹H-MRSI can provide an adjunctive tool to examine in vivo target engagement when there is a strong mechanism of action.

Author Contributions

N.A.J. and B.S.S. were involved in conception and design of the study, data acquisition and analysis and drafting the manuscript and figures. F.D.A., J.S., F.P., T.S., A.M., D.P., A.D., A.C., T.W., and D.M. were involved in data acquisition and analysis. R.A.P. and C.J.W. were the study statisticians and were also involved in the study conception and

design. I.M., F.B., C.G.W.-K., and J.C. were involved in the conception and design of the study.

Acknowledgments

The authors would like to thank all the participants of the MS-SMART trial. Particularly, thanks to Marios Yiannakis, Almudena Garcia Gomez, Rebecca Samson, and Marcello Moccia. They would also like to thank the MS-SMART investigators: Sebastien Ourselin, Marie Braisher, Tiggy Beyene, Vanessa Bassan, Alvin Zapata (Queen Square Multiple Sclerosis Centre, University College London and University College London Hospitals NHS Foundation Trust, London, UK); Siddharthan Chandran, Peter Connick, Dawn Lyle, James Cameron, Daisy Mollison, Shuna Colville, Baljean Dhillon (Anne Rowling Regenerative Neurology Clinic, The University of Edinburgh, Royal Infirmary of Edinburgh, NHS Lothian, Edinburgh, UK); Moira Ross, Gina Cranswick, Allan Walker, Lorraine Smith (Edinburgh Clinical Trials Unit [ECTU], Usher Institute, University of Edinburgh, Edinburgh, UK); Gavin Giovannoni, Sharmilee Gnanapavan (Blizard Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University, Barts Health NHS Trust, London, UK); Richard Nicholas (Imperial College Healthcare NHS Trust, London, UK); Waqar Rashid, Julia Aram (Brighton and Sussex University Hospitals NHS Trust, Brighton, UK); Helen Ford (Leeds General Infirmary, Leeds Teaching Hospitals NHS Trust, Leeds, UK); Sue H Pavitt (Dental Translational and Clinical Research Unit, University of Leeds, Leeds, UK); James Overell (The Queen Elizabeth University Hospital Glasgow, NHS Greater Glasgow and Clyde, Glasgow, UK); Carolyn Young, Heinke Arndt (The Walton Centre NHS Foundation Trust, Liverpool, UK); Martin Duddy, Joe Guadagno (Royal Victoria Infirmary, The Newcastle upon Tyne Hospital NHS Foundation Trust, Newcastle, UK); Nikolaos Evangelou (Queens Medical Centre, Nottingham University Hospital NHS Trust, Nottingham, UK); Matthew Craner, Jacqueline Palace (John Radcliffe Hospital, Oxford University Hospitals NHS Foundation Trust, Oxford, UK); Jeremy Hobart (Derriford Hospital, University Hospitals Plymouth NHS Trust, Plymouth, UK); Basil Sharrack, David Paling (Royal Hallamshire Hospital, Sheffield Teaching Hospitals NHS Foundation Trust, Sheffield, UK); Clive Hawkins, Seema Kalra (Royal Stoke University Hospital, University Hospitals of North Midlands NHS Trust, Stoke-on-Trent, UK); Brendan McLean (Royal Cornwall Hospitals NHS Trust, Truro, UK); Nigel Stallard (Statistics and Epidemiology, Division of Health Sciences, Warwick Medical School, University of Warwick, Coventry, UK); and Roger Bastow (patient representative). MS-SMART is an investigator-led project sponsored by University College London (UCL). This

independent research is awarded by the Efficacy and Mechanism Evaluation Programme (EME 11/30/11) and funded by the Medical Research Council (MRC), the UK Multiple Sclerosis Society, and the National Multiple Sclerosis Society (USA NMSS) and managed by the National Institute for Health Research (NIHR) on behalf of the MRC-NIHR partnership. Additional support comes from the University of Edinburgh; the National Institute for Health Research University College London Hospitals (NIHR-UCLH), Biomedical Research Centre (BRC), and University College London; and NIHR Leeds CRF (DenTCRU). They also acknowledge the support provided the Edinburgh Clinical Trials Unit (ECTU), MS Clinical Trials Network (MS CTN), and the QSMSC NMR/MRI analysis centre. C.J.W. and R.A.P. were supported in this work by NHS Lothian via the Edinburgh Clinical Trials Unit. B.S. is funded by Wings for Life. Riluzole was provided without charge by Sanofi-Genzyme who was not involved in either the trial design, running of the trial, or analysis.

Conflict of Interest

B.S.S., F.D.A., J.S., F.P., A.C., T.W., R.A.P., C.J.W., A.M., D.P., A.D., D.M.C.M., and I.M. have no conflict of interests to declare. N.A.J. is a local principal investigator on commercial MS studies funded by Novartis, Roche, and Sanofi. F.B. serves on the editorial boards of *Brain*, *European Radiology*, *Journal of Neurology*, *Neurosurgery & Psychiatry*, *Neurology*, *Multiple Sclerosis and Neuroradiology*, and serves as consultant for Bayer Shering Pharma, Sanofi-Aventis, Biogen-Idec, TEVA Pharmaceuticals, Genzyme, Merck-Serono, Novartis, Roche, Synthon, Jansen Research, and Lundbeck. C.A.G.W.-K. has received research grants (PI and co-applicant) from Spinal Research, Craig H. Neilsen Foundation, EPSRC, Wings for Life, UK MS Society, Horizon2020, NIHR/MRC, MRC and is a shareholder of Queen Square Analytics. In the last 3 years, J.C. has received support from the Efficacy and Evaluation (EME) Programme, a Medical Research Council (MRC) and National Institute for Health Research (NIHR) partnership and the Health Technology Assessment (HTA) Programme (NIHR), the UK MS Society, the US National MS Society, and the Rosetrees Trust. He is supported in part by the National Institute for Health Research, University College London Hospitals, Biomedical Research Centre, London, UK. He has been a local principal investigator for a trial in MS funded by the Canadian MS society. A local principal investigator for commercial trials funded by: Actelion, Biogen, Novartis and Roche; has received an investigator grant from Novartis; and has taken part in advisory boards/consultancy for Azadyne, Biogen, Celgene, Janssen, MedDay, Merck, NervGen, Novartis, and Roche.

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