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# Fragile X syndrome: a pilot proton magnetic resonance spectroscopy study in premutation carriers

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## Abstract

**Purpose:** There is increasing evidence that neurodevelopmental differences in people with Fragile X syndrome (FraX) may be explained by differences in glutamatergic metabolism. Premutation carriers of FraX were originally considered to be unaffected although several recent reports demonstrate neuroanatomical, cognitive, and emotional differences from controls. However there are few studies on brain metabolism in premutation carriers of FraX.

**Methods:** We used proton magnetic resonance spectroscopy to compare neuronal integrity of a number of brain metabolites including N-Acetyl Aspartate, Creatine + Phosphocreatinine, Choline, myoInositol, and Glutamate containing substances (Glx) in 17 male premutation carriers of FraX and 16 male healthy control individuals.

**Results:** There was no significant between-group difference in the concentration of any measured brain metabolites. However there was a differential increase in N-acetyl aspartate with aging in premutation FraX individuals compared to controls.

**Conclusions:** This is the first <sup>1</sup>H-MRS study to examine premutation FraX individuals. Although we demonstrated no difference in the concentration of any of the metabolites examined between the groups, this may be due to the large age ranges included in the two samples. The differential increase in NAA levels with aging may reflect an abnormal synaptic pruning process.

**Keywords:** Fragile X, Premutation carriers, MRS, NAA

## Background

Expanded trinucleotide repeats are associated with several disabling neuropsychiatric and neurological conditions, such as Huntington's disease, myotonic dystrophy, Friedreich ataxia, spinal and bulbar palsy, and Fragile X syndrome (FraX). The clinical phenotype of FraX is caused by an expansion of a single trinucleotide repeat sequence (CGG) in the 5' untranslated region of the Fragile X Mental Retardation 1 (FMR-1) gene on the X chromosome. Expansion of the FMR1 gene to more than 200 CGG repeats (full mutation) is accompanied by

methylation of FMR-1 and loss of FMR-1 protein (FMRP) production [1,2]. Premutation carriers of FraX have 55 to 200 CGG trinucleotide repeats with diminished production of FMRP in blood [3], and elevated levels of FMR-1 mRNA [4,5]. Normal controls have less than 55 CGG repeats.

The cognitive and behavioral phenotype of the full mutation of FraX has been described by many authors [6,7]. In brief, the cognitive phenotype of males usually includes a moderate to severe intellectual disability [8,9]; deficits in executive function, short-term memory, attentional control, and arithmetic and visuo-spatial processing [10]; and the behavioral phenotype includes gaze aversion, anxiety, hyperactivity, and social-interaction deficits [11]. Females with FraX are less severely affected, owing to the second, unaffected X chromosome. They may be of average intelligence or have a

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mild intellectual disability and often have executive function deficits [12]. They are also at an increased risk for mood disorders and social anxiety [8,13].

Contrary to initial beliefs that premutation carriers of FraX were unaffected, there is increasing evidence that they have a variety of cognitive deficits, differences in brain anatomy and that some develop Fragile X-associated tremor/ataxia syndrome (FXTAS). For example, we reported that adult male premutation carriers display a wide range of executive function, memory, attention, language and perceptual abnormalities [14], and have reduced regional grey matter volume in a number of brain regions including the cerebellum, amygdalo-hippocampal complex, caudate nucleus, and parietal lobe [15]. FXTAS is clinically characterized by cerebellar ataxia, tremor, parkinsonism, and mild cognitive decline, and occurs in 40 % to 45 % of male premutation carriers of FraX aged 50 years or older [4,16]. Age-related cognitive decline has also been documented however in male premutation carriers over 50 years of age without FXTAS, with particular age-related deficits in executive function noted [17]. Female premutation carriers of FraX similarly demonstrate abnormalities including a mild form of the physical phenotype of FraX [12,18], elevated levels of follicle stimulating hormone [19], premature ovarian insufficiency (POI) [20,21], and increased emotional problems, with high rates of major depressive disorder [13] and some anxiety disorders, in particular panic disorder and agoraphobia [22,23]. Although less common, FXTAS has also been reported in female premutation carriers of FraX [24]. Hence premutation carriers of FraX have a wide range of physical and cognitive abnormalities, many of which are associated with or become more prominent with aging.

Differences in neuronal integrity/metabolism may help explain some of these cognitive abnormalities in premutation carriers of FraX. One technique used to measure neuronal integrity is *in vivo* proton magnetic resonance spectroscopy ( $^1\text{H-MRS}$ ) [25,26]. This provides spectra which can be used to quantify a range of brain metabolites, including N-acetyl Aspartate (NAA), Creatine + Phosphocreatine (Cr + PCr), Choline (Cho), myo-Inositol (mI), and Glutamate containing substances (Glx, which includes the combined signal from (Glutamate (Glu) and Glutamine (Gln)).

NAA is present at high concentrations in both gray and white matter. Its synthesis is closely correlated with mitochondrial energy metabolism - and so NAA is often used as a measure of neuronal density and/or mitochondrial function [26-30]. In contrast Cr + PCr and Cho are used as measures of (respectively) phosphate metabolism and membrane turnover [25,26,31-33], while mI is associated with glial cell structure and proliferation [34].

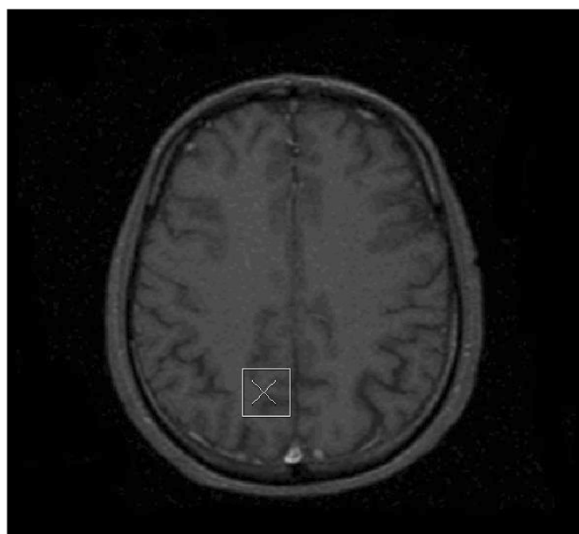
Glutamate is the major excitatory neurotransmitter and is converted into glutamine by glutamine synthetase [35]. It has been suggested that neurological and psychiatric symptoms associated with FraX may be a consequence of an exaggerated response to metabotropic glutamate receptor (mGluR) activation due to an absence/reduction of FMRP [36]. FMRP modulates dendritic maturation and synaptic plasticity and one of the mechanisms postulated for this effect is its inhibition of the metabotropic Glu receptors (mGluR), mGluR1 and mGluR5 mediated mRNA translation in dendrites [37,38]. In mouse models the mGluR5 antagonist 2-methyl-6-phenylethynyl-pyridine (MPEP) has been shown to reverse behavioral phenotypes (including hyperactivity, seizures, pre-pulse inhibition deficits, repetitive behaviors) and to lead to remarkable improvements in synaptic plasticity and spine morphology [39]. Furthermore, a recent human study investigated AFQ056, a receptor subtype-selective inhibitor of mGluR5, and noted an improvement in behavioral symptoms in 30 male individuals with FraX [40].

Hence there is increasing evidence that abnormalities in glutamatergic metabolism may underpin neurodevelopmental and/or behavioral abnormalities in people with FraX. Relatively few studies, however, have investigated neuronal integrity in premutation FraX carriers using  $^1\text{H-MRS}$  with only two case series (both consisted of two FXTAS individuals respectively) published to our knowledge [41,42]. Also nobody has reported on glutamate containing substances. In this study, we therefore used  $^1\text{H-MRS}$  to assess neuronal integrity in the parietal lobe of premutation carriers (without FXTAS) and controls. Furthermore, we investigated age-related differences in neuronal integrity between the groups. We chose the right parietal lobe (Figure 1) as our region of interest, as we have previously demonstrated developmental differences in this brain region and it provides good signal-to-noise [15]. We chose individuals without FXTAS, as FXTAS affects only some premutation carriers over 50 years of age, is associated with significant cognitive decline and hence the inclusion of these individuals may have impacted on the validity of our results.

## Method

### Subjects

We included 17 male premutation carriers of FraX (mean age  $50 \pm 15$ , range 19–70 years) who were recruited from genetic services throughout Britain (Guy's Hospital London; Kennedy-Galton Centre, Harrow, London; St. James Hospital, Leeds; Wessex Hospital, Southampton) on the basis of their genotype (that is, not phenotype). The mean, SD, and age range ( $47 \pm 17$ , range, 20–70 years) of the 16 healthy control subjects did not differ from the premutation carriers. Similarly



**Figure 1** Position of voxel in the right parietal lobe.

they did not differ in IQ or in handedness and were recruited locally through advertisement and underwent the same investigations as the premutation carriers of FraX. All participants gave informed consent and/or assent (as approved by the Institute of Psychiatry and the South London and Maudsley NHS Trust research ethics committee and the individual local research ethics committees attached to the genetic centers where subjects were recruited).

All participants in the study underwent routine blood tests and a structured physical and psychiatric examination [43,44], (for the presence of DSM-IV axis 1 or 2 disorder: [45]). Full scale intelligence quotient (IQ) was measured by the Wechsler Adult Intelligence Scale [46], and handedness was determined using Annett's questionnaire [47]. We used a variety of psychometric tests to further assess psychopathology including depression - Beck Depression Inventory (BDI-II) [48], anxiety - Beck Anxiety Inventory (BAI) [49] and the Hamilton Anxiety Scale (HAS) [50], and obsessiveness - Yale-Brown obsessive compulsive scale (Y-BOCS) [51].

Individuals were excluded if they had a history of major psychiatric disorder (for example, psychosis), autism spectrum disorder, head injury, epilepsy, toxic exposure, abnormalities in routine blood tests, drug or alcohol misuse, clinical abnormality on routine MRI, or other genetic disorders. All subjects were familiarized with the MRI scanner before imaging and no sedation was used during the scanning process.

#### Blood/cheek swab testing

Polymerase chain reaction (PCR) analysis [52], confirmed premutation FraX carrier (55–200 CGG trinucleotide repeats), and control (<55 CGG trinucleotide

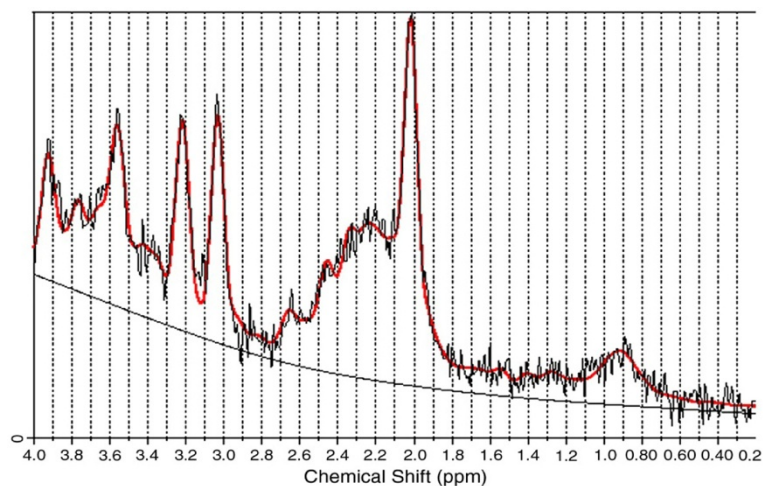
repeats) status and a 'Fragile X Size Polymorphism Assay' kit (Applied Systems) measured the precise CGG trinucleotide repeat number.

#### MRI and MRS acquisition

All 17 male premutation FraX subjects, and 16 male controls underwent MRI scanning on the same 1.5-T GE Signa NV/i MR system (General Electric, Milwaukee, WI, USA) at the Maudsley Hospital, London. A 3D fast inversion-recovery prepared spoiled gradient acquisition in the steady state (IR-FSPGR) pulse sequence was acquired from all subjects with inversion time = 450 ms, echo time = 2.8 ms, and repetition time = 13.8 ms using acquisition parameters chosen using a contrast simulation tool [53]. There were 124 contiguous coronal slices acquired with a slice thickness of 1.5 mm and a matrix of 256x256, resulting in an in-plane resolution of 0.859x0.859 mm<sup>2</sup>.

Single-voxel <sup>1</sup>H MR spectroscopy was performed in the same scanning session using a point-resolved spectroscopy (PRESS) sequence with repetition time = 3 s, echo time = 35 ms, and 160 averages with automated shimming and water suppression and excellent reproducibility [54] and used to obtain spectra from each voxel after CHESS water suppression with high signal to noise ratio and clearly resolved NAA, Cho, mI, and Cr + PCr peaks among other metabolites. MR spectra were acquired from a 6 mL volume prescribed over the right parietal lobe using co-ordinates derived from the coronal IR-FSPGR images (Figure 1). The water suppression and shimming were optimized using a standard automated pre-scan, and *in-vivo* metabolite levels for NAA, Cr + PCr, Glx, Cho, and mI were measured using LCModel software (LCModel version 6.1) [55]. Each spectra was reviewed to ensure adequate signal to noise ratio ( $\geq 6$  as determined by LCModel) and line width (maximum 10 Hz), as well as the absence of artifacts. The Cramer Rao lower bound was chosen to be < 20 to ensure a good quality fit for each peak. LCModel uses a linear combination of model spectra of metabolite solutions *in vitro* to analyze the major resonances of *in vivo* spectra. In this case, a basis set acquired on the scanner consisting of alanine, aspartate, creatine, gamma-aminobutyric acid (GABA), glutamine, glutamate, glycerophosphocholine, mI, lactate, NAA, N-acetyl-aspartylglutamate (NAAg), scyllo-inositol, and taurine, was used, together with a baseline function. Lipids and macromolecules were not estimated. An example LCModel output is given in Figure 2

To ensure that differences in tissue composition did not account for metabolic differences between subject groups the tissue composition of each <sup>1</sup>H-MRS voxel was analyzed using SPM (Statistical Parametric Mapping) software (available at <http://www.fil.ion.ucl.ac.uk>).



**Figure 2** An example of typical MR spectra from the right parietal lobe. The *in vivo* data and fitted baseline are shown in black, with the LCModel fit overlaid in red.

uk/spm) to determine the percentage of grey and white matter and CSF composition from the IR-FSPGR images within the MRS voxel. The metabolite levels were then corrected for the amount of cerebrospinal fluid in the spectroscopy voxel.

As expected, many of the metabolite peaks that were included in the LC model had a Cramer-Rao lower bound of > 20. However, NAA, Cr + PCr, Cho, mI, and

Glx all had a Cramer-Rao lower bound < 20 for the parietal voxel of interest, and concentrations were therefore derived from these metabolite peaks.

All spectroscopy analysis was carried out blind to subject status.

### Statistical analysis

#### Spectroscopy

All spectroscopy data were normally distributed. We compared mean differences in metabolite concentrations between premutation carriers of FraX and controls using independent t-tests. We also correlated (within each group) age and metabolite concentrations. We then investigated group differences in brain aging by transforming the relevant Pearson's *r* coefficient into Fisher's Z-score to test the significance of the difference between correlations, where a  $Z_{\text{observed}} \geq \pm 1.96$  is significant [56].

**Table 1** Psychometric data

	Premutation FraX Carriers (n = 17) Mean (SD)	Healthy controls (n = 16) Mean (SD)	P
Age (years)	50 (15)	47 (17)	0.547
Full Scale IQ	114 (13)	115 (17)	0.882
Verbal IQ	113 (16)	116 (21)	0.669
<i>Subcategories</i>	51 (9)	53 (14)	0.570
Vocabulary	26 (4)	26 (5)	0.947
Comprehension	21 (3)	21 (5)	0.832
Similarities	113 (12)	110 (14)	0.552
<i>Performance IQ</i>			
Block design	35.50 (8.00)	32.82 (8.34)	0.413
Object Assembly	28.81 (6.02)	25.91 (6.39)	0.249
BDI	5 (5)	5 (4)	0.746
BAI	5 (6)	3 (2)	0.276
HAS	0 (0)	0 (0)	1.000
Y-BOCS - rituals	2 (4)	2 (4)	0.910
Y-BOCS - obsessions	3 (4)	3 (4)	0.910
CGG	87 (18)	29 (6)	< 0.00001

BAI, Beck Anxiety Inventory, BDI, Beck Depression Inventory, CGG, Cytosine-guanine-guanine, HAS, Hamilton Anxiety Scale, Y-BOCS, Yale Brown Obsessive Compulsive Scale.

**Table 2** Spectroscopy data

	Premutation FraX Carriers (n = 17) Mean (SD)	Healthy controls (n = 16) Mean (SD)	P
<i>Voxel contents</i>			
Grey matter volume %	42.4 (12.8)	40.5 (7.8)	0.605
White matter volume %	52.1 (14.1)	54.1 (8.8)	0.627
CSF matter volume %	5.5 (3.0)	5.4 (1.9)	0.928
Choline (mM)	1.23 (0.16)	1.20 (0.20)	0.598
Creatine + phosphocreatinine (mM)	6.17 (0.39)	5.90 (0.41)	0.062
Myoinositol (mM)	3.90 (0.40)	3.83 (0.53)	0.665
NAA (mM)	6.21 (0.42)	6.09 (0.81)	0.600
Glx (mM)	9.95 (0.17)	9.91 (0.28)	0.430

All spectroscopy data attained from voxel in right parietal lobe. Glx, Glutamate/Glutamine; NAA, N-acetyl aspartate.

**Table 3 Correlation of brain metabolites and age in premutation FraX carriers and controls**

	Correlation with age		Difference between the two group z
	Premutation FraX Carriers (n = 17) r	Healthy controls (n = 16) r	
Choline	0.002	-0.210	0.548
Creatine + phosphocreatinine	0.274	0.077	0.530
Myoinositol	0.375	0.243	0.379
NAA	0.303	-0.412	2.345*
Glx	-0.024	-0.585	1.678

The difference in correlation co-efficients between premutation FraX carriers and controls is expressed where  $Z \geq \pm 1.96$  is significant ( $P < 0.05$ ).

**Results**

**Demographic profile**

There was no between group difference in age or IQ at the time of MRI acquisition between premutation FraX carriers and controls. Similarly no difference was noted between the groups in symptoms of depression, anxiety, or obsessive compulsive disorder (Table 1).

**Spectroscopy (Tables 2 and 3)**

There was no difference in the content of grey matter, white matter or cerebrospinal fluid (CSF) in the voxel of interest between premutation FraX individuals and controls. Similarly no difference was noted in the concentration of any of the metabolites measured.

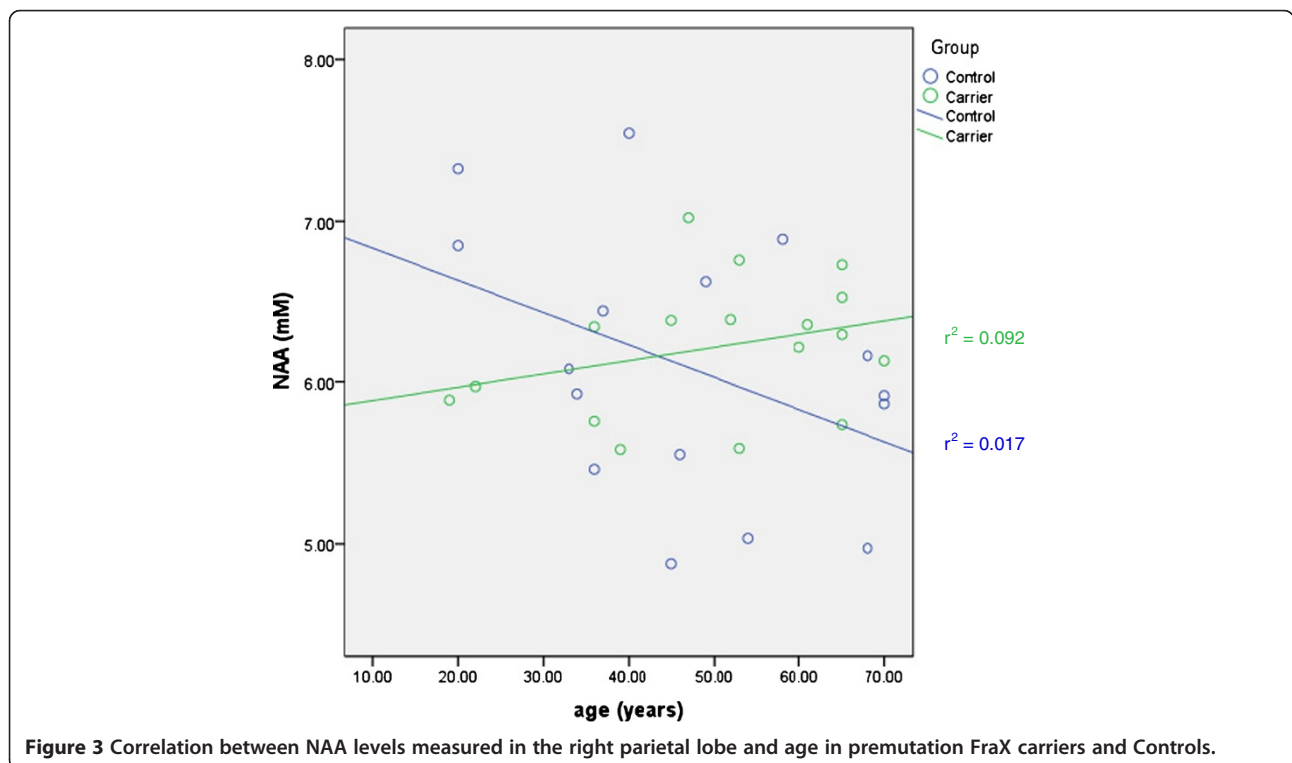
A differential increase in NAA levels with aging within premutation FraX carriers was found compared to controls ( $Z = 2.345$ ) (Figure 3). A non-significant differential increase in Glx levels with aging within premutation carriers compared to controls was noted ( $z = 1.678$ ) (Figure 4). When we examined individuals between 30 and 60 years of age only ( $n = 18$ ), this differential increase in Glx levels was significant ( $z = 2.110$ ).

**Discussion**

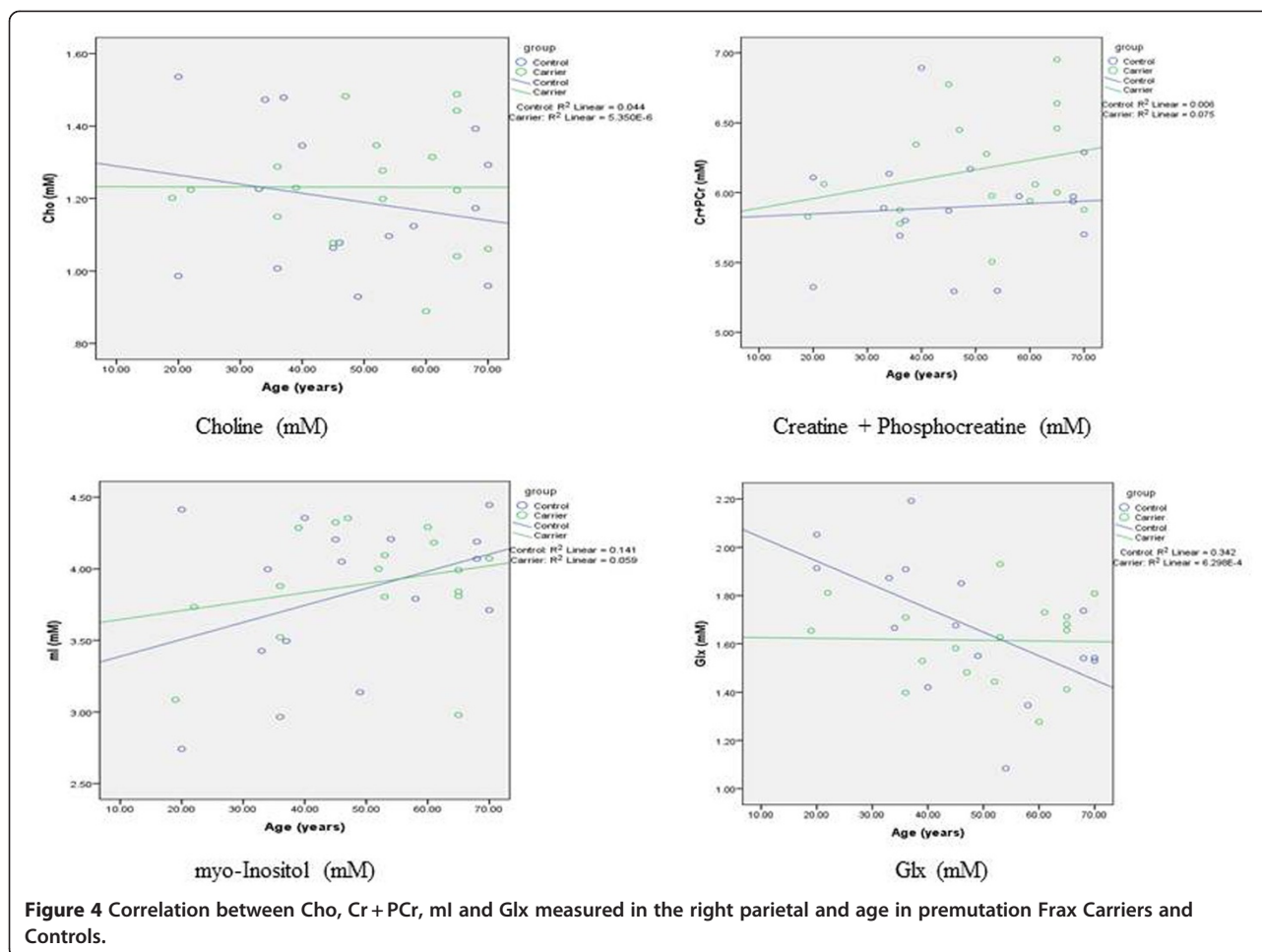
This is the first MRI spectroscopy study to compare a group of pre-mutation FraX individuals to healthy controls. We demonstrated an age-related increase in NAA in the pre-mutation FraX individuals compared to healthy controls. We found no mean difference in the concentration of any of the metabolites examined between the two groups, although this may be due to the large age ranges included in the two samples.

Two previous studies (examining four individuals in total) with FXTAS gave conflicting results in relation to NAA levels with two individuals having reduced cerebellar NAA/Cr + PCr levels and two individuals having normal NAA/Cr + PCr levels [41,42]. There have been no previous MR spectroscopy studies to our knowledge to date examining full mutation FraX individuals.

NAA is present at high concentrations in both grey and white matter and its' synthesis is closely correlated with mitochondrial energy metabolism - therefore NAA is often used as a measure of neuronal density and/or



**Figure 3 Correlation between NAA levels measured in the right parietal lobe and age in premutation FraX carriers and Controls.**



mitochondrial function [26-30]. NAA levels in healthy controls usually increase gradually with age in grey and white matter until approximately the first and third decades respectively before declining gradually thereafter [57,58]. Thus, increased NAA levels, with subsequent down-regulation, are normally associated with healthy cerebral maturation [59]. Morphological studies of healthy neural development are generally consistent with these spectroscopic findings, reporting an initial rapid overproduction of neural synapses in early to late childhood [60], with subsequent synapse elimination late in childhood and adolescence [61], followed by a slow reduction in synaptic density thereafter [61,62]. Our finding of a differential increase in NAA concentration with aging in premutation carriers of FraX is consistent with previous research suggesting an abnormal maturation process; for example increased cell packing secondary to greater synaptic density. This suggestion is supported by prior work in mice [63], *Drosophila* [64], and humans [65,66], that demonstrated synaptic alterations in the absence of FMRP. Alternatively, the greater age-related increase in NAA in premutation carriers of FraX may

reflect a hyper-metabolic state in the mitochondria with a subsequent increased risk of oxidative damage and neuronal apoptosis [67].

We detected no difference in Glx levels and found no age-related changes in these metabolites between the FraX and control groups, however we were unable to examine these metabolites (that is, Glu/Gln) individually as these can only be reliably examined at magnetic field strengths > 3 T due to the multiple overlap of the resonances at field strengths < 3 T. Therefore future studies, at higher magnetic field strengths that can examine these metabolites individually are merited. Indeed, future pharmaco-therapeutic strategies for FraX may focus on Glu, with evidence that the mGluR5 antagonist, MPEP, abolishes the audiogenic seizure phenotype in *FMR1* knock-out mice [68], decreases the mushroom body defects (fused  $\beta$ -lobes) [69], and as described above, ameliorates several clinical features in mouse models. These findings have been replicated in multiple animal models with many phenotypes and have led to several human Phase II trials that are ongoing.

In addition to a medium field strength (1.5 T), our study was also relatively small and so may not have had sufficient power to detect small group differences. However, it is the largest study to date in this population, and we have reported case-control differences in similar sample sizes in people with other neurodevelopmental disorders. We did not obtain spectra from other brain regions (due to time constraints) and thus we were unable to examine the neuronal integrity of limbic regions and cerebellum for example. Also, this is a cross-sectional study, and therefore we are only able to describe age-related differences - and not individual changes over time. Hence, there may have been undetectable age-related confounders (for example, health differences) affecting our results, although all individuals had no recent health difficulties at the time of scanning. Nevertheless, this pragmatic study design allows analysis across a wide age range (52 years), which would not be achievable in a longitudinal brain imaging study. Whilst we found no difference in NAA levels between the two groups, NAA levels varied with age in both groups and therefore it is possible that if we examined a narrower age range and had greater numbers of individuals in the study, differences in mean NAA levels may have been detected between premutation FraX individuals and controls. Consequently our finding of no difference in the levels of NAA should be interpreted with caution. Similarly, given the large age range and relatively small sample size, our findings of no difference in the levels of other metabolites should be interpreted with caution.

## Conclusion

This is the first <sup>1</sup>H-MRS study to examine premutation FraX individuals. We detected a differential increase in NAA levels with aging. This finding provides initial evidence for an abnormal maturation process in premutation FraX - perhaps reflecting increased cell packing secondary to abnormalities in synaptic pruning and synaptic density; and/or differences in mitochondrial metabolism.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

BH, ED, CM, and AS were involved in the data acquisition and image analysis. BH, ED, KM, MC, AS, and DM were involved in the study conception and design. BH, MC, ED, AS, KM, and DM, were involved in data interpretation. All authors were involved in approval of the article to be published.

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