# Establishing reliable MR spectroscopy techniques for measuring GABA and Glutathione in the human brain

Maiken Kirkegaard Brix

Thesis for the Degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
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UNIVERSITY OF BERGEN

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# Preface

The PhD started as a project derived from the Bergen fMRI Group with the title: "Cognitive and neuronal correlates of Autism Spectrum Disorder: Structural and functional MR imaging". The aim of this project was to study structural and functional changes associated with autism spectrum disorder (ASD) using multimodal magnetic resonance imaging (MRI).

After some time, I came to the understanding that the magnetic resonance spectroscopy (MRS) technique was not as straight forward as I expected it to be. This counted in particular for the more advanced γ-aminobutyric acid (GABA) edited MEscher-GArwood Point RESolved Spectroscopy (MEGA-PRESS) sequence – which had an important part in the ASD project. MEGA-PRESS with all its pitfalls and possibilities, intrigued me, and my focus started slowly shifting from a MRI/neuropsychiatric view to a more MRS/methodical view. I found that more reproducibility studies on basic MRS parameters in MEGA-PRESS were needed. The GABA edited MEGA-PRESS reproducibility study is a result of this. In addition, the increased interest in GABA edited MEGA-PRESS in our group led to a collaboration with other research cites worldwide.

After the work on GABA edited MEGA-PRESS, expanding the focus to glutathione edited MEGA-PRESS was the natural next step to pursue.

The continuation of the work in this thesis would be to bring back the optimized MRS sequences to the neurologists, the psychiatrists/psychologists and the neuroscientists for them to explore important research questions regarding disease and health. My contribution is a better way of interpreting the MRS results and communicating whether the results are reliable or not.

A few comments:

- When MRS is described, it refers to $^{1}$H brain MRS at a 3.0 T scanner if nothing else is mentioned.

- Glutathione exists in two forms, reduced (GSH) and oxidized (GSSG). When glutathione is mentioned in this work, it refers to reduced glutathione, in line with common practice in the literature.
- Most of the GSH measurements and the initial analysis in the third study of this thesis were performed during the last two weeks before Christmas 2016, hence the name: The “Christmas phantom” study.

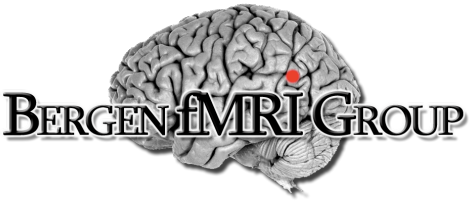
# Scientific environment

The project is derived from Bergen fMRI Group in cooperation with the Radiology Department at Haukeland University Hospital. The Bergen fMRI Group is an interdisciplinary research group at the University of Bergen and Haukeland University Hospital with interests in fMRI, MRS and related MR methods. A MRS interest group within the Bergen fMRI Group have been particularly involved in this project, with the joint leaders of the fMRI group Karsten Specth and Renate Grüner, Lars Ersland, Alex R Craven, Gerhard Dwyer, Cecilie Rygh, Marco Hirnstein, Kristiina Kampus and Kenneth Hugdahl as the most frequent participants.

International collaborators are Ralph Noeske, Senior Scientist in GE and Felix Raschke at the University of Nottingham, Division of Radiological and Imaging Science. Mark Mikkelsen and Georg Oeltzschner at the Johns Hopkins Medicine Department of Radiology and Radiological Science in Baltimore.

My main supervisor is Mona K Beyer, neuroradiologist at the Department of Radiology and Nuclear Medicine, Oslo University Hospital. Co-supervisors are Physicist Lars Ersland at the Department of Clinical Engineering, Haukeland University Hospital and professor Kenneth Hugdahl, former leader of the Bergen fMRI group.

Researchers involved to the ASD part of the project are: Child psychiatrists Maj Britt Posserud and head of Department of Clinical Psychology Åsa Hammar. They have been professional advisers on ASD, reviewing the documentation of the clinical assessment leading to an ASD diagnosis and supervising the clinical interviews. Professor Emeritus in medical microbial ecology Tore Midtvedt and leader of the BioMed Clinics Research Lab Hanne B Walker, have been deeply involved in the ASD project by recruiting children with ASD.

| | |
|---|---|
|  | Department of Clinical Medicine (K1), University of Bergen, Norway |
|  | Bergen fMRI group, Norway |
|  | Haukeland University Hospital, Bergen, Norway |

# Acknowledgements

I would like to express my sincere appreciation to those who have contributed to this thesis and supported me during the PhD program. Without your help, encouragement and flexibility, I would never have been able to finish my PhD.

The work has been performed at the Department of Radiology at Haukeland University Hospital, Bergen. The financial support, for which I am very grateful, was provided through The Western Norway Regional Health Authority and grants from the ERC to Kenneth Hugdahl.

I would like to thank my PhD advisors, Mona K Beyer, Lars Ersland and Professor Kenneth Hugdahl, for supporting me these past years.

My main supervisor, Mona K Beyer, is one of the smartest, most organized, effective and kindest person I know, and I have been extremely lucky to have a supervisor who cared so much about my work and myself. Her email response time is on average 30 min (if she is not on a plane or somewhere remote with no WiFi), and she answers all questions and queries with realistic optimism and great enthusiasm.

Lars Ersland have had the frustrating task trying to teach a computer ignorant Linux, script writing and advanced MRS analyses. I have learned so incredibly many things that I never thought that I would learn. We have also had some great trips to Cardiff, San Diego, Chicago, Honolulu etc. together, not to forget the occasional fishing trips as well. I owe to you this PhD and some buckets of coffee to go with it.

I have been very fortunate to have Kenneth Hugdahl as a co-supervisor. Kenneth Hugdahl is an immense force to be reckoned with, not only professionally for his outstanding knowledge and broad network, but also as a pleasant and enthusiastic person with the ability to move projects forward.

I also thank Alex R Craven for contributing with his brilliant ideas and elegant programming skills, often connected to his database backbone(☺), Renate Grüner for

general problem solving and efficiency and Ralph Noeske for having an answer to all my MRS questions.

I highly appreciate the management and my close colleagues at the Department of Radiology, my future workplace, and Jenny- the coolest person I know, colleagues until retirement takes us and friends for life!

I owe a lot of thanks to my co-authors on the articles included in my thesis: Gerard E Dwyer, C. John Evans, Tore Midtvedt and Hanne B Walker.

Thank you, all the radiographers contributing to the MR scanning with great care and dedication; Eva Øksnes, Turid Randa, Trond Øvreaas, Roger Barndon, Christel Jansen and Tor Fjørtoft.

A special thanks to my family for their love, help and support. Stig, my rock solid, loving husband for being so patient and for always believing in me. My two super fantastic children Ragnar and Mathilde, for all those hugs and kisses when PhD-life has treated me poorly. My parents and my sister Maja for a happy childhood, for letting me be me, and for letting your phone be a help hotline for all imaginable problems.

Last but not the least, my deepest gratitude to the participants' voluntarily taking part in the ASD and reproducibility studies. Your effort is highly valued. Without you, none of these studies would have been possible.

# Abstract

**Background:**

Proton MR spectroscopy (MRS) is a well-established method for measuring the relative concentration of a wide range of metabolites in the human brain noninvasively. Lately, more advanced spectroscopic techniques, such as MEGA-PRESS, have emerged enabling us to measure low concentrated metabolites with complex peak splitting patterns. Examples of such metabolites are the main inhibitory brain neurotransmitter, γ-aminobutyric acid (GABA), and the main brain antioxidant, glutathione (GSH). Impairment of both GABA and GSH have been implicated in the pathophysiology of several psychiatric and neurodegenerative disorders, including schizophrenia, bipolar disorder, autism spectrum disorder (ASD), multiple sclerosis, Alzheimer's disease, amyotrophic lateral sclerosis and Parkinson disease. An accurate and reliable quantification of these metabolites *in vivo* is therefore of utmost interest and clinical relevance.

The PhD started with an ASD focus, setting out to examine brain MRS measurable differences between boys with ASD and controls. The focus, however, soon shifted to the methodological aspect of MRS, with a desire to contribute in establishing reliable MRS techniques for measuring GABA and GSH in the human brain.

**Aims:**

1.) The aim of the ASD study was to explore the excitatory/inhibitory hypothesis in children with ASD by looking for imbalances in brain metabolites in boys with ASD compared with typically developing controls with standard and advanced MRS techniques.
2.) Validating GABA and GSH edited MEGA-PRESS, and comparing these sequences to the standard single voxel measurements; short TE STEAM and PRESS sequence.

**Methods:**

Four different studies were performed, all on a 3.0 T GE MRI scanner.

1. *The ASD study:* 14 boys with ASD and 24 age-matched controls were examined with both the GABA edited MEGA-PRESS and PRESS sequence. Autism symptom severity were reported by the Autism Spectrum Screening Questionnaire (ASSQ).
2. *The GABA reprod study:* Two 20 min long GABA edited MEGA-PRESS acquisition were performed in 21 healthy young male volunteers. The participants were scanned twice with identical protocols. By applying a time-windowing approach, within-and between-session reproducibility was calculated.
3. *The "Christmas phantom" study:* 122 GSH edited MEGA-PRESS and PRESS spectra of a phantom containing GSH were acquired over a time period of 11 days. The resulting decaying GSH curve (GSH oxidizes to GSSG) were modelled. A 1-year-after follow-up acquisition for both sequences was also performed.
4. *The GSH reprod study:* GSH edited MEGA-PRESS and short TE STEAM and PRESS acquisitions were performed in 36 healthy volunteers. The participants were scanned twice with identical protocols, one week apart. The time-windowing approach was applied for within- and between-session reproducibility for GSH edited MEGA-PRESS. Differences between quantified GSH levels between males and females were examined, and the three different methods of measuring GSH were evaluated.

**Main Results:**

1. There was a significant negative correlation in the ASD group between ASSQ and GABA levels, however there was no significant difference between the ASD group and the control group in MEGA-PRESS measured GABA levels.

2. Increasing the number of repetitions in GABA edited MEGA-PRESS showed improvements for within- and between-session reproducibility up to about 218 paired

repetitions (scan length ~ 13 min). Gannet combined with LCModel proved the best method processing the GABA data.

3. Both GSH edited MEGA-PRESS and PRESS were able to measure the degradation of GSH in the phantom, however the modelled GSH edited MEGA-PRESS degradation curve was more accurate than PRESS.

4. Between-session variability of GSH edited MEGA-PRESS stabilised at around 128 paired repetitions (~8 min). There were no significant correlations between GSH measured with MEGA-PRESS, STEAM and PRESS, and no differences in measured GSH levels between males and females.

**Conclusion:**

In line with other studies, the ASD participants have GABA values that seem to change with their clinical severity although there was no group difference with healthy controls. For both GABA and GSH, it is possible to acquire reproducible MEGA-PRESS measurements. GSH edited MEGA-PRESS measurements have somewhat higher coefficient of variation (meaning lower reproducibility), but stabilises at a shorter scan length than GABA edited MEGA-PRESS. MEGA-PRESS is more accurate that both PRESS and STEAM in measuring GSH for *in vivo* measurements. This is also reflected in its *in vitro* quantification, where the PRESS measurements fit of GSH seem to include oxidised GSH.

# List of publications

1. **Brix MK**, Ersland L, Hugdahl K, Grüner R, Posserud MB, Hammar Å, Craven AR, Noeske R, Evans JC, Walker HB, Midtveit T, Beyer MK: Brain MR Spectroscopy in Autism Spectrum Disorder –the GABA excitatory/inhibitory imbalance theory revisited. ***Frontiers in Human Neuroscience*** 2015. **9**: p. 365.
2. **Brix MK**, Ersland L, Hugdahl K, Dwyer GE, Grüner R, Noeske R, Beyer MK, Craven AR: Within- and between-session reproducibility of GABA measurements with MR spectroscopy. ***J Magn Reson Imaging*** 2017. **46**(2): 421-430.
3. **Brix MK**, Dwyer GE, Grüner R, Noeske R, Craven AR, Ersland L: MEGA-PRESS and PRESS measure oxidation of glutathione in a phantom. ***Submitted***
4. **Brix MK**, Craven AR, Hugdahl K, Grüner R, Noeske R, Beyer MK, Ersland L: Reproducibility of GSH measurements in MR spectroscopy. Manuscript in preparation.

# Other publications

1. Hugdahl K, Beyer MK, **Brix MK**, Ersland L: Autism Spectrum Disorder, functional MRI and MR spectroscopy: Possibilities and challenges. ***Microbial Ecology in Health & Disease*** 2012 **printed volumes 1-21**: 1651-2235
2. Randa TI, Øksnes E, **Brix MK**: Sederingsfri MR på barn med autism. Published in ***Hold Pusten*** (The scientific journal of The Norwegian Society of Radiographers) 2014.
3. Mikkelsen M, Barker P, Bhattacharyya PK, **Brix MK**, Buur PF, Cecil KM, Chan KL, Chen DYT, Craven AR, Cuypers K, Dacko M, Duncan NW, Dydak U, Edmondson DA, Ende G, Ersland L, Gao F, Greenhouse I, Harris AD, He N, Heba S, Hoggard N, Hsu T-W, Jansen JFA, Kangarlu A, Lange T, Lebel RM, Li Y, Lin C-YE, Liou J-K, Lirng J-F, Liu F, Ma R, Maes C, Moreno-Ortega M, Murray SO, Noah Sean, Noeske R, Noseworthy MD, Oeltzschner G, Prisciandaro JJ, Puts NAJ, Roberts TPL, Sack M, Sailasuta N, Saleh MG, Schallmo M-P, Simard N, Swinnen S, Tegenthoiff M, Truong P, Wang G, Wilkinson ID, Wittsack H-J, Xu H, Zhang C, Zipunnikov V, Zöllner, Edden RAE: Big GABA: Edited MR spectroscopy at 24 research sites. ***Neuroimage*** 2017 **159**: 32-45.

# ABBREVIATIONS & SYMBOLS

| Abbreviation | Description |
|---|---|
| $^{1}H$ | $^{1}$Hydrogen |
| 2HG | 2-hydroxygluterate |
| ACC | Anterior cingulate cortex |
| ADHD | Attention deficit/hyperactivity disorder |
| ADI-R | Autism Diagnostic Interview-Revised |
| ADOS | Autism Diagnostic Observation Schedule |
| ALS | Amyotrophic lateral sclerosis |
| ASD | Autism spectrum disorder |
| ASSQ | Autism Spectrum Screening Questionnaire |
| BASING | BAnd Selective INversion with Gradient Dephasing |
| BW | Bandwidth |
| CHESS | CHEmical Shift-Selective |
| Cr | Creatine |
| CRLB | Cramér-Rao lower bounds |
| CSDE | Chemical shift displacement error |
| CSF | Cerebro spinalfluid |
| CSI | Chemical shift imaging |
| DAWBA | Development and Well- Being Assessment |
| DTI | Diffusion tension imaging |
| DSM V | Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition |
| E/I | Excitatory/inhibitory |
| EEG | Electroencephalography |
| fMRI | Functional magnetic resonance imaging |
| fMRS | Functional magnetic resonance spectroscopy |
| FWHM | Full width at half maximum |
| GABA | γ-aminobutyric acid |
| GABA+ | GABA including associated macromolecules |
| GAD | Glutamic acid decarboxylase |
| Glx | Glutamate and glutamine |
| Glu | Glutamate |
| GSH | Glutathione (reduced) |
| GSSG | Glutathione (oxidized) |
| HERMES | Hadamard Encoding and Reconstruction of MEGA-Edited Spectroscopy |
| Hz | Hertz |
| i.u. | institutional units |
| M | Molar |
| MHz | Megahertz |
| MEGA-PRESS | MEscher-GArwood Point RESolved |
| min | Minutes |
| mI | Myo-inositol |

| | |
|---|---|
| ml | Milliliters |
| MM | Macromolecules |
| mm | Millimetres |
| mM | Millimolar |
| mmol/$cm^3$ | Millimole/cubic centimeter |
| MR | Magnetic Resonance |
| MRI | Magnetic Resonance Imaging |
| MRS | Magnetic resonance spectroscopy |
| msec | Milliseconds |
| NAA | N- acetylaspartate |
| NAAG | N-acetyl aspartyl glutamate |
| NMR | Nuclear magnetic resonance |
| OCD | Obsessive compulsive disorder |
| OVS | Outer volume suppression |
| ppm | Parts per million |
| PRESS | Point REsolved Spectroscopy Sequence |
| PRIAM | Parallel Reconstruction In Accelerated Multivoxel |
| PTSD | Post traumatic stress disease |
| REC | Regional Committee for Medical and Health Research Ethics |
| RF | Radiofrequency |
| ROS | Reactive oxygen species |
| SAD | Social anxiety disorder |
| sec | Seconds |
| SDQ | Strengths and Difficulties Questionnaire |
| SNR | Signal to noise |
| STEAM | STimulated Echo Acquisition Mode |
| SVS | Single voxel spectroscopy |
| T | Tesla |
| TA | Acquisition time |
| tCho | Total choline (phoshocholine and glycerophosphocholine) |
| tCr | Total creatine (creatine + phosphocreatine) |
| TE | Echo time |
| TM | Mixing time |
| TMS | Tetramethylsilane |
| tNAA | N-acetylaspartate + N-acetyl aspartyl glutamate |
| TR | Repetition time |
| VAPOR | Variable power and optimized relaxation delays |
| VOI | Volume of interest |
| WASI | Wechsler Abbreviated Scale of Intelligence |
| WET | Water excitation technique |

| Symbols | Description |
|---|---|
| $\mu$ | Magnetic moment |
| $\omega$ | Larmor frequency of sample compound (rad $s^{-1}$) |
| $\gamma$ | Gyromagnetic ration (rad $T^{-1}$ $s^{-1}$) |
| $B_0$ | Static magnetic field (T) |
| $B_1$ | Magnetic radiofrequency field (T or $\mu$T) |
| $\delta$ | Chemical shift (ppm) |
| $J$ | Scalar coupling constant (Hz) |
| $T1$ | Longitudinal relaxation time (msec or sec) |
| $T2$ | Transverse relaxation time (msec or sec) |
| $N$ | Number of … |

# 1. INTRODUCTION

## 1.1. $^{1}$H Magnetic Resonance Spectroscopy: Basic Principles

From being a radiology modality considered in demand but highly unnecessary and without any documented value by a national committee (Lønning I-rapporten) in 1987, magnetic resonance imaging (MRI) is now regarded an invaluable diagnostic tool in medicine. In 1983, 200 MRI scanners were installed worldwide, and the first MRI machine in Norway was installed in 1986. In 1999, the number of MRI scanners in Norway was 39, and today the total number is 153.

MRI magnetic field strength is measured in tesla (T). Most clinical scanners in use today have a field strength of either 1.5 T or 3.0 T. Scanners with ultrahigh field strength are available, but only used in research, where 9.4 T (1) is available for humans (Siemens is currently manufacturing a 11.7 T whole body MRI magnet) and 21.0 T (2) for animals. Using a magnet with higher magnetic field strength is an advantage in MRI due to the higher spectral resolution and the increased signal-to noise ratio (SNR). However, higher field strength gives rise to more artefacts and the technology is more expensive. Currently almost all MRI applications takes advantage of 3.0 T, and for brain imaging, 3.0 T is preferred. There are currently 22 3.0 T scanners in Norway.

***Figure 1:*** *3.0 T GE Discovery MR750 (Milwaukee, US) with a 32-channel GE head coil. (Photograph by Roger Barndon)*

Magnetic resonance spectroscopy (MRS) is a MRI technique applied both in research and as a clinical resource tool, to detect abnormalities that may or may not yet be visible on conventional MRI scans. MRS is mostly used in relation to brain examinations, but MRS has also found its appliance in the prostate, mamma and musculoskeletal system. The MRS utility in the everyday clinical practice at a Radiology Department is limited, however, in evaluating diagnosis such as brain tumours and several inherited metabolic disorders (e.g. Canavans disease), its value is indisputable. Single voxel spectroscopy (SVS), where MRS is measured from a small region of interest (ROI), typically 2 x 2 x 2 $cm^3$, is more common in clinical use than multi voxel spectroscopy. The advanced editing techniques (e.g. MEscher-GArwood Point RESolved Spectroscopy (MEGA-PRESS)) described in detail later in this thesis are not yet available in the clinic.

### 1.1.1. Nuclear Magnetic Resonance

MRI is based upon the principle of nuclear magnetic resonance (NMR). The atomic nucleus consists of neutrons and protons which hold an intrinsic property called spin. Atomic nuclei with even numbers of protons and neutrons have no observable spin, and all the other atoms with odd numbers have a non-zero spin. All molecules with a non-zero spin have a magnetic moment (μ) and are NMR-active. The magnetic moment of the nucleus makes it behave like a small bar magnet.

Examples of NMR-active nuclei are $^{1}$Hydrogen ($^{1}$H), $^{13}$Carbon, $^{31}$Phosphorus, $^{15}$Nitrogen, $^{29}$Silicon. $^{1}$H has a strong magnetic moment and also exists naturally in large amounts in biological tissue. That is why $^{1}$H is the most commonly used nucleus spin in both clinical and research MRI to generate MRI signals.

When placed in an external magnetic field, two important things happen to spins.

1) The spins precess about the magnetic field at a frequency (in the MHz range) defined by the Larmor equation.

   $\omega = \gamma B_0$

   Whereby γ is the gyromagnetic ratio (in MHz/T) and $B_0$ is the magnetic field strength (in T). The gyromagnetic ratio is a constant for a given nucleus and equals 42.6 MHz/T for hydrogen ($^{1}$H).

2) The spins align with the magnetic field, either parallel (low energy state) or antiparallel (high energy state). More precise; the presence of a strong magnetic field slightly favours the parallel spin orientation. The difference between the parallel and the antiparallel state is not that big, and the excess number of protons that align parallel within a 3.0 T field is only 9 per million. However a 2 mm x 2 mm x 5 mm voxel contains approximately $1.338 \times 10^{21}$ protons. The result is a net magnetization vector parallel to the external magnetic field.

For magnetization to generate an MR signal, it must exist in the transverse plane. Generation of transverse magnetization is accomplished by applying a second magnetic field ($B_1$) perpendicular to the system in form of a radiofrequency (RF) pulse (90°) at the Larmor frequency with a specific strength (amplitude) and for a short period of time. When magnetization is in the transverse plane, the Larmor precession creates a magnetic field that can be detected by a RF coil.

In addition to a strong magnetic field and radio wave transmitters and receivers, MR Scanners for *in vivo* human imaging also include magnetic field gradients to localize the signals in space, and computer software to process and analyse the received signals.

Traditionally MRI is primarily concerned with the acquisition of structural tissue images. Varying acquisition parameters gives different tissue contrasts reflecting the bulk magnetic properties of the tissue they contain. The most important parameters are:

- TR (repetition time): Time between excitation pulses. It reflects amount of recovery of longitudinal magnetization.
- TE (echo time): The time from the centre of the 90° pulse to the centre of the echo
- TI (inversion time): The time between the 180° inverting pulse and the 90° pulse in an inversion recovery sequence.
- Flip angle: The angle to which the net magnetization vector is rotated to the main magnetic field direction during application of a RF pulse.

### 1.1.2. Magnetic Resonance Spectroscopy

Structural MRI almost always measures water and fat signals. The amounts of water and fat in body tissue is high (in the molar (M) range), and is consequently a good signal source. MRS on the other hand, acquires signals from molecules with much lower concentration than water and fat (in the millimolar (mM) range). The signal

generated in MRS is therefore of considerably smaller amplitude and it also needs to be separated from the significantly stronger signal from water and fat.

Whereas MRI provides spatial maps or images of containing information regarding tissue density and composition, MRS is a technique that allows quantification of biochemical compounds to be performed *in vivo*. This makes MRS an important method for examining the cell metabolism of tissues and organs. Metabolites determined with MRS are present in concentrations characteristic of healthy tissue. However, stress, disorders or diseases may shift the concentration of metabolites. It is possible to display these changes, or shifts, in concentration with MRS.

*Chemical shift:*

The metabolites can be differentiated from each other since they resonate at slightly different frequencies based on their local chemical environments. The atomic nuclei are "shielded" from $B_0$ at different levels by the electron cloud surrounding them. In other words, the electron reduces the magnetic field strength as experienced by the nucleus.

To be able to compare spectra measured on MR scanners with different field-strength (e.g.: 1.5 T vs 3.0 T vs 7.0 T), the MRS frequency shift is defined relative to the signal of a reference molecule, tetramethylsilane (TMS), in parts per million (ppm). TMS has been chosen for its sharp MRS signal which does not interfere with other resonances signals. Resonance frequency depends on $B_0$, but ppm is independent of $B_0$.

The chemical shift ($\delta$) of the nuclei is defined as the difference between the Larmor frequency of the nuclei and the reference molecule TMS.

The spectral display for chemical shifts shows the frequency axis from right to left. Signals from metabolite nuclei with stronger shielding and lower resonance frequency is located to the right while signals from nuclei with weaker shielding and higher resonance frequency are located to the left (Figure 2).

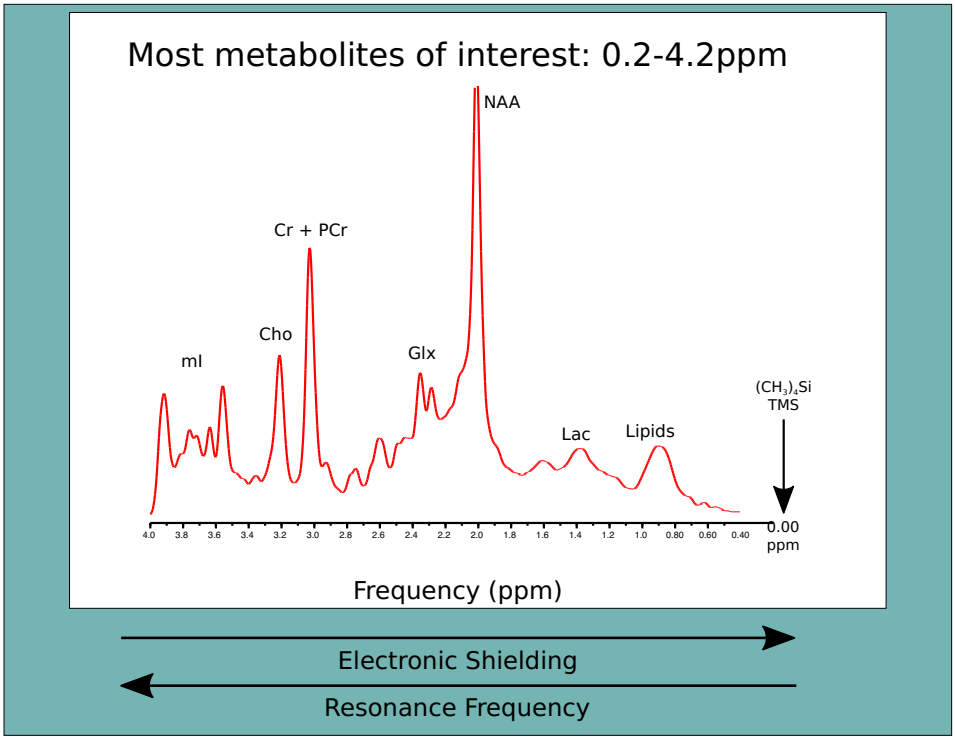



***Figure 2:*** *The figure shows the direction of the electronic shielding and the resonance frequency in ppm in a MRS spectrum.*

*Coupling:*

MR active nuclei also experience another effect; "J-coupling" also called "scalar coupling". Coupling (*J*, measured in Hz) occurs when protons within a molecule are influenced by neighbouring protons through sharing of electrons. The J-coupling effect is always mutual; if nucleus A affects the precession frequency of nucleus B through J-coupling, then nucleus B affects nucleus A.

The result is splitting of the spectral peak, and modulation of peak phase and intensity as a function of TE (J-evolution/scalar-evolution). Compared to the chemical shift, J-coupling does not depend on the strength of the external magnetic field.

*Shimming:*

Good spectroscopic results require high homogeneity of the $B_0$ field across the volume of interest (VOI) to ensure adequate spectral resolution and good SNR, and to avoid linewidth- broadening due to field inhomogeneity. For this reason, shimming of the main magnetic field is especially important in MRS experiments. Shimming involves adjusting the electrical current in shim coils making the magnetic field within the VOI as homogeneous as possible in the presence of the object of examination in the MR Scanner.

*The MRS spectrum*

The end result is the MRS spectrum which is composed of signals from multiple metabolites including broader bands resonances from lipids and macromolecules (MM), see Figure 3. Each metabolite has a unique chemical signature in the spectrum with a highly- reproducible frequency distribution. The relative areas under each peak are roughly proportional to the number of nuclei contributing to the signal.

In the most favourable case, a peak can be validly associated with an unique metabolite. However, peak area measurements in MRS are complicated by resonance overlap, baseline distortions, and non-ideal lineshapes. Measurements will also depend on factors such as relaxation times, pulse sequence used, and scanner

hardware (e.g., receiver gain, coil loading, gradient linearity).

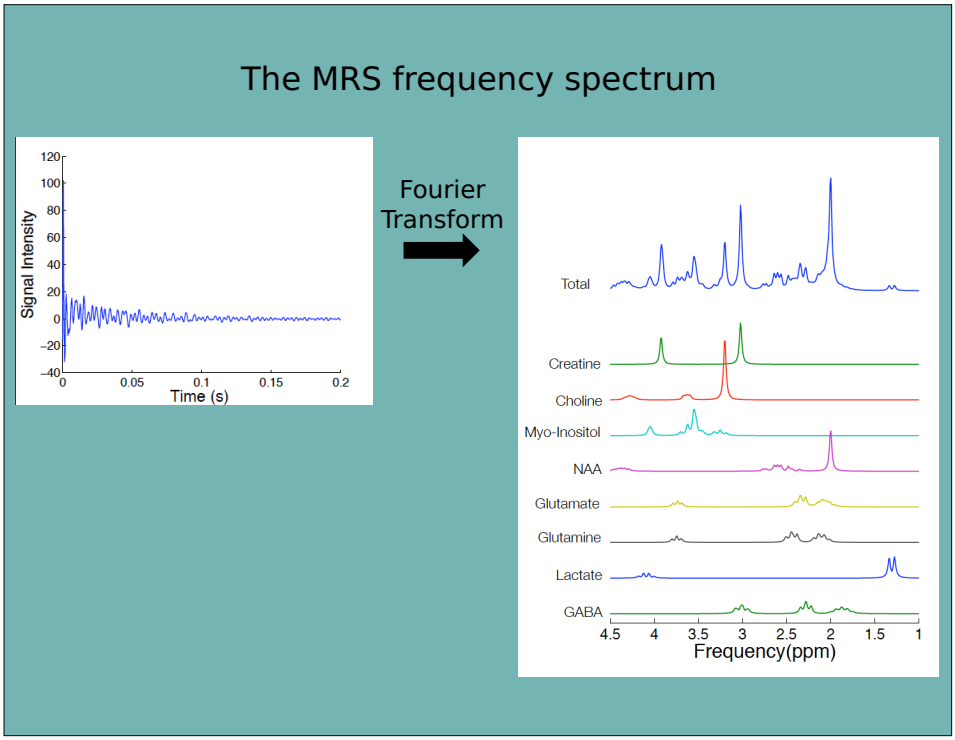



***Figure 3: MRS spectrum in the time and frequency domain.*** *From the time domain with time in sec on the x-axis and signal intensity on the y axis, the MRS spectrum undergoes Fouriers Transform and ends up in the frequency domain where frequency in ppm is on the x-axis and signal intensity is on the Y axis. The final spectrum is composed of signals from multiple metabolites, and each metabolite is identified by a unique and highly reproducible frequency distribution. (Figure with minor alterations: https://www.mc.vanderbilt.edu/documents /fmri/files/2013_Phys352A_MRS(1).pdf)*

Lengthening TE simplifies both the baseline and the pattern of peaks in the spectra. Metabolites with both short and long T2 relaxation-times are observed at short TEs (30 msec - 35 msec). At long TEs (e.g. 144 or 288 msec), only metabolites with a long T2 are seen, resulting in a spectrum with primarily n-acetylaspartate (NAA), creatine (Cr), choline (Cho) and lactate (Lac), See Table 1.

***Table 1:*** *Principal brain metabolites*

| Metabolite | | Frequency (ppm) | Short T2 | Long T2 | *In vivo* brain concentration | Role |
|---|---|---|---|---|---|---|
| **GABA** | γ-aminobutyric acid | 1.89, 2.28 & 3.01 | * | | ~ 1 mM | Intracellular neurotransmitter marker |
| **GSH** | Glutathione | 2.15, 2.55, 2.93, 2.98, 3.77& 4.56 | * | | ~ 1–3 mM | Main brain antioxidant |
| **mI** | Myoinosytol | 3.6 | * | | ~ 4-8 mM | Glial marker |
| **tCho** | Phoshocholine and glycerophospho-choline | 3.2 | * | * | ~ 1-2 mM | Cell membrane metabolism marker |
| **tCr (Cr + PCR)** | Creatine and phosphocreatine | 3.0 & 3.9 | * | * | ~ 6-12 mM | Energy metabolism marker, often served as a reference peak as it is ~ constant |
| **Glx (Glu, Gln + GABA)** | Glutamate, Glutamine GABA, | 2.1-2.5 | * | | ~ 14-16 mM | Intracellular neurotransmitter marker |
| **tNAA** | N-acetyl aspartate, and N-acetyl-aspartyl-glutamate | 2.0 | * | * | ~ 7-16mM | Healthy neuron marker |
| **Lac** | Lactate | 1.3 | * | | < 1mM | Increased with anaerobe metabolism |

### 1.1.3. Brain metabolites

Resonance frequency, *in vivo* brain concentration and role of the brain metabolites tNAA, mI, Glx, tCr, tCho and Lac are summed up in Table 1. γ-aminobutyric acid (GABA) and glutathione (GSH) are described more thoroughly below.

*GABA:*

GABA is the main inhibitory neurotransmitter in the mammalian brain and is present in more than 30-40% of all synapses; only glutamate (Glu), the main excitatory neurotransmitter, is more widely distributed (3). Healthy brain function relies on GABAergic inhibition, and understanding the role of inhibitory processes in normal brain function has become a core interest within basic and clinical neuroscience. The balance between excitatory and inhibitory (E/I) neurotransmission is tightly regulated. An imbalance in neurotransmitter excitation and inhibition appears to be involved in many of neuropsychiatric disorders such as:

- Anxiety (4)
- Obsessive compulsive disorder (OCD) (5)
- Substance addiction (6)
- Depression (7-11)
- Schizophrenia (12), and in particular auditory hallucinations (13)
- Primary insomnia (14)
- Autism spectrum disorder (ASD) (15)

And neurological diseases such as:

- Parkinson's disease (16)
- Amyotrophic lateral sclerosis (ALS) (17, 18)
- Diabetic neuropathy (19).

The GABA molecule resonate as a quintet at 1.89 ppm, and a triplet at 2.28 ppm and 3.01 ppm. It is usually the 3.01 ppm peak that is used for quantification. All three

GABA peaks are overlapped by metabolites with higher concentration, mainly Cr. Editing technique is one way of resolving the GABA signal from the overlapping Cr signal.

An additional challenge regarding conventional GABA-editing is the co-editing of a signal arising from MM at 3.0 ppm (J-coupled with a resonance at 1.7 ppm). This signal superimposes on the GABA doublet and may contribute to as much as 60% of the quantified value of GABA (20).

The concentration of GABA in the brain is relatively low, ranging from 0.5 to 1.4 mM in healthy adults (21). A common assumption is that white matter has a GABA concentration that is half that of grey matter (22). It is also assumed that GABA levels within grey or white matter do not vary across the brain - an assumption which is most likely an oversimplification (23).

*GSH:*

GSH is the major cellular antioxidant important for detoxification and elimination of environmental toxins and free radicals that may cause damage to cellular functions by oxidizing lipids, proteins and DNA. In addition, GSH also plays an important role for the immune system and in cell differentiation, proliferation and apoptosis (24-26).

Glutathione exists in two forms, reduced (GSH) and oxidized (GSSG). Oxidized glutathione is two reduced glutathione molecules bound together at the sulphur atoms.

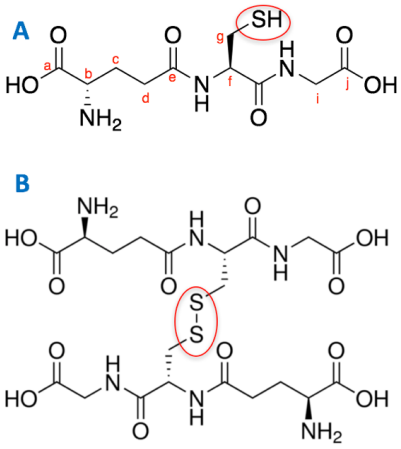



***Figure 4: Glutathione (GSH).*** *A: The reduced glutathione; GSH. The thiol group serves as an electron donor to other molecules, such as free radicals, to neutralize them. B: The oxidized glutathione; GSSG. After donating an electron- two GSH have reacted to form GSSG.*

Oxidative stress caused by a disrupted GSH antioxidant function, has been implicated in the pathophysiology of numerous psychiatric and neurodegenerative disorders, including:

- Schizophrenia (27-29)
- Bipolar disorder (29-32)
- Multiple sclerosis (33, 34)
- Alzheimer's disease (34)
- ASD (35)
- ALS (36)
- Parkinson disease (37, 38)

As for GABA, and other metabolites with coupled spin-systems, the GSH spectrum has several broad low-amplitude signals that are not fully resolved from the other metabolites in the *in vivo* spectrum. Again, spectral editing schemes can be applied. The GSH molecule resonate at 2.15 ppm, 2.55 ppm, 2.93 ppm, 2.98 ppm, 3.77 ppm

and 4.56 ppm. The concentration of GSH in the healthy brain is 1–3 mM (39), with higher levels in grey matter than white matter (40, 41).

### 1.1.4. MRS acquisition methods

A more detailed description of the MRS sequences applied in the four studies are is found in the methods section (3.1)

*Single voxel spectroscopy (SVS)*

SVS measure the MRS spectrum of a single selected small VOI. Three mutually orthogonal slices are stimulated sequentially for localisation, and the VOI is the result of the intersection of the three slices. Only the spins in this voxel are measured.

The two most common pulse sequences for excitation and localization in SVS are STimulated Echo Acquisition Mode (STEAM) and Point REsolved Spectroscopy Sequence (PRESS). The sequences differ in the radiofrequency pulses used to generate echo's (Stimulated echo in STEAM and spin echo in PRESS), while the gradient pulses applied to achieve voxel localization are the same.

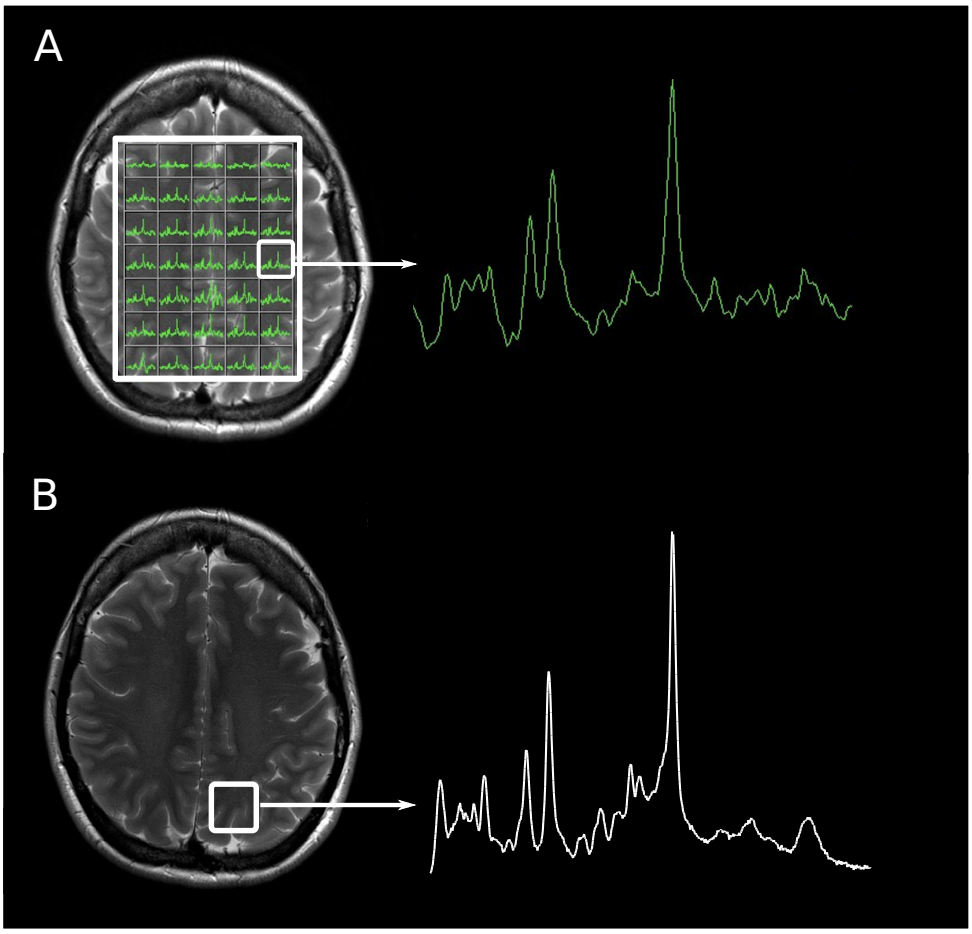



***Figure 5: Single voxel vs Multi-voxel MRS**. A: Multi-voxel chemical shift imaging (CSI): more time consuming, but better assessment of entire larger lesions and spatial resolution for smaller organs and lesions.) Not (yet) available for Glu, GABA and GSH. B: Single voxel spectroscopy (SVS): Most widely used, fast and easy, but limited application for large or inhomogeneous lesions.*

*Multi-voxel chemical shift imaging (CSI)*

CSI have a larger total coverage area and a higher spatial resolution than SVS sequences, however there are several disadvantages with this technique, including;

1) Both the pre-scan preparation for the technician and the imaging time is longer.
2) It is difficult achieving a shim that is homogenous over the entire ROI.

3) The SNR and spectral quality for each individual voxel is lower.
4) There is a substantial spectral cross voxel contamination. About 24 % (42) of the signal in every voxel in a 2D CSI acquisition derives from its neighbour voxels.
5) Editing sequences for measuring substances like GABA and GSH are yet not available for CSI.

CSI is based on a repetition SVS with added spatial phase encoding, and can be either 2D or 3D. In a true 3D CSI, the number of phase encoding steps equals the total number voxels acquired. A matrix of 8x8x8 voxels with a TR of 2.0 sec will take 2x8x8x8 = 1024 sec, or approximately 17 min to perform.

*MRS editing techniques*

There are three approaches to separate spectrally overlapping resonances:

1) Increasing the $B_0$ and with this making the signals stronger and more spread out (higher spectral resolution).
2) Adding a second frequency dimension to the MR acquisition allowing the signals more space to spread out. (2D spectroscopy techniques such as JPRESS and COSY).
3) Reducing the information content of the spectrum by editing techniques.

The third strategy is referred to as “editing” the spectrum. All editing sequences have two features in common: the localisation of signal (usually PRESS), and a mechanism for reducing the information content of the spectrum. The most common editing approaches takes advantage of known J-coupling relationships within molecules of interest to separate from the overlapping and more concentrated molecules, e.g.: MEGA-PRESS (43) and BAnd Selective INversion with Gradient Dephasing (BASING) (44).

Metabolites that can be edited include GABA, GSH, ascorbic acid, Lac, aspartate, N-acetyl aspartyl glutamate (NAAG), 2-hydroxygluterate (2HG), Glu, glycine and serine.

Recent development has seen advanced editing approaches that can separate not only one, but two or even more J-coupled metabolites simultaneously. Double MEGA-editing is such an example (45). A Hadamard encoding scheme (Hadamard Encoding and Reconstruction of MEGA-Edited Spectroscopy (HERMES)) (46) in the frequency domain allows the simultaneous acquisition of even multiple metabolites within a single editing sequence. The method has been applied for the measurement of NAA, NAAG and aspartate, (47) GABA and GSH (48). It is also possible to combine HERMES with a dual voxel excitation scheme (Parallel Reconstruction In Accelerated Multivoxel (PRIAM)) and acquire two J-coupled metabolites from two brain regions simultaneous (49) which is equivalent to a fourfold net acceleration.

## 1.2. Autism Spectrum Disorder

### 1.2.1. Background

ASD is a developmental disorder defined by impaired social communication and interaction, and by restricted, repetitive patterns of behaviour, interests or activities (50). The ASD phenotype comprises a spectrum of symptoms varying in their presentation, the course and outcome, and in their responses to therapy. The estimated prevalence of ASD in the Norwegian population ranges from 0.44 – 0.87% (51, 52) with 4-5 times as many boys as girls diagnosed with the disorder (53).

No single cause has been identified in ASD, and, given the complexity of the disorder and the wide variety of symptoms and the varying severity, there are probably many interacting causes. What we do know for certain is that ASD is highly heritable. The relative risk of a second child having this diagnosis is 20-50 times higher than the population based rate (54). For ASD in general, from twin and family studies, the heritability estimates have been determined to be around 90% (55). Parents and siblings often show mild manifestations of ASD (~ 20-45%), including delayed

language, difficulties with social aspects of language, delayed social development, absence of close friendships, and a perfectionist or rigid personality style (56).

Children with ASD show less attention to social stimuli, smile less and have less eye contact. They often fail to develop age-appropriate peer relationships, and struggle with the specific characteristics of social boundaries that are mostly invisible and context dependent. Another symptom is extreme resistance to change. Children with ASD usually prefer established behaviour and set environments. As a reaction to stress they can show a wide range of stereotypic behaviours and movements. The unusual social development normally becomes apparent early in childhood and clinical signs are usually present by age 3 years (50).

There are a number of comorbidities and symptom complexes found in patients with ASD, and these create an extra challenge for individuals with ASD and their families, educators, and health professionals.

A study by Gjevik et al (57) examining 71 6- to 18-year old Norwegian children with ASD for other child psychiatric disorders found that 72 % of participants had at least one comorbid disorder, and 41 % had two or more. The most common overlaps were for anxiety disorders diagnosed in 42 % of the children and attention-deficit/hyperactivity disorder (ADHD) diagnosed in 31 % of the children. (Anxiety disorder including specific phobias, social phobia and obsessive-compulsive disorder). Depressive disorder was diagnosed in 10 % of the children and tics were diagnosed in 11 % of the children. These results are consistent with other similar studies (58). The question is whether these comorbidities are true comorbidities, or just a part of the ASD syndrome.

An estimated 30 % of ASD patients also have epilepsy, and as many as 85 % of children with ASD have subclinical epileptiform activity recorded on scalp electroencephalography (EEG) (59, 60).

There are no definitive biological markers of ASD and the diagnosis is based on the assessment of behaviour patterns and the observations of the child’s expressions and

demeanour by applying different mapping tools. Examples of such tools are the Autism Diagnostic Observation Schedule (ADOS) and the Autism Diagnostic Interview-Revised (ADI-R). In Norway, the diagnostic evaluation is done by a multidisciplinary team consisting amongst others psychologist and psychiatrist.

ASD is considered a lifelong chronic disorder, and there exists no known cure. At present, the main goal for treatment is to reduce associated clinical and cognitive impairments in order to lessen family stress and maximize the individuals ability to function in its environment (61).

### 1.2.2. ASD & MRS

Despite the promise of MRS in understanding the pathophysiology of ASD through measuring *in vivo* biochemical and metabolite concentration levels, the scientific literature in this area is replete with contradictory results. A summary of the main findings in the ASD & MRS literature with focus on GABA, Glu and GSH follows.

*GABA and Glu*

GABA and Glu MRS applications have been directed towards addressing the hyper-glutaminergic (62) or hypo-GABAergic (63) dysfunction hypothesis of ASD.

The literature indicates that an E/I imbalance in persons with ASD could lead to excessive stimulation of the glutamate system resulting in “excitotoxicity" in regions of the brain responsible for language, social interaction, and multisensory perception (64, 65). The increased prevalence of seizure disorders in ASD could also be explained by such neural hyper excitability (59).

Several studies provide evidence for cellular abnormalities that may contribute to E/I imbalance in ASD; animal models (66-68), post-mortem studies (69-75) and studies of gene function and expression (54, 76, 77).

The signals from Glu are difficult to distinguish from glutamine even at high-field scanners, short TE and long acquisition time, and different editing techniques, thus

the two metabolites are usually measured and quantified together as Glx (78). (Included in the Glx signal is also minor contributions from GABA and GSH). There are no compelling evidence in support of changed Glx levels in ASD, both higher and lower Glx levels have been reported (79). However, in older groups with ASD, Glx levels tend to be increased, while younger groups with ASD tend to have reduced Glx or no changes (80).

Four studies (not including the present study in the thesis) have been published measuring GABA levels in children with ASD at 3.0 T applying the MEGA-PRESS editing technique. Reduced GABA/tCr concentration in ASD compared to typically developing controls (TD) (81-84) were reported in the following ROIs; frontal lobe, left motor cortex, left auditory cortex and right sensorimotor cortex, and no significant difference in GABA/tCr were found in ROIs in the left auditory cortex and the middle occipital cortex (82, 84).

*GSH:*

Another pathophysiological hypothesis of ASD is the redox/methylation theory suggesting an imbalance between oxidative stress and the antioxidant system leading to neuronal damage in genetically predisposed individuals (85). Disturbance in GSH homeostasis has been found in other psychiatric and neurological disorders, including schizophrenia (29, 86), bipolar disorder (87), Parkinson's disease and Alzheimer's disease (88, 89). The redox/methylation theory is supported by reduced glutathione-mediated redox status in blood samples (90-94) and post-mortem findings (35, 95) in individuals with ASD.

To this date, only two MRS studies measuring GSH with adequate techniques have been published (35, 96). One study found reduced GSH levels in the cerebellum and temporal cortex of the brains of individuals with ASD compared with age-matched control subjects, with no differences in the frontal, parietal and occipital cortices (35). While the other study found no group differences in GSH concentrations in either the dorsolateral prefrontal cortex and the dorsal ACC (96). Two other studies, Durieux 2015 (97) and Endres 2017 (96), found no group differences in GSH concentrations

in either the basal ganglia, the dorsomedial prefrontal cortex, the dorsolateral prefrontal cortex and the ACC. However, the MRS data in these studies were acquired with a simple PRESS sequence which is probably not adequate for measuring GSH, and the results are most likely not to be trusted.

*tNAA, tCr, tCho, mI:*

Several studies have shown decreased tNAA, tCr, tCho and mI levels (98). There is however inconsistency in the literature and the results vary. Some studies even found a substantial increase in the above-mentioned metabolites. Widespread reduction of tNAA, tCr, tCho and mI in ASD may reflect dysfunction, loss or immaturity of neurons (99) and glial cells (100) and reduced axon density (101).

It should be mentioned that a field-strength of 1.5 T has been applied in 80 % of these MRS investigations. MR systems at 3.0 T have several advantages over 1.5 T systems in MRS when it comes to increased SNR and better spectral resolution, and some challenges such as larger chemical shift and higher sensitivity to magnetic susceptibility effects. These differences make it difficult for a direct comparison between results from a 1.5 T and a 3.0 T scanner system.

# 2. SPECIFIC BACKGROUND AND AIMS OF THE STUDY

## 2.1. Specific background

Neurological and psychiatric disorders are now recognised as leading causes of morbidity and disability, and rank among the most costly disorders to affect humans (102). The underlying mechanisms of many of these disorders are largely unknown, and identifying the underlying pathophysiology is imperative and may lead to major health benefits. Evidence implicate that an imbalance in excitatory Glu and inhibitory GABA activity in the brain may be a shared pathophysiological mechanism in disorders, such as ASD and schizophrenia. Another metabolite that has come in focus lately is the powerful brain antioxidant GSH. A decrease in cell GSH levels results in increased vulnerability to oxidative stress, and is thought to be implicated in the aetiology of inflammatory, immune- and neurodegenerative diseases. The advanced MRS technique MEGA-PRESS provides the opportunity to investigate both GABA and GSH levels non-invasively in the brain. The same MRS technique may also be applied for the measurements of Lac and 2HG – metabolites that are highly interesting in clinical settings.

It is necessary to validate the MEGA-PRESS editing technique for both GABA and GSH to be able to trust the measurements we achieve. This has not always been done adequately in the past years and the results which we refer from previous research are therefore potentially not reliable. Unvalidated MRS techniques will contribute to MRS's poor reputation by some clinicians, and might even prolong the time until some of these advanced MRS techniques are introduced to clinical practice.

This project involves characterisation of neurochemical changes associated with ASD, by applying the GABA edited MEGA-PRESS technique. In addition, reproducibility studies of GABA and GSH using the same technique have been performed for the value of future studies. The projects also include phantom studies for validating data acquisition and data analysing. MRS processing and analysing

tools have been developed and improved parallel to the project both within the fMRI group but also with international co-operators.

## 2.2. Aims of the study

**PAPER I:** GABA edited MEGA-PRESS in ASD;

In the first publication, MRS was applied to children with ASD and healthy controls using the PRESS and MEGA-PRESS sequences in a voxel placed in the left ACC. The aim was to look for imbalances in metabolites in the ASD group and to replicate previously published results regarding the Glu/GABA E/I imbalance theory.

**PAPER II:** GABA edited MEGA-PRESS reproducibility;

In the second study, the aim was to validate GABA edited MEGA-PRESS results in a replication-study in a group of healthy adult volunteers. The reliability of metabolite concentration estimates was systematically examined both within- and between-measurements by increasing scan time. The effect on reproducibility of voxel placement and application of different analysing tools were also tested.

**PAPER III:** The GSH "Christmas phantom" study;

In the third study, the aim was to measure changes in GSH concentration using the PRESS and MEGA-PRESS sequences by tracking the natural oxidation of GSH in a phantom. The accuracy of the two sequences would also be evaluated.

**PAPER IV:** GSH edited MEGA-PRESS reproducibility;

In the fourth study, the aim was to validate GSH edited MEGA-PRESS by a similar fashion as the GABA reproducibility study. A short TE PRESS and STEAM sequence were also included to look at how well GSH measurements from the three MRS techniques agree. An additional element in this study is that both males and females were included in order to look at gender differences.

# 3. MATERIALS AND METHODS

The different MRS techniques applied in the four papers are described in details in section 3.1. Lipid suppression, water suppression, data pre-processing and spectrum quality control are also addressed in this section. Section 3.2 contains a description of participants and data collection for the four studies including an account of the MR protocol. Section 3.3 describes the statistical methods used.

## 3.1. MRS

*PRESS*

The single voxel PRESS sequence uses one slice selective 90˚ pulse followed by two slice-selective 180˚ pulses to create a spin echo.

The pulse sequence structure in the PRESS sequence results in relative high minimum achievable TE, since multiple RF-pulses and waiting for the spin echo's takes time. TEs of 30-35 msec are routinely used in PRESS. Long TEs is, however, applicable for measuring metabolites with a long TE, and TE of 144 msec is particularly useful as it inverts Lac at 1.3 ppm.

There exists a potential for tissue heating in the PRESS sequence. This is caused by the multiple 180º-pulses which in some instances might exceed the specific absorption rate (SAR) limit.

A standard PRESS sequence (TE = 35 msec) was included in the ASD study to measure the metabolites tNAA, tCr, tCho and mI. PRESS (TE = 30 msec) was also included in the two GSH- studies to test the accuracy and reproducibility of PRESS measured GSH values.

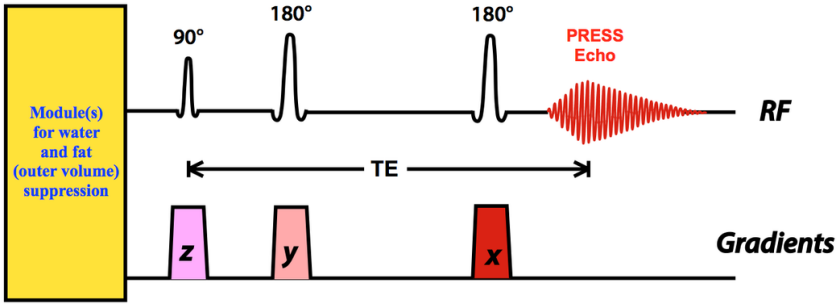



***Figure 6:*** *The PRESS sequence. The PRESS sequence consists of a slice selective 90° excitation pulse and two 180° refocusing pulses. The time interval between the two 180° pulses are TE/2. The time between the 90° pulse and the first 180° pulse as short as possible, but sufficiently long to allow the gradient pulses to be played put. RF = Radio Frequency pulse, x= gradient in x-plane, y = gradient in y-plane and z = gradient in z-plane. Courtesy of Allen D. Elster, MRIquestions.com.*

*STEAM*

The single voxel STEAM sequence consists of three selective 90° pulses to produce a stimulated echo. STEAM allows for short TEs (down to ~ 7 msec) making it possible to detect short T2 metabolites such as Glu, GSH and mI. The use of 90˚ pulses allows for sharper slice profile, higher bandwidth, and lower tissue energy deposition. However, the STEAM signal is only 50 % as large as for PRESS.

In the GSH reproducibility study, a short TE STEAM sequence (TE = 7 msec) was applied in order to examine the GSH reproducibility compared to a MEGA-PRESS and PRESS.

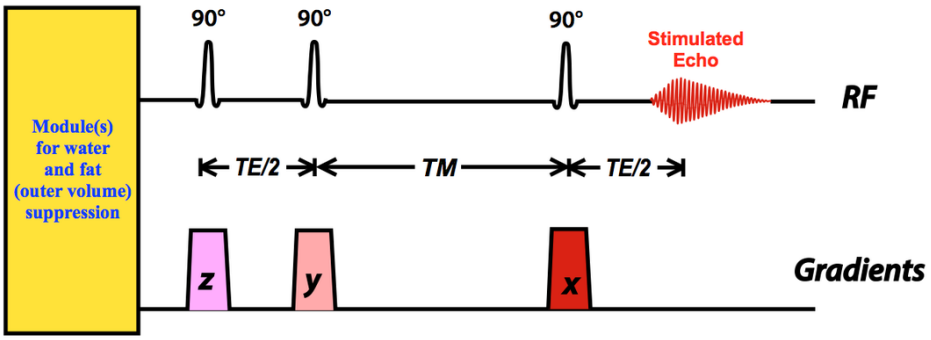



***Figure** 7: The STEAM sequence. The STEAM sequence consists of three 90° slice selective pulses. The interval between the second and third pulses, mixing time (TM), is kept to a minimum. T2 decay does not occur during TM, and TM is therefore not included in TE. RF = Radio Frequency pulse, x= gradient in x-plane, y = gradient in y-plane and z = gradient in z-plane. Courtesy of Allen D. Elster, MRIquestions.com.*

The main difference between STEAM and PRESS is that the spin-echo-based PRESS sequence has twice the SNR, while STEAM has the ability to obtain shorter TEs (103). Short TE STEAM is preferable for observing resonances with shorter T2s, while long TE PRESS (with its superior SNR) should be used for resonances with longer T2s (such as tCho, tCr, tNAA and Lac).

*MEGA-PRESS*

As mentioned in section 1.1.3, MRS signals from molecules such as GABA and GSH are overlapping and obstructed in conventional brain MRS by signals from other compounds which are present at much higher concentrations. In order to obtain signals from GABA and GSH, the spectral editing technique of MEGA-PRESS can be applied. The method takes advantage of J-coupling properties in the metabolite of interest and suppresses signals from unwanted compounds. A description of GABA and GSH edited MEGA-PRESS follows, see also Figure 8.

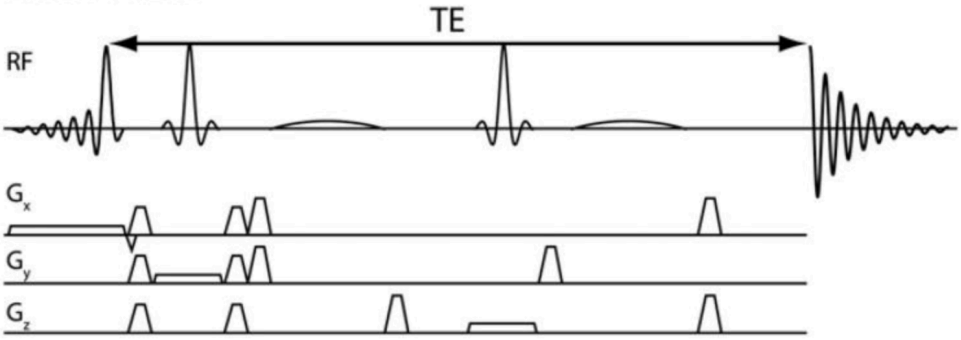



***Figure 8: The MEGA-PRESS sequence:*** *The MEGA-PRESS sequence consists of the acquisition of two datasets ("ON" and "OFF"). Two frequency-selective editing pulses are incorporated into a single voxel PRESS sequence. For the "ON" spectra the editing pulses are applied at 1.9 ppm for GABA editing and 4.56 ppm for GSH editing, while for the "OFF" spectra the editing pulses are omitted (or more commonly, applied at a frequency that has no impact on the GABA or GSH signals. (Mullins et al., 2014 NeuroImage, reprinted with permission.)*

*GABA*

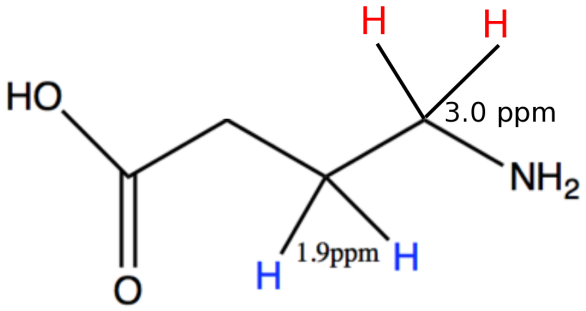



***Figure 9: The GABA molecule***

In GABA edited MEGA-PRESS, the editing inversion pulses are applied to the C-3 protons of GABA at 1.9 ppm. Due to the J-coupling the C-4 protons of GABA at 3.02 ppm are affected while other metabolite peaks like the strong Cr peak remain unaffected. This is called the EDIT-ON spectra. In a second acquisition scheme called EDIT-OFF, the same pulse is applied symmetrically to the other side of the

water peak at 7.5 ppm (4,7 ppm + (4.7 ppm – 1.9 ppm)), this is done to reduce baseline artefacts in the edited spectrum. The inversion pulse in the EDIT-OFF spectra does not affect the J-coupling in the GABA molecule, and the J-coupling evolves freely throughout the TE. EDIT-ON and EDIT-OFF spectra are acquired in an interleaved fashion several times, a total of 256 spectra (128 EDIT-ON spectra and 128 EDIT-OFF spectra) or more is common.

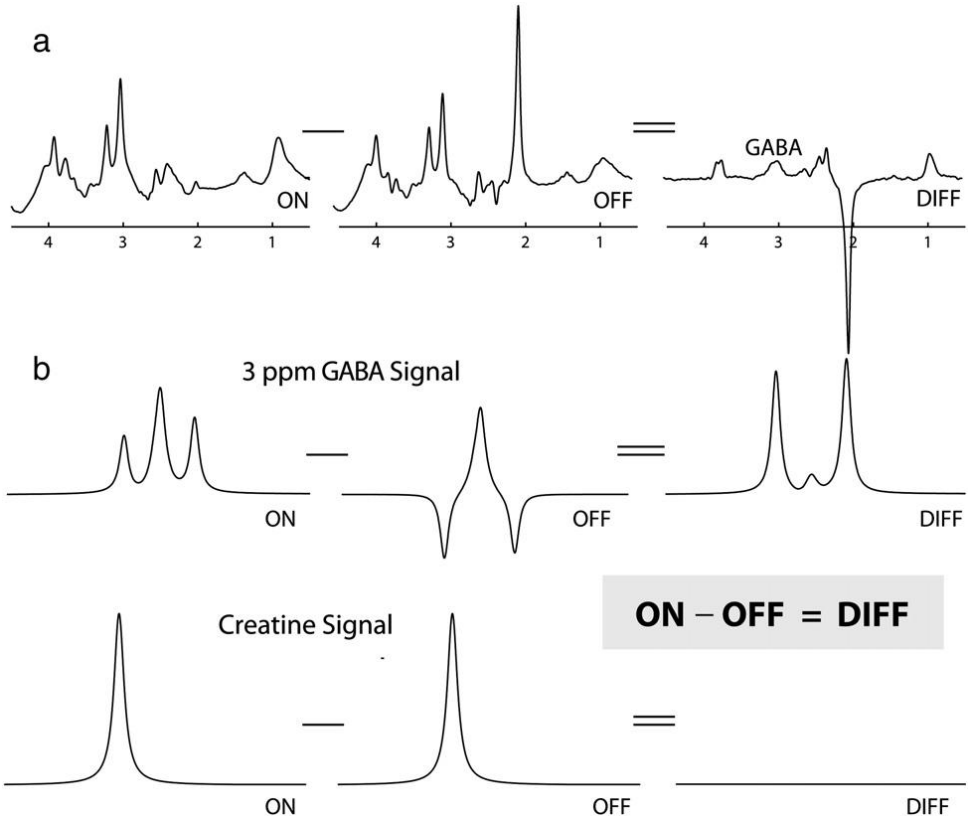



***Figure 10:*** *Schematic diagram of MEGA-PRESS editing for GABA* ***(a)*** *Editing pulses applied at 1.9 ppm modulate the shape of the GABA signals at 3.0 ppm* ***(b)****. Subtracting scans acquired without these pulses (labelled OFF) from scans acquired with the editing pulses (ON) removes overlying Cr signals from the edited spectrum, revealing the GABA signal in the difference spectrum (labelled DIFF).* ***(b)*** *The effect of editing pulses on signals at 3 ppm signal is shown. (Mullins et al., 2014 NeuroImage, reprinted with permission.)*

The EDIT-ON and EDIT-OFF spectra are then summed up, and the resulting difference spectra is derived from the difference between the two. The difference

spectrum does not have the strong singlet signal from Cr, allowing quantification of the GABA peak at 3.02 ppm. Important to notice is that this peak also includes signals from coedited macromolecules (MM) and the measured signal is therefore considered as GABA+ (GABA + MM).

*GSH*

In GSH edited MEGA-PRESS, the editing pulses in EDIT-ON are applied to the cysteine alpha proton at 4.56 ppm. The observed signal due to J-coupling originates from the beta protons at 2.93 ppm and 2.97 ppm.

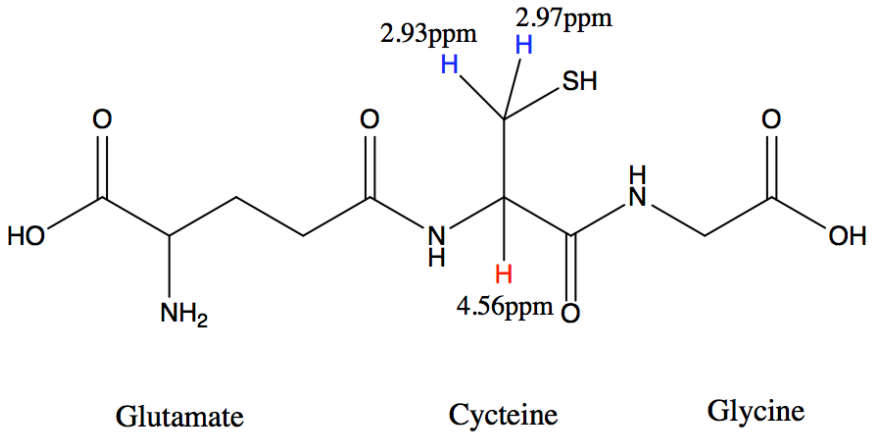



***Figure 11: The GSH molecule***

In GSH edited MEGA-PRESS, the editing pulse is very close to water (editing pulse: 4.56 ppm, water peak: 4.68 ppm), so applying the EDIT-OFF pulse symmetric to water gives little meaning, and could even partly affect the cysteine alpha proton at 4.56 ppm and ruin the difference spectra. In the GSH reproducibility study – the editing pulse in the OFF spectra was set to 20 ppm- and by this not affecting any of the metabolites we are measuring.

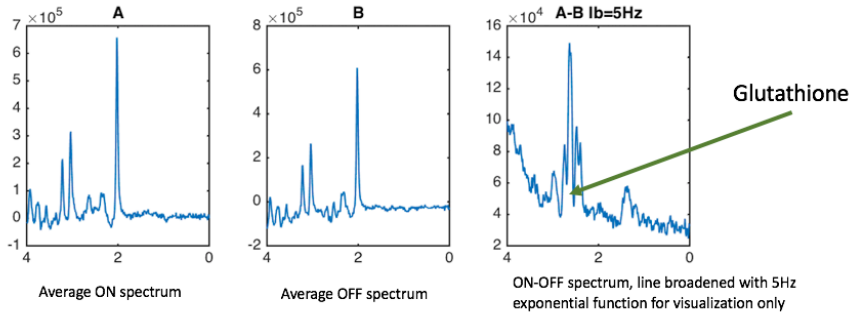



***Figure 12: GSH edited MEGA-PRESS:*** *Average ON spectrum minus average OFF spectrum gives a difference spectrum with a GSH peak ~ 3.0 ppm. The difference spectrum has been line broadened with a 5 kHz exponential function for visualization only.*

The ON and OFF spectra are generally collected interleaved to limit the impact of participant and hardware instabilities. The editing efficiency is a measure of how much of the theoretically observable signal can be measured. The editing efficiency for GABA edited MEGA-PRESS at TE 68 msec is about 0.4 - 0.5 (22, 104) and close to 0.74 (105) for GSH edited MEGA-PRESS at TE 131 msec.

*Lipid suppression:*

Fat from voxels obtained near the scalp tissue can distort the baseline of the MR spectra so that metabolites cannot be detected. Lipids resonate at 1.3 ppm.

The most common method to eliminate unwanted fat signal is using outer volume suppression (OVS) techniques. OVS bands utilizes spatially (but not frequency) selective pulses and dephasing gradients to reduce or eliminate signals from all tissues (not just lipids). OVS bands are usually sufficient lipid suppression for brain MRS.

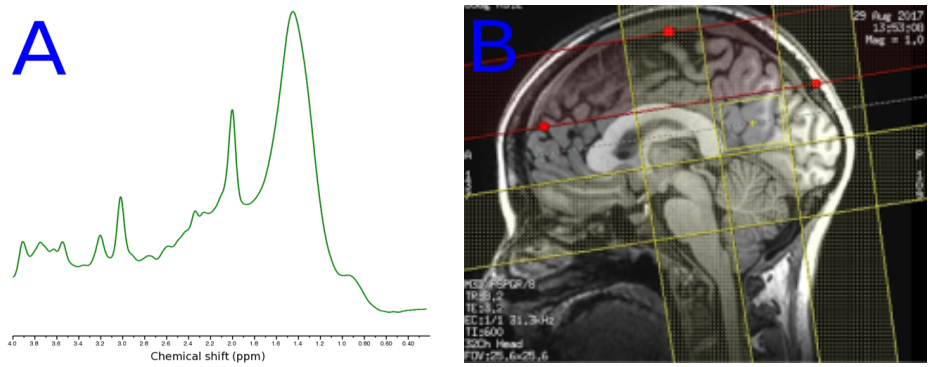



***Figure 13: (A) MRS spectrum with lipid contamination & (B) OVS bands:*** *(A) A short TE PRESS spectra with severe lipid contamination. Head movement has caused the voxel to partly cover a region with lipids. (B) An illustratory example of how OVS bands can be applied for lipid suppression in single voxel spectroscopy.*

*Water suppression*

The most commonly used method for water suppression is to apply water peak frequency selective RF pulses prior to MRS signal excitation to minimize the longitudinal magnetization of water at the time of excitation. CHEmical Shift-Selective (CHESS) (106) RF pulses tip the longitudinal magnetization of water into the transverse plane, where it gets dephased by a gradient crusher while the magnetization of other frequencies remains unaffected. Multiple CHESS pulses with optimized flip angles and delays can be used to give good water suppression over a range of $B_1$ field strengths ($B_1$ inhomogeneity) values and water T1 relaxation times (which is important for the suppression of both brain water and cerebrospinal fluid (CSF)).

Other examples of water suppression  are the Water suppression Enhanced through T1 effects (WET) (107) scheme, which employs three to four CHESS pulses of optimized flip angle and the VAriable POwer and optimized Relaxation delays (VAPOR) (108) scheme that uses six to eight CHESS pulses of variable flip angle and timing.

Water suppression in STEAM, PRESS and MEGA-PRESS sequences in GE systems is currently done with three CHESS pulses at three different flip angels. (Default flip angles: 105°, 80°, 145°.)

Averaging is a process of repeating the sequence of RF pulses, gradient pulses and the signal acquisition a specified number of times, in order to add more signal. Adding signal increases the SNR in proportion to √N where N is the number of repetitions that are averaged. A substantial number of repetitions, typically 64, 96, 128, 192, 256 or more, is necessary to obtain a high quality averaged spectrum. Several different expressions have been used in the MRS literature to describe the number of averages applied in the acquisition of the MRS spectra. Scans, averages, sums of averages, sums of signals, repetitions all mean the same thing. In sequences like PRESS and STEAM, the nomenclature is fairly straightforward. In MEGA-PRESS, however, it is important to specify whether one means e.g. 256 ON + 256 OFF repetitions or 256 repetitions in total.

*Data pre-processing and spectrum quality control*

Data pre-processing in MRS include:

- Coil combination (phase correct and combine data from the individual coils from either the 8- or the 32- channel phased array receive head coil).
- Automatic frequency and phase correction of the single-scan data.
- Water removal.
- Zero-filling (to improve resolution).
- Elimination of residual eddy current effects (109).
- Applying an apodization filter (exponential line broadening) to remove noise from the detector channels.

Some steps are done in the time domain while others are done in the frequency domain, and this varies in the different software packages.

If LCModel is to be used for analysis, the apodization filter should not be applied and

the data needs to be in the time domain. Fourier's transformation and the reverse Fourier's transformation can be applied to transform between the frequency and the time domain data.

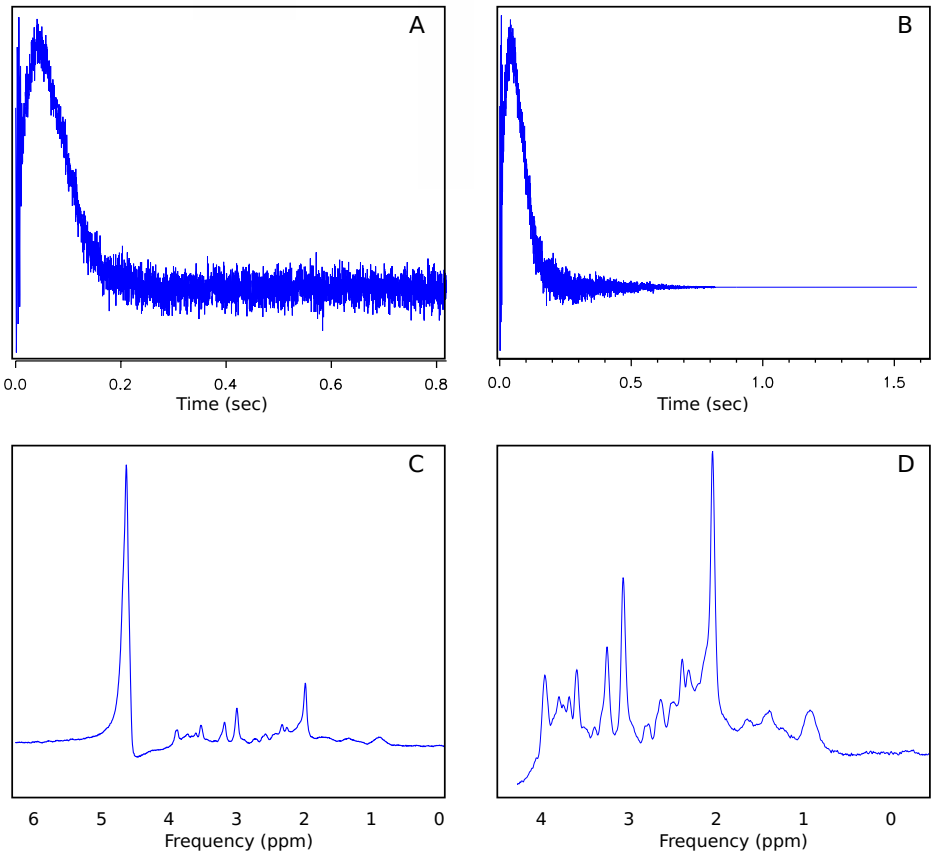



***Figure 43: MRS Pre-processing*****:** *(A) Raw time domain data. (B) Time domain data after zerofilling and apodization. (C) Data in the frequency domain, a quite solid water peak remains even after water suppressed MRS acquisitions. (D) The finally processed MRS spectrum in the frequency domain.*

Data are visually inspected for spectral artefacts (e.g. non-constant baselines and subtraction errors in MEGA-PRESS,) and unwanted signals (especially lipid contamination). Wide peak shapes reflect poor shimming or patient motion. There should be no trouble separating the main Cho (at 3.2 ppm) and Cr (at 3.0 ppm) peaks.

*Quality parameters for data acquisition are:*

Signal-to-noise ratio (SNR): The amplitude of the metabolite over the amplitude of the noise. The minimum acceptable SNR in an *in vivo* spectrum is usually set to 10. The different analysing packages have different approaches on defining the noise signal. In Gannet, the modelled tNAA signal in the averaged OFF spectrum is divided by the SD of the detrended noise signal between 10 and 11 ppm.

Spectral linewidth: How broad the peaks are in the resulting spectra- the full-width half-maximum (FWHM). After shimming, a spectral line width FWHM < 8-12 Hz is considered acceptable values in *in vivo* spectrum depending on the region examined. The different software packages have different approaches also here. In Gannet, the spectral linewidth from the modelled unsuppressed water signal or a modelled metabolite (tCr/tNAA/GABA) signal are measured directly.

*Estimation of fitting reliability are:*

The appearance of the residuals, the quality of the baseline fit and % Cramer-Rao Lower Bound (CRLB) are estimates on how well the MR spectra are fitted. CRLB are error estimates of the quantitative fitting procedure and includes the effects of the SNR and the inherent limits of the fitting model. CRLB < 20 % is considered acceptable.

## 3.2. Participants and Data Collection

### 3.2.1. PAPER I: GABA edited MEGA-PRESS in ASD

*Participants*

The study was approved by the Regional Committee for Medical and Health Research Ethics in Western Norway (REK 2011/565). Written informed consent was retrieved from the boy's parents or legal representatives.

Initially, 20 boys fulfilling the ASD diagnosis criteria according to the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-V) criteria (110) were

included. 30 age matched controls (boys) were recruited from the National Registry as control participants.

Trained psychology students assessed all participants with the two sub-test of the Wechsler Abbreviated Scale of Intelligence (WASI) (111) . The ASD section of the structured interview questionnaire Development and Well- Being Assessment (DAWBA) was performed in order to exclude presence of ASD symptoms in the TD group (112). A mental health screening questionnaire from a local Child Study including the Strengths and Difficulties Questionnaire (SDQ) (113) and the Autism Spectrum Screening Questionnaire (ASSQ) (114) were also included.

Exclusion criteria in both groups were braces (for the MR investigation), genetic abnormalities and gestation age <36 weeks. In addition, epilepsy and autistic traits and other neuropsychiatric disorders were exclusion criteria in the TD group.

Acceptable MEGA-PRESS/ PRESS spectra were obtained in 14 boys in the ASD group and 21/24 boys in the TD group. (Six boys in each group were excluded for various reasons. See the Paper I for more details.)

See Table 2 for a description of the participants, including age, and ASSQ-score. (ASSQ-score is only applicable for the ASD group.)

*Table 2: Demographics*

| | | *N* | *Mean age ± SD* | *ASSQ-score* |
|---|---|---|---|---|
| *ASD* | PRESS | 14 | 10.2 ± 1.9 | 23.5 ± 8.4 |
| | MEGA-PRESS | 14 | 10.2 ± 1.9 | 23.5 ± 8.4 |
| *TD* | PRESS | 24 | 10.2 ± 1.8 | NA |
| | MEGA-PRESS | 21 | 10.2 ± 1.8 | NA |

*MR protocol:*

Brain MRI scans were acquired using a 3.0 T GE Signa HDXt MR scanner (Milwaukee, USA) equipped with an 8-channel head coil. The following MR protocol was applied:

1) 3D FSPGR T1 weighted anatomical scan. Number of slices = 188, slice thickness = 1.0 mm, repetition time (TR) = 7.8 msec, TE = 2.95 msec, inversion time (TI) = 500 msec, field of view = 260 × 260 $mm^2$, flip angle = 14°, matrix = 192 × 192 giving a total acquisition time (TA) = 7:28 min.
2) PRESS with TR = 1500 msec and TE = 35 msec, 128 repetitions giving TA = 3.48 min.
3) MEGA-PRESS with TR = 1500 msec and TE = 68 msec, 128 paired repetitions of 4096 datapoints (TA ~ 7 min) at 5 kHZ spectral width with a phase cycling of 8 paired repetitions. A 16 msec sinc weighted Gaussian editing pulse was applied at 1.9 ppm (ON) and 7.46 ppm (OFF) in interleaved scans.

Both the PRESS and MEGA-PRESS acquisitions were obtained from a 21 ml voxel in the left ACC.

### 3.2.2. PAPER II: GABA edited MEGA-PRESS reproducibility

*Participants:*

Twenty-one healthy male volunteers (age 32 ± 6 (SD)) were recruited in accordance with local ethical guidelines. Written informed consent was retrieved.

Participants were instructed to abstain from caffeine, alcohol, and nicotine, exercise and sex for the twelve hours prior to each MR examination due to possible influences on GABA levels.

*MR protocol:*

Brain MRI scans were acquired using a 3.0 T GE Discovery MR750 (Milwaukee, US) with a 32-channel GE head coil. The following MR protocol was applied:

1) 3D FSPGR T1 weighted anatomical scan. Number of slices = 188, slice thickness = 1.0 mm, TR = 7.8 msec, TE = 2.95 msec, TI = 450 msec, field of view = 256 × 256 $mm^2$, flip angle = 12°, matrix = 256 × 256 giving TA = 7:10 min.
2) MEGA-PRESS with TR = 1800 msec and TE = 68 msec, 328 paired repetitions of 4096 datapoints (TA~20 minutes) at 5 kHZ spectral width with a phase cycling of 8 paired repetitions. A 20 msec sinc weighted Gaussian editing pulse was applied at 1.9 ppm (ON) and 7.46 ppm (OFF) in interleaved scans.

Two voxel placements; one from a 21 ml volume in the middle ACC and the other from a 22 ml volume in the left inferior frontal gyrus (Broca's region). The order of the two brain regions was randomized (balanced), to avoid any bias.

All participants were scanned twice with identical protocols, at exactly the same time of the day, one week apart.

*In vitro* phantom scans were included as an indicator for performance under ideal conditions. The phantom MEGA-PRESS acquisitions were performed using the same sequence parameters as for in-vivo scans, but with a smaller voxel size (12 ml)

The metabolite solution was based on the GE "braino" MRS-HD-Sphere spectroscopy phantom used in Schirmer and Auer (115), with the following metabolite concentrations: 10 mM NAA, 10 mM Cr, 3 mM Cho, 7.5 mM mI, 10 mM Glu, 5 mM Lac, with the addition of GABA at a concentration of 2.0 mM.

### 3.2.3. PAPER III: The GSH "Christmas phantom" study.

*Phantom preparation:*

The metabolite solution was based on the GE "braino" MRS-HD-Sphere phantom used in Schirmer and Auer (115) with the following metabolite concentration: 12.5 mM NAA, 10.0 mM Cr, 3.0 mM Cho, 7.5 mM mI, 12.5 mM Glut, 5.1 mM Lac, 2.0 mM GABA and 3.0 mM GSH.

*MR protocol:*

MRI scans were acquired using a 3.0 T GE Discovery MR750 (Milwaukee, US) with an 8-channel GE head coil. The following MR protocol was applied:

1) MEGA-PRESS with TR = 1800 ms, TE = 131 ms and 328 paired repetitions (328 ON and 328 OFF scans) of 4096 datapoints (TA = 20:24 min) at 5 kHz spectral width, phase cycling of 8 paired repetitions, 20 ms sinc weighted Gaussian editing pulse was applied at 4.56 ppm (ON) and 20 ppm (OFF) in interleaved scans.
2) PRESS with TR = 3000 ms and TE = 30 ms, 256 repetitions giving a TA of 14:00 min.

In both acquisitions, a fixed voxel size was 35 x 20 x 25 $mm^3$ was used. The voxel was positioned in the centre of the phantom using a T1 weighted image acquired before the MRS acquisition. A total of 122 MEGA-PRESS and PRESS spectra were acquired during a time period of 11 days. One year follow-up acquisitions with the same sequence parameters were performed on the same phantom. The decaying GSH signals measured from both MEGA-PRESS and PRESS data were then modelled in Matlab.

Acquisitions were also made on a similar phantom without GSH and a pure GSSG phantom. The phantom without GSH was used as a zero reference to make sure that neither MEGA-PRESS nor PRESS quantified any GSH when no GSH was present. The pure GSSG phantom was used to establish T2 relaxation time for GSSG.

### 3.2.4. PAPER IV: GSH edited MEGA-PRESS reproducibility

*Participants*

The study was approved by the Regional Committee for Medical and Health Research Ethics in Western Norway (REK 2017/516). 41 healthy volunteers (aged 25 ± 4.2 (SD), twenty females) were recruited. Written informed consent was retrieved.

Participants were instructed to abstain from strenuous exercise 6 hours and alcohol and nicotine twelve hours prior to each MR examination due to possible influences on GSH levels.

*MR protocol:*

Brain MRI scans were acquired using a 3.0 T GE Discovery MR750 (Milwaukee, US) with a 32-channel GE head coil. The following MR protocol was applied:

1) 3D FSPGR T1 weighted anatomical scan. Number of slices = 188, slice thickness = 1.0 mm, TR = 8.2 msec, TE = 3.2 msec, TI = 600 msec, field of view = 256 × 256 $mm^2$, flip angle = 8°, matrix = 256 × 256 giving TA = 11:44 min.
2) MEGA-PRESS with TE = 131 msec and TR = 1800 msec, 192 paired repetitions of 4096 datapoints (TA = 12:14 min) at 5 kHZ spectral width with a phase cycling of 8 paired repetitions. A 16 msec sinc weighted Gaussian editing pulse was applied at 4.56 ppm (ON) and 20 ppm (OFF) in interleaved scans.
3) PRESS with TE = 30 msec and TR = 1800 msec, 128 repetitions giving TA = 4:34 min.
4) STEAM with TE = 7 msec, TR = 1800 msec and TM = 8.784 msec, 128 repetitions giving TA = 4:34 min.

MEGA-PRESS, PRESS and STEAM were performed at two different voxel placements; a 20.6 ml volume in the middle OCC and a 20.6 ml volume in the left ACC. The order of the two brain regions was randomized (balanced), to avoid any

bias. All participants were scanned twice with identical protocols, at exactly the same time of the day, one week apart.

## 3.3. Statistical Methods

Demographic, ASSQ, Intellectual ability (WASI) and MRS data were analysed using SPSS (version 12.01) (IBM Corp. USA) statistical analysis software package.

Normally distributed data were analysed with either Student's t-test. When the data were not normally distributed, a log-transformation was performed, and if the data were still not normally distributed, the nonparametric Mann-Whitney U test was applied.

Coefficients of variation (CVs) analysis were performed with Excel (Microsoft Office 2010), and effect size and power analyses were acquired with G*Power 3.1. All significance levels were set to 0.05.

A time window approach was applied in both the GABA and the GSH reproducibility analyses. The acquisitions were subdivided to produce datasets equivalent to various shorter scan lengths (16, 32, 48, 64… and 192 paired repetitions). These datasets were then applied for both the within-and between-session analyses.

# 4. MAIN RESULTS

**PAPER I:** GABA edited MEGA-PRESS in ASD;

There was a significant negative correlation in the ASD group between ASSQ and GABA levels, however no significant difference between ASD and typically developing controls in GABA levels was found.

**PAPER II:** GABA edited MEGA-PRESS reproducibility;

Increasing the number of repetitions showed improvements for within- and between-session reproducibility up to about 218 repetitions. (CV ranging from 4 to 14 %). Gannet combined with LCModel approach proved the best method. (CV = 4 – 5 %). Measurements from the ACC region had higher CVs than the inferior frontal gyrus (Broca's region). (CV = 6 – 14 % vs 4 – 7 %), indicating that measurements from Broca's region are more reproducible.

**PAPER III:** The GSH "Christmas phantom" study

Both MEGA-PRESS and PRESS showed degradation of the measured GSH signal. The modelled curves gave for t = 0, GSH = 2.9 institutional units (i.u.) for MEGA-PRESS and GSH = 2.3 i.u. for PRESS (start concentration of GSH was 3.0 mM). T $\rightarrow \infty$ gave ~ 0 i.u. for MEGA-PRESS and 0.7 i.u. for PRESS. After ~1 year, the concentration of GSH in the phantom measured by MEGA-PRESS was 0 i.u. and for PRESS 0.6 i.u. Assuming that PRESS also includes GSSG in its GSH fit, the theoretical PRESS measured GSH value from the 1 year follow-up phantom was calculated to be 0.7 mM.

**PAPER IV:** GSH edited MEGA-PRESS reproducibility

Between-session variability of GSH edited MEGA-PRESS stabilised at around 128 paired repetitions (~8 min, CV ~ 11 – 12 %). Between-session CV-values for the PRESS sequence were 20 – 26 % and for STEAM 16 – 20 %. CV values for within-session variability of MEGA-PRESS edited GSH measurements dropped to ~ 4 % at

128-144 paired repetitions There were no correlations between GSH values measured with MEGA-PRESS, STEAM and PRESS, and no differences in measured GSH levels between males and females.

# 5. DISCUSSION

To simplify terms for the discussion; the GABA edited MEGA-PRESS in ASD study will be referred to as the ASD study and the GABA edited MEGA-PRESS reproducibility study and the GSH edited MEGA-PRESS reproducibility study will be referred to as the GABA reprod and GSH reprod study, respectively (or just the reprod studies where both studies are involved) in the discussion section.

The discussion section is divided into methodological considerations and discussion of the results. In methodological considerations, the data collection in the four studies are discussed with emphasis on inclusion/exclusion criteria, voxel size and placements. The reason for the somewhat illogical study order (first ASD study, then the reprod studies) is also explained. A special section is dedicated to the discussion of the MRS method in general, and the MEGA-PRESS in particular. What is actually measured in GABA edited MEGA-PRESS is also addressed. As the subtitle implies, in discussion of the results, the results of the four studies are discussed.

## 5.1. Methodological Considerations

### 5.1.1. Data collection: The ASD study

*Inclusion/exclusion criteria*

The ASD diagnosis displays a significant clinical heterogeneity, and defining inclusion and exclusion criteria when planning an ASD study is challenging. The researcher wants to study the core elements of ASD, not its comorbidities, but perhaps the comorbidities are an integrated part of the ASD symptom complex? The inclusion/exclusion criteria in the ASD study were comparable to similar studies, with two exceptions. Firstly; females are not included in the study. There are 4-5 times as many boys as girls with the diagnosis (53), and the clinical presentation of the disorder is different in females than males (116). Girls have social and personal attributes that masks or compensate for ASD symptoms to a greater extent than what boys have. Biological factors may protect girls from the condition to develop in the

first place (117). In order to make the ASD group as homogenous as possible and avoid statistical power issues by including girls, it was decided to only study boys. Secondly, epilepsy was not an exclusion criterion in our study. ASD and epilepsy are conditions that are highly connected, and by excluding children with epilepsy, the ASD group would not be representative of children with ASD in general.

14 of 20 boys in the ASD group managed to carry out the MR examination with acceptable MRS data. The MRS data of these 14 boys were of same quality as the MRS data of the included TD boys. There definitely exists a selection bias in whom of the boys managed to perform the MR examination, and who did not. For example, boys with ASD extremely sensitive to loud noises had greater difficulties in completing the MR examination. A success rate of 14/20 also makes it questionable whether MRS is truly a feasible investigation tool for future clinical application in this group.

*The VOI*

Setting up MRS experiments is also challenging when it comes to choosing which region of the brain one wants to examine. The MEGA-PRESS technique for both GABA and GSH editing is not yet available as multi-voxel spectroscopy, and the chosen VOI needs to be rather large in order to acquire enough data to get acceptable SNR. As confirmed in the GABA reprod study, GABA edited MEGA-PRESS from one VOI takes ~12 min. This greatly limits how many brain regions that can be examined in one experiment.

The left ACC region was chosen as VOI in the ASD study for its involvement in higher cognitive and intellectual functioning. The frontal lobe is one of the more difficult regions in the brain to acquire MRS data from, mostly due to problems with air from the paranasal sinuses. The GABA reproducibility study did however show that reliable GABA measurements can be achieved in this area.

### 5.1.2. Data collection: The reprod studies

*Inclusion/exclusion criteria*

Inclusion and exclusion criteria in the GABA and GSH reprod study were more straight forward. Females were not included in the GABA reprod study. There are mixed results in the literature whether gender affects GABA levels (118, 119) and it was therefore decided, for a pure reproducibility purpose, to only study males. In the GSH reprod study, however, females were included. As far as we are aware of, there is only one previous study examining gender differences in GSH edited MEGA-PRESS (120). With twice the sample size as the GABA reprod study, the GSH reprod study became then not only a reproducibility study, but also a study where potential gender differences could be examined.

*The VOI*

Even though there is good reason to assume a degree of transferability of results between the GABA or GSH reprod studies from one ROI to another, the results from the reproducibility studies will most likely only apply for the regions examined. The ACC and auditory cortex regions are of particular interest for the extended research group (fMRI group), and were therefore chosen. In the GSH reprod study, a voxel placement in the occipital lobe was also included. This is a brain region where achieved MEGA-PRESS quality is known to be good, and the region has also previously been used in GABA edited MEGA-PRESS reproducibility studies (121, 122).

With regard to voxel size, a 27 ml voxel was applied in the ASD study while voxels of ~ 21 ml are applied in the reprod studies. A voxel size of 27 ml for GABA edited MEGA-PRESS have been previously considered best practice (22), however a somewhat smaller voxel gives adequate signal and is also easier to position in the brain. In addition, a smaller voxel gives less partial volume effects.

### 5.1.3. MRS limitations

One of the main limitations of MRS is the need for large voxel sizes resulting in poor spatial resolution. Because *in vivo* metabolites are in the mM concentration range, voxel sizes must be sufficiently large to obtain enough signal to generate a high-quality spectrum. As mentioned earlier, multi-voxel spectroscopy is not yet available for GABA and GSH edited MEGA-PRESS, and it is too time consuming to cover the entire brain with single voxels. The achievable spatial coverage is in other words also poor.

It is crucial that the participant lie still in the scanner since head movements shift the region where MRS is obtained. This may be to regions of no interest, or to a region that contains fat, CSF or air, which will ruin the MRS spectra. It is even more important that the participant lie still when performing MEGA-PRESS than regular PRESS for several reasons:

- The scan times for MEGA-PRESS acquisitions are longer.
- It is the difference spectra that are used for quantification.
- The preciseness of the editing pulse is affected due to motion related frequency changes.

Another limitation is the chemical shift displacement error (CSDE) which describes the inaccuracy of the slice localization. CSDE is proportional to $B_0$ and inverse proportional to the bandwidth (BW) of the RF pulse excitation. PRESS and STEAM are localization sequences with narrow BW while the newer SVSs LASER and semi-LASER acquire a localization method (adiabatic) with wider BW.

In addition to more accurate volume selection, and by this avoiding CSDE, adiabatic pulses compensate effectively for $B_1$ inhomogeneity and can provide more SNR at high magnetic fields. Following this, GABA and GSH edited MEGA –LASER is thought to replace MEGA-PRESS in the future.

### 5.1.4. GABA+: What are we actually measuring?

The spectral editing techniques applied in our studies does not separate the GABA signal from MM. A considerable MM component is also affected by the editing pulse which means that the resultant GABA+ measurements includes of as much as ~60% MM. There are several different approaches to overcome the unwanted MM coediting, the most popular being placing symmetrical editing pulses about the MM resonance at 1.7 ppm (ON/OFF 1.9/1.5 ppm) (123). However, the reproducibility of the MM-suppressed GABA edited acquisition is poorer than the standard GABA-edited acquisition since MM-suppressed GABA measurements seem more susceptible to $B_0$ field changes resulting from scanner drift and participant head motion (124).

Another concern in interpretation of GABA measurements from spectral editing techniques is what the measurements actually represent. There are three pools of GABA in the CNS, all with different roles, and some of the GABA pools may be more tightly bound (less "visible" for GABA edited MEGA-PRESS) than others (125).

- *Cytoplasmic GABA* exists throughout the neurons. The 67kD enzyme glutamic acid decarboxylase (GAD) that catalyses the decarboxylation of Glu to GABA and $CO_2$ is tonically active and this GABA pool is believed to have a role in the cells metabolism.
- *Vesicular GABA* exists in the vesicles in the axon terminals. The 65kD GAD enzyme which catalyses the formation of GABA here is phasically active, and this GABA pool plays a role in inhibitory synaptic neurotransmission.
- Free extracellular GABA acting on extra synaptic GABA receptors. The third GABA pool is less studied that the two others, nevertheless, this pool appears to have a tonic, neuromodulatory role in cortical inhibition.

The ratio of vesicular to cytosolic GABA has not been directly measured. It is most likely dynamic and highly dependent on cellular activity and the availability of

precursors (126). All this implies that measured GABA concentration cannot simply be regarded as the same as neurotransmitter GABA. Changes in metabolism and even modulation of blood flow must be taken into consideration when interpreting the GABA measurements.

However, several studies have demonstrated the relationship between behaviour and spectral edited GABA measurements (127-129), which means that the GABA measurements at least correlates in one way or another with the neurotransmitter and neuromodulator pools of GABA in the cortex.

### 5.1.5. The order of the studies

An important methodological limitation of the thesis is that the GABA edited MEGA-PRESS reproducibility study came after the GABA edited MEGA-PRESS had been applied in the ASD study. The acquisition parameters in the MR protocol were different in the two reprod studies and in the ASD study. The ASD sample were boys aged 6-13 years, while the reprod studies sample were young healthy men aged 18-35 years. GABA edited MEGA-PRESS was set up on our MR machine just before the start-up of the ASD study. A GABA reprod study on healthy male adults, but with the same acquisition parameters as the ASD was performed, but the quality of the spectra from this study was markedly poorer than the ASD study. Another GABA edited MEGA-PRESS reproducibility study was performed, but again – the data quality was poor. A scanner upgrade followed with the installation of a new magnet and updated software - a “forklift upgrade”. Testing the reproducibility of a study done before the scanner upgrade had no meaning, and it was therefore decided to design the next reproducibility study to match GABA edited MEGA-PRESS appliances in future studies. Updated acquisition parameters were therefore used in the GABA edited MEGA-PRESS reproducibility study, which was then basically the third reprod study.

Both the quality of the acquired MRS spectra and the quality of the fit of the MRS spectra in the ASD study were checked as described in earlier sections. It would,

however have been preferable to have done a reproducibility study before the ASD study in order to assure the quality of the method properly. This is one of the main lessons learned in this thesis; not to include a novel MR sequence in study protocols without having tested it thoroughly on beforehand. Nonetheless, our group would probably not be this advanced in the MRS editing techniques if the GABA edited MEGA-PRESS sequence had not been included in the ASD study.

## 5.2. Discussion of Results

### 5.2.1. The ASD Study

There were no statistically significant differences between the two groups (ASD/TD) in GABA+ and GABA+/tCr levels.

A few studies have shown a reduction in the GABA concentration in individuals with ASD, compared to healthy controls, at a couple of different voxel placements in the brain. There are reasons to believe that these differences, given that they exist, are not present globally throughout the brain. Two studies have shown reduced GABA/tCr concentration in the ASD group compared to healthy controls in the right sensorimotor cortex, but no significant differences were found in the middle occipital cortex. One study showed significant reduced GABA/tCr concentration in the ASD group compared to healthy controls in the left motor cortex and the left auditory, but no significant differences were found in the left auditory cortex. One final study – the Harada study- found no differences in GABA/tCr concentration in the lenticular nuclei, but they did find lower GABA/tCr in the ASD group compared to healthy controls in the left frontal lobe. The frontal voxel position in their study is comparable to the voxel position applied in our ASD study. However, as pointed out in the Paper I, there were several concerns which may question the results in the HARADA study: Most of the children were sedated with triclofos sodium (a GABA agonist), the subjects had a wide age range, and there was no information whether the included subjects were boys or girls. We do not know how these factors affects MRS measured GABA concentration.

The ROIs described in the previous section are the only regions in the brain where these types of GABA measurements have been performed in children with ASD. The numbers of participants with ASD with measurements from each ROIs are also fairly low ranging from a total of 8 (auditory cortex (82)) to 48 (sensorimotor cortex (82, 84)).

GABA levels fluctuate and are dependent on cell metabolism, neuronal activity and blood flow. There is a need to replicate previous findings in larger participant-groups with a more optimized MRS sequence.

The GABA measurements described in the previous studies are snapshot-measurements from a relatively large brain region, and it is therefore difficult to interpret if there is any functional relevance of these changes. Using MRS to show functionally specific correlations between GABA levels and behaviour in individuals with ASD may prove valuable for future studies in the area. For example, Puts et al 2016 (84), found associations between GABA levels in children with ASD and tactile behavioural abnormalities present in this group.

All MRS studies on children and adults with ASD suffer from selection bias; only high- functioning individuals with ASD will be able to complete a MR examination with acceptable quality of MRS spectra. It is a reasonable assumption that an eventual difference in measured GABA levels would be larger between more affected ASDs (more severe ASD symptoms) and healthy controls. ASSQ is a screening questionnaire applied worldwide for registering ASD symptom severity. A high score means more ASD symptoms, and a score below 17 makes the ASD diagnosis rather unlikely (130). In the ASD study we did find a negative correlation between measured GABA levels and ASSQ score. The higher the ASSQ score the lower GABA levels. The findings nicely agree with the E/I theory in ASD.

However, one should take caution when interpreting correlations, since correlations do not allow for causality. Although it was statistically significant, the correlation did not survive multiple comparison due to small sample size, which is a limitation of the results.

An interesting question when looking at the results in the ASD study is, for whom does these results apply? Are they transferable to a female ASD population or other age groups? A safe answer would be that they apply to high-functioning boys.

### 5.2.2. The Reprod Studies & the “Christmas phantom” study

#### 5.2.2.1. Optimal number of repetitions

The optimal number of repetitions for GABA edited MEGA-PRESS derived from the GABA reprod paper is 220 paired repetitions, however a CV of under 8 was achieved for both the ACC and the Broca region at around 160 paired repetitions. When looking at the early work on GABA edited MEGA-PRESS, 128 paired repetitions was the standard (8, 131) and this was also the number of paired repetitions applied in the ASD study. The paper by Mullins et al (22) did not discuss the effect of scan length, and did not contain any recommendations on how many repetitions that is ideal. A previous study from our group (122) found, in line with the GABA reprod results, that the optimal scan length was 256 paired repetitions (with TR 1500 msec gives TA~13 min) in a 27 ml voxel placed in the occipital lobe. The most recent studies measuring GABA with MEGA-PRESS uses between 256-288 (132, 133) paired repetitions.

220 paired repetitions give a scan length of roughly 13 min depending on TR time, which can be challenging for patients. However, GABA edited MEGA-PRESS with this scan length have been applied in several group studies with different patient groups such as schizophrenia (134), depression (9), ADHD (135), ALS (18), diabetic neuropathy (19) and neurofibromatosis.

The optimal number of repetitions for GSH edited MEGA-PRESS were found to 128 paired repetitions (TA ~ 8 min), in other words much shorter that for the GABA edited MEGA-PRESS. However, the CV never was never lower than 10. This may imply that there might be more work to do to optimize the GSH edited MEGA-PRESS technique, or that GSH levels fluctuate more than GABA levels.

Averaging GABA/GSH quantification results over a group is one thing, using the quantification results from one single individual is another thing. On a single subject basis, the MRS measured signals from GABA and GSH signals are too low and disease unspecific, and the intra-and interindividual variations are too high. These sequences are therefore only suitable for group comparisons and not for diagnostic purposes at the moment.

#### 5.2.2.2. Comparing GSH edited MEGA-PRESS with PRESS and STEAM

Between-session CVs from the GSH reprod study revealed that PRESS and STEAM had far worse reproducibility in both brain regions measured (20 – 26 % for PRESS and 16 – 20 % for STEAM). There were no correlations between GSH levels measured with MEGA-PRESS, PRESS or STEAM, implying that the three methods might actually not be measuring the same thing.

It is important at this point to acknowledge the differences in the two measuring techniques. Where MEGA-PRESS is directly measuring a distinct peak through the editing approach, PRESS and STEAM relies on a model assumption of concentrations (prior information). It is actually not possible to visually inspect the GSH signal in the PRESS (and STEAM) spectra.

The results from the decaying GSH measurements by MEGA-PRESS and PRESS in the "Christmas phantom" study showed that the GSH PRESS fit also included the product of oxidation – GSSG. What guarantees do we have that the PRESS measurement, or STEAM for that matter, are not including other metabolites with similar resonance frequencies the *in vivo* fitted result of GSH.

A concern with GSH edited MEGA-PRESS is the relative long TE (131 msec) required to achieve an optimal editing efficiency. This combined with the rather short T2 of GSH (T2 = 67 msec at 4T (136)) gives rise to considerable signal loss compared to short-TE PRESS. This is also evident from the GSH reprod study where

SNR from MEGA-PRESS measurements from the middle OCC and left ACC were 34.3 and 16.1 while SNR from PRESS were 56.1 and 38.2 respectively. This is a trade-off that has to be acknowledged. However, what use is it to apply a sequence with higher SNR if we are not certain that it is only measuring the signal from the metabolite we are interested in!

# 6. CONCLUSIONS

*The ASD Study:*

The aim of the ASD study was to explore the E/I hypothesis in children with ASD using both the PRESS and MEGA-PRESS sequence. A negative correlation was found between autism severity, as measured by the ASSQ, and GABA in the ASD group. However, no group level differences in GABA was found between the ASD and TD group. The ASD study underlines that the severity of autism symptoms has to be taken into account when examining the E/I hypothesis in ASD.

*The GABA reprod study:*

The aim of the GABA reprod study was to examine the within-and between-session reproducibility of the MEGA-PRESS sequence, focusing on optimizing the scan lengths *in vivo*. Reliable GABA measurements can be achieved in the frontal lobe when using appropriate scan parameters and corresponding experimental design.

*The GSH "Christmas phantom" study:*

The aim of the "Christmas phantom" study was to examine how well GSH edited MEGA-PRESS and PRESS was able to measure the degradation of GSH in a phantom. Both methods managed to measure the degradation, but the modelled degradation curve for the MEGA-PRESS data was more accurate.

*The GSH reprod study:*

The aim of the GSH reprod was to examine the GSH measured reproducibility of the MEGA-PRESS, STEAM and PRESS sequence *in vivo*. In addition, gender differences were examined. Reliable GSH measurements were found for MEGA-PRESS. No significant correlations were found in measured GSH levels between the three MRS sequences. There were no gender differences in the brain regions examined.

The work in this thesis has expanded the foundation of the MEGA-PRESS sequence, bringing it one step further towards increased reproducibility and reliability of measured GABA and GSH levels. The GABA and GSH reprod study gives good evidence of which number of repetitions that should be selected when designing new GABA or GSH edited MEGA-PRESS studies. Another result that may be of importance when designing GSH reproducibility studies is that there seems be no differences in measured GSH levels in males and females, even when the use of hormonal contraceptives is taken into account. Lastly, results from the "Christmas phantom" study and the GSH reprod study supports the argument that short TE single voxel sequences STEAM and PRESS are not accurate enough to measure GSH *in vitro* and *in vivo*. The more advances sequences, such as the MEGA-PRESS, are needed.

With the existing MRS sequences, more work needs to be done when it comes to which brain regions that are examined. More work also needs to be done examining the effects age and gender may have on the measured metabolite levels. The newer more advanced HERMES and PRIAM sequences with multi editing and dual voxel acquisitions, which might be the future of edited MRS techniques, needs to undergo the same level of validation before they are applied in research.

The local effort with MEGA-PRESS in Bergen has contributed to a broad collaboration between several leading MRS groups worldwide. This includes the involvement in a multi-site project (the Big GABA project (124) with an agenda to validate, push forward and further develop MRS measurements of GABA using the MEGA-PRESS sequence. The international collaboration will continue in the future, including editing of more metabolites.

MRS is still by some considered an "investigational technique" and "the forever just promising technique" 30 years after the first promising papers on MRS in acute stroke, multiple sclerosis and brain tumours were published. Though some clinicians are desperate if MRS spectra are not acquired as a part of the standard MRI tumour investigation, others could not care less. The question is: Will the newer more

advanced MRS techniques, such as edited MEGA-PRESS, revolutionize MRS and forever convince the doubters that MRS has an important role in the field of neuroradiology? Probably not, or at least not for GABA and GSH edited MEGA-PRESS. 2HG and Lac edited MEGA-PRESS, on the other hand, might be of considerable value in clinical MRI brain protocols in the future.

# 7. FUTURE PERSPECTIVES

As mentioned in chapter 1, *in vivo* measurement of GABA and GSH is currently an area of great interest in modern neuroimaging, and with this a constant stream of methodological development of MRS has evolved. A tighter collaboration between the leading MRS cites in the world, as initiated by for example the Big GABA project, will make it easier to compare data between different studies across different research cites and vendors.

At present, dual editing and dual voxel excitation is the state of the art in MRS. The possibility of measuring GABA and GSH simultaneously is a promising development in the MRS field. Both metabolites are of great interest in a wide range of neuropsychiatric disorders, neurodevelopmental disorders and neurological disorders. It is an enchanting prospect for many researchers to get both metabolites (at two voxel placement) at the same time. This is, however, probably just the beginning. Multi-editing techniques are currently under development with an ultimate goal of whole brain coverage with CSI type techniques.

Another bonus in improving the MEGA-PRESS editing sequence and building international consensus for standardized study design and acquisition protocols for MEGA-PRESS parameters, is the transferability to other metabolites that can be edited. The most interesting metabolites in a clinical aspect is Lac and 2HG.

Elevated Lac indicates anaerobic glycolysis, and the appearance of an obvious Lac peak strongly suggests a pathological process such as ischemia, tumour, trauma, infection or mitochondrial disease. Small increases in Lac may reflect subclinical inflammation, impairment of oxidative metabolism, or increased neural activity (137). The Lac doublet at 1.33 ppm is usually significantly overlapped by MM and lipids. To overcome this, longer TEs (TE 144 msec and TE 188 msec) are often used in clinical settings. A problem with this is that the signals are lost with longer TE, something which may lead to false negative results.

In Lac edited MEGA-PRESS, the editing pulse is applied to the methane spin at 4.1 ppm and the signal is measured from the methyl spin at 1.32 ppm. TE is also long (140 msec), but since all the overlapping signals are removed, enough signal still remains for quantification.

2HG is a tumour biomarker, and is present at very low levels in healthy brain tissue. It may be present in the mM range in tumours with a isocitrate dehydrogenase mutation (grade 2 and grade 3 gliomas in adults.) Interestingly – the editing pulse is applied at the 1.9 ppm peak – same as with GABA editing. The 2HG peak measured is the 4.01 ppm peak.

Individuals that are diagnosed with ASD are, even if they share the same diagnosis, affected very differently. In what way do researchers expect that the GABA edited MEGA-PRESS, or similar sequences, can help to, first of all; diagnosing ASD, and second of all; understanding the variations of the diagnosis, and why there seem to be a difference in how it often expresses itself differently in men versus females? The most optimistic amongst ASD researchers believe that these sequences will have an important role in the future in the diagnosis of ASD and other neurodevelopmental and psychiatric diseases. The more pessimistic researcher would argue that the method at most can be applied in research settings only – and have no use in clinical settings. In the middle, there is a more moderate view which claims that GABA-edited MEGA-PRESS measurements may become a biomarker to identify which ASD subjects that might have effect of treatment with for example GABA analogue drugs like gabapentin and monitoring treatment. The GABA edited MEGA-PRESS method has been known for more than 20 years. There has, however, not been performed enough studies using GABA edited MEGA-PRESS´ appliance and utilities in ASD, to assert the role of this method. There is a severe lack of studies with both males and females with ASD, at different ages, and with different symptoms. Such studies, where also multiple brain regions are examined, need to be conducted in order to see if there are any common denominators.

As of today, Haukeland University Hospital is the only Hospital in Norway using the MEGA-PRESS sequence. Expanded use of the MEGA-PRESS sequence and similar advanced MRS techniques at more research cites might just be what is needed in further developing the understanding of diseases like ASD. Without learning more about what MRS can/cannot show, we will never know.

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# I

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# "Brain MR spectroscopy in autism spectrum disorder—the GABA excitatory/inhibitory imbalance theory revisited"

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Magnetic resonance spectroscopy (MRS) from voxels placed in the left anterior cingulate cortex (ACC) was measured from 14 boys with Autism Spectrum Disorder (ASD) and 24 gender and age-matched typically developing (TD) control group. Our main aims were to compare the concentration of γ-aminobutyric acid (GABA) between the two groups, and to investigate the relationship between brain metabolites and autism symptom severity in the ASD group. We did find a significant negative correlation in the ASD group between Autism Spectrum Screening Questionnaire (ASSQ) and GABA+/Cr, which may imply that severity of symptoms in ASD is associated with differences in the level of GABA in the brain, supporting the excitatory/inhibitory (E/I) imbalance theory. However we did not find a significant difference between the two groups in GABA levels.

**Keywords: ASD, GABA, MRS, MEGA-PRESS, ASSQ**

## Introduction

Autism Spectrum Disorder (ASD) is a pervasive developmental disorder characterized by deficits in social communication and social interaction and by restricted, repetitive patterns of behavior, interests or activities. Symptoms must be present in an early developmental period (before 3 years of age), but they do not necessarily become

**Abbreviations:** ASD, Autism Spectrum Disorder; MRS, Magnetic Resonance Spectroscopy; ppm, Parts per million; NAA, *N*-acetylaspartate; Glu, Glutamate; Gln, Glutamine; MI, Myo-inositol; Cho, Choline; Cr, Creatine; PCr, Phosphocreatine; GABA, Gamma-aminobutyric acid; MM, Macromolecules; GABA+, GABA including associated MM; E/I, Excitatory/inhibitory; TD, Typically developing; SIB, Unaffected siblings; Glx, Glutamate and glutamine; ACC, Anterior cingulate cortex; ASSQ, Autism Spectrum Screening Questionnaire; WASI, Wechsler Abbreviated Scale of Intelligence; DAWBA, Development and Well-Being Assessment; SDQ, Strengths and Difficulties Questionnaire; ADHD, Attention deficit/hyperactivity disorder; ADD, Oppositional defiant disorder; TR, Repetition time; TE, Echo time; CRLB, Cramér-Rao lower bounds; CSF, Cerebro spinalfluid; Gray, Gray matter; White, White matter.

fully manifest until social demands exceed limited capacities (American Psychiatric Association, 2013). The estimated prevalence of ASD in the Norwegian population ranges from 0.44 to 0.87% (Heiervang et al., 2007; Posserud et al., 2010; Surén et al., 2012) with four times as many boys as girls diagnosed with the disorder (Baron-Cohen et al., 2009b). Autism is now called "spectrum disorder" because of the recognition that its manifestation and severity displays great heterogeneity depending on intellectual ability, associated symptoms, possible etiology and developmental level (American Psychiatric Association, 2013). Although there is clearly a genetic basis to ASD, the majority of cases have unknown causes (Abrahams and Geschwind, 2008; Geschwind, 2008). It is, moreover, now widely accepted that ASD is a neurobiological disorder, but specific biological markers are yet to be established (McPheeters et al., 2011; Warren et al., 2011).

Magnetic resonance spectroscopy (MRS) has made it possible to study the concentration of biochemical substances in the healthy and diseased brain (Soares and Law, 2009). By measuring from a volume element (MRS voxel) in specific regions of interest, metabolite concentrations can be estimated due to differences in spectral resonances from the main water peak. Using the PRESS (Point RESolved Spectroscopy) single-voxel spectroscopy sequence (Bottomley, 1987), metabolites such as *N*-acetylaspartate (NAA), glutamate (Glu), glutamine (Gln), myo-inositol (MI), choline (Cho) and creatine (Cr), can be measured. Unfortunately, other important metabolites such as γ-aminobutyric acid (GABA) are not detectable using conventional MRS, due to spectral overlap with more abundant metabolites at 3.02 parts per million (ppm). One way to measure the concentration of GABA is with a spectral editing technique such as BASING (Star-Lack et al., 1997) or MEGA-PRESS (Mescher et al., 1998). A pair of frequency selective inversion pulses within a standard PRESS sequence allows discrimination between overlapping coupled and uncoupled spins. For GABA editing the editing pulses are applied to the C-3 protons of GABA at 1.9 ppm. Due to the spin-spin coupling the C-4 protons of GABA at 3.02 ppm are affected while other metabolite peaks like the strong Cr peak remains unaffected. Subtracting the spectrum from a second acquisition scheme without these editing pulses (or applied symmetric to the water signal, e.g., at 7.5 ppm), will give a difference spectrum without the strong singlet signal from Cr, allowing quantification of the GABA peak at 3.02 ppm. As these GABA protons are also coupled to macromolecules (MM) at 1.7 ppm this peak consists of GABA and an unknown contribution of MM signal and is therefore named GABA+ (GABA+ MM). This is in line with current best practices (Mullins et al., 2014) and hence compatible with the bulk of existing research including studies on ASD (Gaetz et al., 2014; Rojas et al., 2014).

Glutamate is the major excitatory neurotransmitter and GABA is the major inhibitory neurotransmitter in the brain, and probably all areas receive input from both of these neurotransmitters. The balanced interaction between excitatory and inhibitory neurotransmission is tightly regulated (Carlson, 2001) and is essential for controlling cognition, learning, memory and emotional behaviors. Several studies support the idea that imbalance in the glutamate/GABAergic system could be present in a wide range of disorders with quite different clinical appearances, like Downs syndrome, epilepsy, neurofibromatosis and schizophrenia (Ramamoorthi and Lin, 2011).

Lately increasing evidence have emerged suggesting that also ASD may be associated with abnormalities in the glutamate and GABA system including neurotransmitters, receptors and enzymes (Pizzarelli and Cherubini, 2011) often referred to as the excitatory/inhibitory (E/I) imbalance theory. It has been hypothesized that the E/I ratio in the cortex is unusually high, either due to increased glutamate—or because of decreased GABAergic signaling (Rubenstein and Merzenich, 2003). An E/I imbalance might explain the typical ASD symptom of hypersensitivity to sensory stimuli, including aversion to loud noises, tactile stimulation, and bright lights (Kanner, 1943; Baron-Cohen et al., 2009a).

GABAergic dysfunction in ASD has been proposed in animal models (Gogolla et al., 2009; Chao et al., 2010), post mortem studies and *in vivo* human studies; see overview Coghlan et al. (2012). The E/I imbalance hypothesis is also consistent with the observation that rates of epilepsy are higher in the autism population than in the general population (Gillberg and Billstedt, 2000). Another important finding is that gamma oscillations are reduced in ASD patients (Grice et al., 2001; Brown et al., 2005; Wilson et al., 2007). Gamma oscillations a re generated by GABAergic neurons (Pizzarelli and Cherubini, 2011), and are involved in sensory binding and higher cognitive functions (Lisman and Idiart, 1995).

When starting this study, there was, to our knowledge, only one previous study using the MEGA-PRESS sequence to measure the concentration of GABA in the brain of children with ASD compared with normal controls, and our study was designed to further explore the E/I theory. Harada et al. (2011b) reported significantly lower GABA+/NAA and GABA+/Glu ratio in a voxel placed in the left frontal lobe in a population of children with ASD aged 2–11 years compared to a typically developing (TD) control group ($n$ = ASD/TD: 12/10). Since then two more studies have been published. Gaetz et al. (2014) had three different spatially localized voxels; in the left motor cortex ($n$ = 17/15), left auditory cortex ($n$ = 15/11) and the left visual cortex ($n$ = 8/10), respectively. They found that the GABA/Cr ratio was significantly reduced in the motor and auditory cortex, but not significantly different in the visual areas in ASD children aged 11.5 ± 2.7 years compared to TD. The last study, Rojas et al. (2014), found reduced GABA/Cr ratio in a voxel placed in the left auditory cortex in children with ASD aged 14 ± 5 years compared with their unaffected siblings (SIB) aged 12 ± 6 years, and TD aged 12 ± 5 years ($n$ = ASD/SIB/TD: 17/14/17).

To explore the excitatory/inhibitory imbalance theory our aim was to study children aged 6–13 with ASD without sedation using both PRESS and MEGA-PRESS sequence. Since studies in healthy individuals have shown significantly

higher concentrations of GABA, Glutamate + Glutamine (Glx), and Glu in males compared to females (O'Gorman et al., 2011), only boys were included in the current study.

MRS measured metabolite concentrations are normally presented as ratios of water, total Cr, NAA or in the case of GABA, Glx have also been used. The concentrations obtained from MEGA-PRESS use water as an internal concentration reference, meaning that all our GABA+ data are scaled to water. We have chosen to display our results as GABA+ and GABA+/Cr since there are reports from previous studies that there exist group differences between ASD and TD in both NAA (Aoki et al., 2012) and Glx (Horder et al., 2013). Gaetz et al. (2014) and Rojas et al. (2014) also used GABA+/Cr to present their results.

We assessed GABA and other brain metabolite levels from a voxel in the left anterior cingulate cortex (ACC) using a voxel size of $30 \times 30 \times 30$ mm$^3$ (27 ml) see **Figure 1**.

Although closer to the midline of the brain, this voxel placement partly overlaps the voxel placement in the Harada et al. (2011b) study. This was one of the few locations in the frontal lobe where it was possible to fit the relatively large voxel without including bone or cerebrospinal fluid (CSF) in these children. The dorsal portion of the ACC has been shown to be involved in higher cognition and intellectual functioning (Bush et al., 2000), and other studies have in particular implicated the left ACC in patients with mental disorders (Minzenberg et al., 2009).

All participants were assessed using the Autism Spectrum Screening Questionnaire (ASSQ) and parts of the Wechsler Abbreviated Scale of Intelligence (WASI) as variations in both autism severity and intellectual level may have an impact on metabolite levels. To explore the possible relationship between autism severity and GABA, our GABA+ measurements were correlated with the ASSQ scores (Ehlers et al., 1999). In accordance with the E/I imbalance hypothesis, we hypothesized that we would find a lower concentration of GABA+ and GABA+/Cr ratio in the ASD group compared with the TD group, and that the GABA+ concentration and the GABA+/Cr ratio would correlate negatively with the ASSQ score.

## Methods

### Participants

The boys in the ASD group were recruited through parents groups, patient organizations, newspaper advertisements, from the educational and psychological counseling service in the municipality, and from patients receiving care at a private clinic. The boys in the TD group were recruited from the National Registry. The boys in the ASD group had been independently assessed and diagnosed by child psychiatric outpatient clinical specialists in Norway. Documentation of the clinical assessment leading to an ASD diagnosis was requested and reviewed by a clinical ASD expert (co-author M.P.) for all ASD boys to confirm the ASD diagnosis and ensure that they fulfilled the diagnosis also according to the DSM-5 criteria (American Psychiatric Association, 2013). In addition the parents/legal representatives in the TD group were interviewed using the ASD section of the structured interview Development and Well- Being Assessment (DAWBA) to exclude presence of ASD in the TD group (Goodman et al., 2000). Height and weight of all participants were measured prior to imaging and the parents filled out a screening questionnaire for mental health including the Strengths and Difficulties Questionnaire (SDQ; Goodman, 1997), the ASSQ (Ehlers et al., 1999), items on learning problems, obsessive compulsive disorders, tics and the DSM-IV criteria for attention deficit/hyperactivity disorder (ADHD) and oppositional defiant disorder (ODD), to rule out presence of neuropsychiatric conditions in TD children (Heiervang et al., 2007; Posserud et al., 2009). The ASSQ has been thoroughly validated as measure of ASD and was furthermore used as measure of autism severity (Ehlers et al., 1999; Posserud et al., 2009).

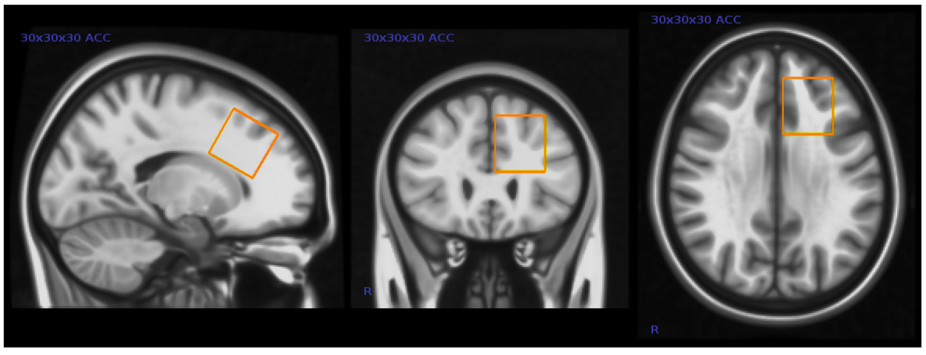


**FIGURE 1 | Placement of magnetic resonance spectroscopy (MRS) voxel in the left anterior cingulate cortex (ACC)**. This figure shows the typical placement of the 3 × 3 × 3 cm MRS voxel in the left ACC.

The two sub-tests Vocabulary and Matrices Reasoning of the WASI as tests of general intellectual capacity and cognitive skills (Wechsler, 1999) were assessed in both groups by trained psychology students. In addition, information regarding ethnicity, other illnesses, medication, and supplements and diets were noted. Exclusion criteria in both groups were braces (for the MR investigation), genetic abnormalities and prematurity (<36 weeks). Epilepsy, autistic traits and other neuropsychiatric disorders were exclusion criteria in the TD group.

We included boys with epilepsy in our ASD group since it is estimated that 30% of ASD patients also have epilepsy, and subclinical epileptiform activity is recorded on scalp EEG in up to 85% of children with ASD (Gillberg and Billstedt, 2000; Yasuhara, 2010). By excluding children with epilepsy we found that our ASD group would not be representative of children with ASD in general.

In the ASD group, 20 boys were initially recruited from which 14 (mean age 10.2 ± 1.9 years), completed the MRI examination with acceptable PRESS and MEGA-PRESS data as per our quality criteria described in the later "MRS data analysis" section. Three boys in the ASD group did not complete the WASI tests but they were nevertheless included for MRS analyses.

TD boys were recruited as control participants. In the TD group, 30 boys were initially recruited. Three of them were excluded prior to the data analyses; one had a large arachnoid cyst, and two scored above the 90-percentile relative to the population norm from the Bergen Child Study (Heiervang et al., 2007) on the screening questionnaires. Three TD boys did not complete the MR examination. This resulted in a total of 24 boys, mean age 10.2 ± 1.8 years in the TD group with acceptable PRESS data, while MEGA-PRESS data was acceptable in 21.

## Ethical Approval

The study was approved by the Regional Committee for Medical and Health Research Ethics, and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from the children's parents/legal representatives.

## Magnetic Resonance Imaging (MRI)

Brain MRI scans were acquired using a 3T GE Signa HDxt MR scanner (GE, Milwaukee, USA) equipped with an 8-channel head coil. The following MR protocol was used for acquisition of brain metabolites:

1. PRESS sequence with TE = 35 ms and TR = 1500 ms, 128 averages giving a total acquisition time (TA) of 3:48 min.
2. MEGA-PRESS with TE = 68 ms and TR = 1500 ms, 128 averages each for edited and unedited parts giving TA = 7 min. The somewhat low number of averages was necessary to minimize scan time with a difficult subject demographic.

GABA-editing was achieved with 16 ms 180° Gaussian editing pulses applied at 1.9 ppm and 7.5 ppm with alternating acquisition, giving the "edit-on" and "edit-off" spectra with the GABA spectrum obtained by taking the difference between the two acquisitions. Water reference lines were acquired as a part of the acquisition both for PRESS and MEGA-PRESS.

The MR scanning protocol also included a 3D T1 weighted anatomical scan (number of slices = 192, slice thickness = 1.0 mm, repetition time (TR) = 7.8 ms, echo time (TE) = 2.95 ms, field of view = 260 × 260 $mm^2$ flip angle = 14°, matrix = 256 × 256) for anatomical imaging and positioning of the MRS voxel. The T1-images from the SPGR sequence were reformatted from sagittal to oblique axial slice direction with slices positioned parallel to the line connecting the lowest edge of the splenium and the rostrum sections of the corpus callosum. The ACC voxel was then positioned on the left side near the midline of the brain between the genu and middle section of the corpus callosum with the lower voxel edge tangential with the upper border of corpus callosum, to avoid CSF, bone and fat contamination.

## MRS Data Analysis

Water-scaled metabolite concentrations from the PRESS and the MEGA-PRESS data were analyzed with the LCModel analysis software version 6.3-1H (Provencher, 1993). A simulated MEGA-PRESS basis set and constrained baseline were used in the fit. For valid PRESS results the standard deviation was required to be %SD < 20% (Cramér-Rao lower bounds/CRLB) and %SD < 20% for the GABA values in the MEGA-PRESS data (**Tables 1**, **2**).

The curve fitting of the GABA peak at 3.02 ppm was visually inspected in each individual. Additional validation was performed using an in-house quality-assurance script implemented in Python.[1] This script validates individual spectra against a typical (group average) spectrum for the region and identifies any aberrations in shape of the spectra or features in the residuals—as would result from artifacts or poor fitting. It additionally checks quantitative metrics such as linewidth, signal-to-noise, and CRLB across the fit. This process is intended to guide and to complement regular visual inspection (**Figure 2**).

## Statistics

Demographic data, ASSQ, intellectual ability (WASI) and MRS data were analyzed using SPSS (version 12.01) (IBM Corp. USA) statistical analysis software package. MRS measurements were non-normally distributed, even after log transformation, and results were thus analyzed using the non-parametric Mann Whitney U-test and Skipped Pearson and Spearman correlation (Pernet et al., 2013) with the level of significance being $p \leq 0.05$. The GABA+/Cr ratio was normally distributed and the Students *t*-test was therefore applied.

## Quantifying Tissue Composition

GABA tissue concentration has been demonstrated to differ between gray matter (GM) and white matter (WM; Petroff et al., 1988). The segmentation of GM, WM and CSF within the MRS voxel was carried out on the 3D-SPGR images. Segmentation was performed using in-house scripts based on

[1]https://www.python.org

**TABLE 1 | SNR (signal to noise ratio) and linewidth expressed as FWHM (Full width at half maximum) in ppm.**

| MEGA-PRESS | ASD (*n* = 14) | TD (*n* = 21) | *P*-value |
|---|---|---|---|
| SNR | 20.7 ± 4.1/20.5 | 19.9 ± 2.6/20.0 | 0.41 |
| FWHM | 0.044 ± 0.014/0.038 | 0.038 ± 0.010/0.033 | 0.26 |
| **PRESS** | **ASD (*n* = 14)** | **TD (*n* = 24)** | ***P*-value** |
| SNR | 26.6 ± 5.3/28.0 | 28.7 ± 2.6/29.0 | 0.39 |
| FWHM | 0.047 ± 0.015/0.046 | 0.044 ± 0.009/0.048 | 0.82 |

**TABLE 2 | % CRLB (Cramer-Rao Lower Bound) for selected metabolites.**

| MEGA-PRESS | ASD (*n* = 14) | TD (*n* = 21) | *P*-value |
|---|---|---|---|
| GABA+ | 0.07 ± 0.015/0.07 | 0.07 ± 0.011/0.06 | 0.70 |
| **PRESS** | **ASD (*n* = 14)** | **TD (*n* = 24)** | ***P*-value** |
| Cr | 0.02 ± 0.006/0.02 | 0.02 ± 0.004/0.02 | 0.94 |
| NAA | 0.02 ± 0.005/0.02 | 0.02 ± 0.003/0.02 | 0.25 |
| MI | 0.04 ± 0.007/0.03 | 0.03 ± 0.006/0.03 | 0.75 |
| Cho | 0.02 ± 0.004/0.02 | 0.02 ± 0.002/0.02 | 0.39 |
| *Glx* | 0.04 ± 0.008/0.04 | 0.04 ± 0.006/0.04 | 0.99 |

SPM's unified segmentation and normalization functionality.[2] The same software was also used to calculate total GM and WM volume and total brain volume.

# Results

## Medication

Five of the 14 boys in the ASD group used psychotropic medication; one was medicated with lamotrigine (antiepilepticum), two with metylphenidate (ADHD medication), one with aripriprazol (antipsychotic medication) and one with metylphenidate and levetiracetam (antiepilepticum). A further two used melatonin. None of the participants in the TD group were on medication.

## Autism Severity

All TD boys scored below the screening cut-off for ASD on the ASSQ, and all the ASD boys scored higher than all the TD boys (mean ASSQ score 23.5 in the ASD group vs. 1.5 in the TD group, $p < 0.001$).

## WASI Two Subtest Format

The results for the WASI total score and scores for the two subtests are shown in **Table 3**.

As can be seen in **Table 1**, the ASD group scored below the TD group on all three WASI variables. The difference is significant for the Vocabulary scores, and approaches significance for the Matrix Reasoning scores ($p = 0.07$). However, the total score including both subtests was not significantly different between groups and both groups were within normal range of intellectual capacity.

[2]http://www.fil.ion.ucl.ac.uk/spm/software/spm8/

## MRS Results

Below is a summary of findings for both the PRESS and MEGA-PRESS sequences in the two groups. **Table 4** there were no statistically significant differences between the two groups (ASD/TD) in GABA+ and GABA+/Cr levels. For comparison with previous studies, analyses were also performed with GABA+/Glx and GABA+/NAA, but no statistically significant differences between the two groups were found. See also scatter plots for the distribution of GABA+ and GABA+/Cr in the two groups (**Figure 3**).

There was a statistically significant negative correlation between the ASSQ score and the GABA+/Cr concentration in the ASD group, (**Figure 4**). The significant negative correlation between GABA+ and ASSQ when using the Pearson correlation coefficient did not hold when the skipped Pearson and skipped Spearman test was applied.

The correlations did not survive correcting for multiple comparisons due to the relative low number of participants. Controlling for WASI did not change the strength or direction of the correlation between ASSQ and GABA, but the statistical significance was lost.

ASSQ is a questionnaire instrument applied to measure autism severity with values above the cut off set at 17 indicating ASD. The TD group all had ASSQ scores below 5. Still, correlation analyses were performed between the ASSQ score and GABA+ and the ratios and none of them were statistic significant.

## Tissue Segmentation

There were no group differences in total brain volumes and segmented tissue volumes in the MRS voxel (**Table 5**). As no systematic differences in tissue composition were observed, these estimates were not considered further in the statistical analysis.

# Discussion

In our correlation analyses we found significant negative correlations in the ASD group between ASSQ score and GABA+/Cr ratio, indicating that lower GABA+/Cr ratio is associated with increasing autism severity.

This is the first paper, to our knowledge, that has examined autism symptom severity and correlated this with metabolite concentration measured from MRS using a MEGA-PRESS sequence. However, our study supports the MEG-study by Cornew et al. (2012) where they did find an association between oscillatory anomalies, which suggest E/I imbalance, and autism symptom severity. The participants in the Cornew study were 50 children, 27 with ASD and 23 age-matched controls, aged 6–15 years.

Our study did not reveal any group-level differences between the ASD and the TD group regarding the concentration of GABA+ and GABA+/Cr. These results do not fully support all the results from the three previous studies (Harada et al., 2011b; Gaetz et al., 2014; Rojas et al., 2014) that have applied MRS MEGA-PRESS to compare brain GABA+ concentrations in children with ASD.

Harada et al. (2011b) found significantly lower GABA+/NAA and GABA+/Glu ratio in the ASD group, with a voxel placed in

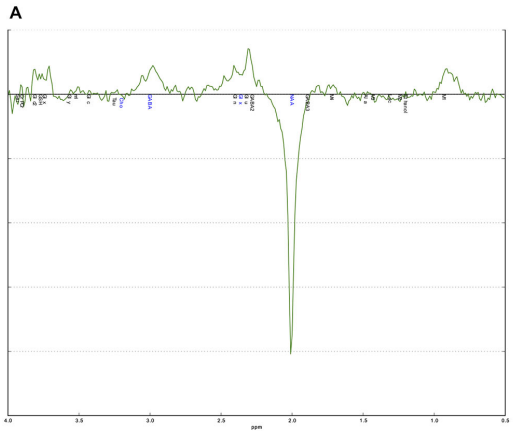


**FIGURE 2 | Examples of a good and a poor quality MEGA-PRESS spectrum**. **(A)** The MEGA-PRESS spectrum shows a clearly visible GABA+ peak at 3.0 ppm. The difference between the data and the fitted curve, the residual, is small indicating a good fit. **(B)** The MEGA-PRESS spectrum is noisy with no clear GABA+ peak at 3.0 ppm. The residual is also large indicating a poor fit.

**TABLE 3 | WASI IQ scores**.

| | ASD (*n* = 11) | TD (*n* = 24) | *P*-value |
|---|---|---|---|
| WASI, total score | 97.6 ± 19.6/95.0 | 108.3 ± 14.3/107.0 | 0.11 |
| Raw score Vocabulary | 30.8 ± 15.4/34.0 | 40.4 ± 9.4/41.5 | 0.04 |
| Raw score Matrix Reasoning | 19.7 ± 8.3/23.0 | 25.8 ± 9.3/26.5 | 0.07 |

*Data are presented as mean ± standard deviation/median value.*

the left frontal lobe, partly overlapping with our voxel placement in the left ACC. Gaetz et al. (2014) found a significantly reduced GABA+/Cr ratio in the ASD group in voxels placed in the left motor and auditory cortex, while Rojas reported reduced GABA+/Cr ratio in children with ASD and their unaffected siblings compared to TD controls in a voxel placed in the left auditory cortex (Rojas et al., 2014).

**TABLE 4 | The summarized results for the quantization of relevant metabolites from the MEGA-PRESS and PRESS sequences**.

| MEGA-PRESS | ASD (*n* = 14) | TD (*n* = 21) | *P*-value |
|---|---|---|---|
| GABA+ | 2.49 ± 0.40/2.53 | 2.66 ± 0.45/2.56 | 0.41 |
| GABA+/Cr* | 0.42 ± 0.066/0.42 | 0.44 ± 0.074/0.43 | 0.30 |
| **PRESS** | **ASD (*n* = 14)** | **TD (*n* = 24)** | ***P*-value** |
| Cr | 6.01 ± 0.54/6.00 | 6.08 ± 0.39/6.05 | 0.71 |
| NAA | 8.61 ± 0.60/8.66 | 8.99 ± 0.73/9.08 | 0.10 |
| MI | 5.18 ± 0.87/5.08 | 4.98 ± 0.54/4.95 | 0.69 |
| Cho | 2.17 ± 0.21/2.16 | 2.19 ± 0.21/2.18 | 0.80 |
| *Glx* | 15.2 ± 1.58/15.00 | 15.49 ± 1.17/15.48 | 0.35 |

*The concentrations obtained from the MEGA-PRESS experiment use water as an internal concentration reference. *Cr from PRESS. Data are presented as mean ± standard deviation/median. Values for metabolites are in Institutional Units.*

An obvious difference between the three studies is that the voxel placements in our study differed from the others. The ACC region was chosen for voxel placement since the area is implicated in the pathophysiology of ASD due to its crucial role in social cognitive processes (Devinsky et al., 1995; Bush et al., 2000; Amodio and Frith, 2006). The ACC region is also known to be involved/affected in patients with psychiatric disorder including schizophrenia, obsessive–compulsive disorder, depression, post-traumatic stress disorder and ASD (Benes, 1993; Rauch et al., 1994; Devinsky et al., 1995; DeVito et al., 2007; Minzenberg et al., 2009).

Several recent studies implicate the ACC region in ASD, and also connected it to imbalances in GABA/Glu. For example, Naaijen et al. (2015) found aberrant fronto-striatal glutamatergic levels, including the ACC in children with ASD. Similarly, Cochran et al. (2015) found that glutamate-GABA interaction and balance was disturbed in ASD subjects, and that this correlated with social cognition measures. Finally, Hall et al. (2015) used an auditory oddball task that requires complex cognition, and measured both electrophysiology responses and MRS glutamate levels from the ACC and found a significant positive correlation between the two indicating a specific connection between an index of glutamate neurotransmitter function and frontal event-related potential. Thus, the ACC region has been shown to be sensitive for subtle changes in metabolite levels during social cognitive and rest periods.

Both Harada et al. (2011b) and Gaetz et al. (2014) showed that there might be regional differences as they found lower concentration of GABA+ in ASD individuals in some areas of the brain and with no group differences in other areas compared to TD individuals. Further studies are needed to investigate the role of voxel placements for GABA+ measurements.

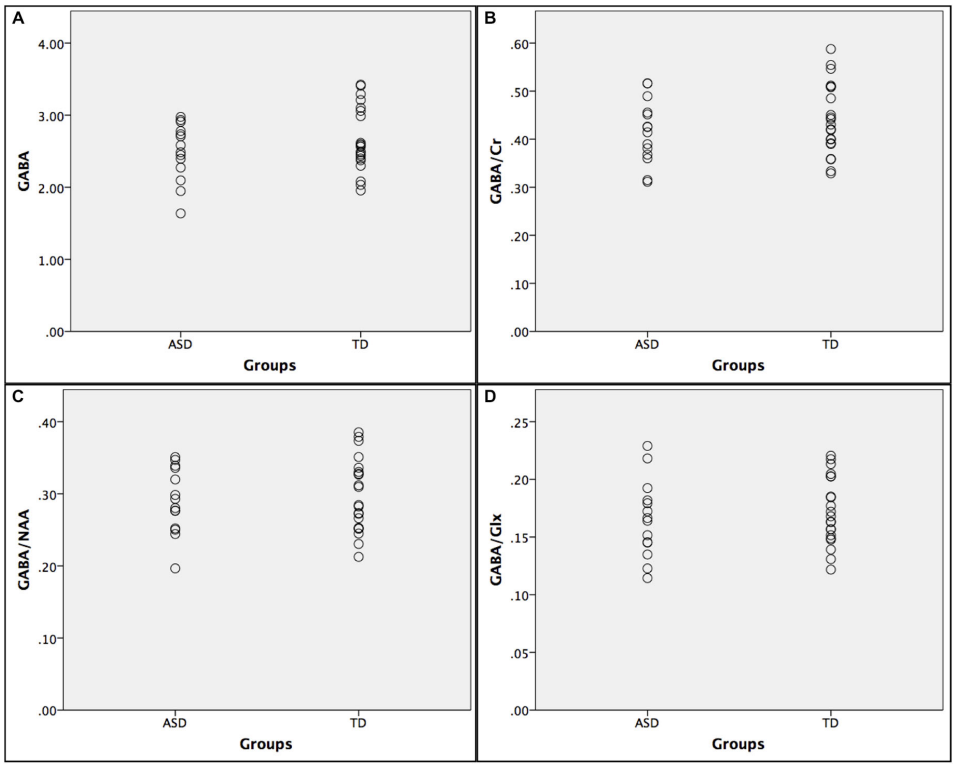


**FIGURE 3 | Scatter plots showing the distribution of GABA+ ratios in the autism spectrum disorder (ASD) and typically developing (TD) groups**. **(A)** GABA+; **(B)** GABA+/Cr; **(C)** GABA+/NAA; and **(D)** GABA+/Glx.

Another possibility is that there are subgroups in the ASD population where differences in GABA+ or the metabolites used in the GABA+ ratio exist, which may explain the heterogeneity in the ASD phenotype. All four studies on ASD children, referred to in this manuscript, including our study, had small sample sizes. The subjects in the Harada paper had a wide age range (Harada et al., 2011b), and there was no information whether the included subjects were boys or girls, while both boys and girls were included in the Gaetz et al. (2014) and Rojas et al. (2014) paper. In addition the Gaetz paper lacked sufficient information about the recruitment of TD. As pointed out earlier, gender may be a significant factor in predicting GABA+ levels (Harada et al., 2011a; O'Gorman et al., 2011), however other studies have not shown this difference (Puts and Edden, 2012). We need more knowledge on how age and gender influences GABA+ levels and we cannot rule out that such group differences contribute to the previous results.

A strength in our study is that we only included boys, and there were no age differences between the two study groups. Moreover, both groups had total mean IQ scores within normal range. Thus we think our results are representative for boys in this age group. The discrepancy between the groups in IQ with lower verbal IQ scores in the ASD group was expected, since it reflects a clinical characteristic of children with ASD (Mayes and Calhoun, 2003).

Controlling for WASI did not change the strength or direction of the correlation between ASSQ and GABA+, indicating that WASI was not a confounder in our analyses. Thus the variation in performance on WASI (IQ) seems secondary compared to a composite measure of ASD symptoms (ASSQ) to describe the variation GABA+/Cr ratio in the ACC. The lower GABA values were associated with more severe ASD symptoms. More in depth neuropsychiatric tests were not included in this study, therefore it is not possible to evaluate performance of functions typically associated with the ACC region such as executive control, theory of mind etc. While there may be some association between regional GABA+ content and neuropsychological performance, further studies are needed to explore these possible confounding factors.

Use of medication differed between the groups; 5 of 14 boys in the ASD group used psychotropic medication and an

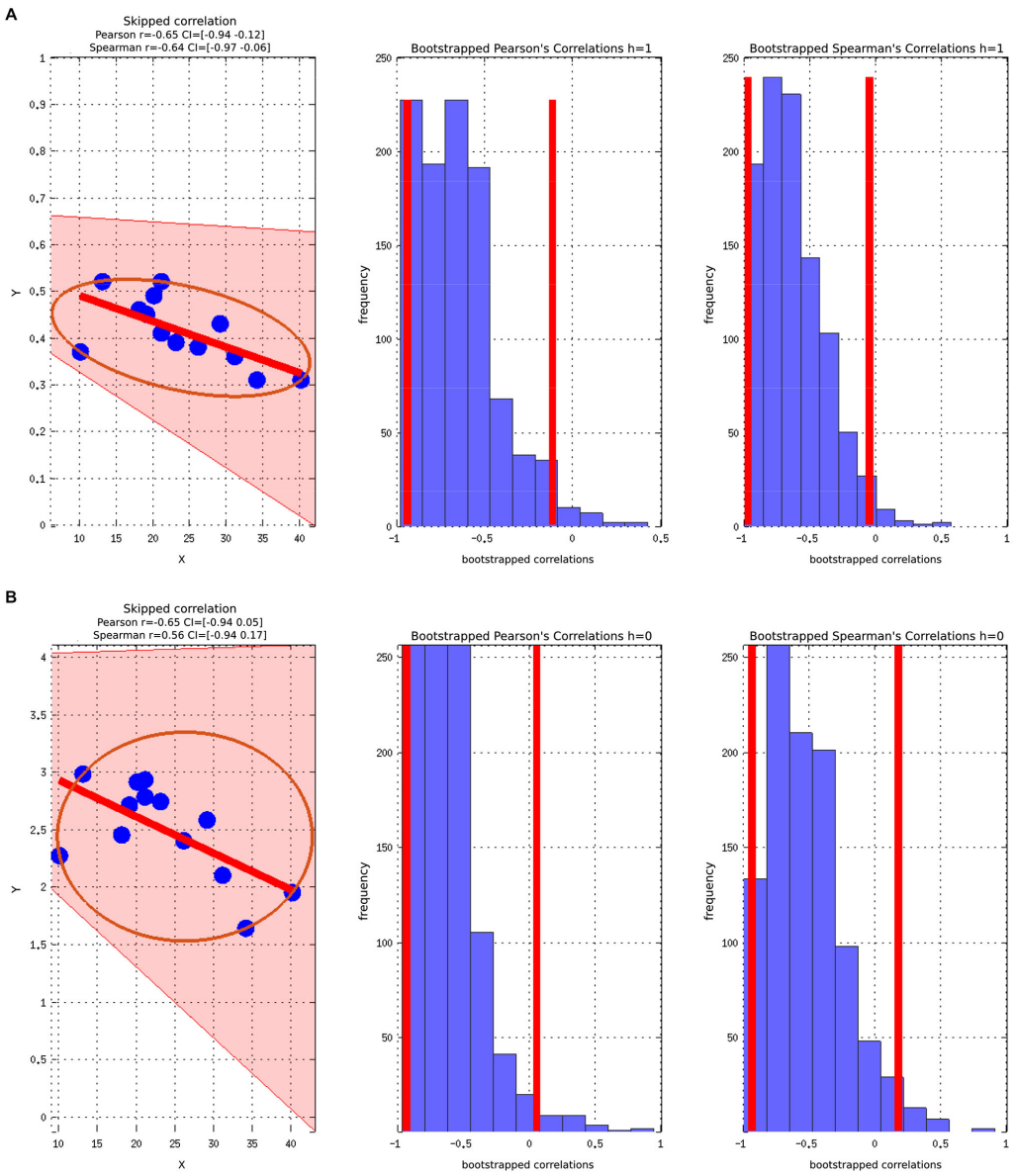


**FIGURE 4 | Skipped Pearson and Spearman correlations with a 95% confidence interval (CI). The figures also include Bootstrapped Pearson and Bootstrapped Spearmans plots**. **(A)** ASSQ vs. GABA+/Cr; **(B)** ASSQ vs. GABA+.

additional two used melatonin, vs. no medications in the TD group. GABA inhibition is a well-known target for therapeutic anticonvulsant treatment, but neither of the two antiepileptic drugs lamotrigine and levetiracetam have GABA enhancement as their main effect (Olsen and Avoli, 1997). An MRS study by Kuzniecky et al. (2002) showed a 25% increase in GABA concentration on five healthy volunteers administered with long-term dosing of lamotrigine measured from a voxel in the occipital

**TABLE 5 | Results for the total brain volume ratios measured by tissue segmentation, and results from tissue segmentation in the MRS voxel.**

| | ASD | TD | *P*-value |
|---|---|---|---|
| Brain volume Gray | 855 ± 72/865 | 882 ± 75/874 | 0.50 |
| Brain volume White | 405 ± 37/406 | 418 ± 42/422 | 0.38 |
| Brain volume total | 1261 ± 99/1277 | 1300 ± 112/1301 | 0.46 |
| Voxel CSF | 5.5 ± 4.7/4.3 | 3.4 ± 2.6/2.5 | 0.20 |
| Voxel Gray | 44.7 ± 5.6/45.2 | 45.2 ± 5.2/43.9 | 0.89 |
| Voxel White | 48.7 ± 10.3/53.0 | 51.3 ± 7.2/53.4 | 0.39 |

*Data are presented as mean ± standard deviation/median. All volumes are in ml.*

lobe. The exact mechanism of levetiracetam in treating epilepsy is unknown, and *in vivo* studies of levetiracetam on whole mice brain preparation haven not detected any differences on the overall GABA and glutamate concentration (Sills et al., 1997).

All analyses were performed both with and without the two boys using antiepileptic drug and it had no significant effect on the results for the group level analyses.

Methylphenidate has a dopaminergic effect similar to amphetamine and cocaine by blocking the dopamine and noradrenaline transporters in pre-synapse, thus increasing the availability of these neurotransmitters in the synaptic cleft. GABA can modify the activity of dopaminergic neurons, and psychostimulants (Pierce and Kalivas, 1997) may also influence the activity of GABAergic neurons directly. One study on female rats found that repeated treatment with methylphenidate significantly increased the expression of GAD65 and GAD667 in the prefrontal cortex thus increasing vesicular GABA levels (Freese et al., 2012). There is limited research available on the effects of methylphenidate on GABA levels in humans; therefore a possible impact on GABA levels cannot be excluded.

An 18F-Fluoroflumazenil PET study on 17 patients with schizophrenia found that aripiprazole administration resulted in increased GABA transmission in the prefrontal cortex (Lee et al., 2013).

Melatonin activates the benzodiazepine-GABAA receptor complex with consequent enhancement of GABAergic activity (Niles, 2006).

A common denominator of the four types of psychotropic medication and melatonin is that they may all increase GABA+ levels, and they may thereby potentially mask an underlying group difference in GABA+ level between the ASD and the TD group. When comparing with the three other MEGA-PRESS studies on children with ASD, we found no information regarding epilepsy or usage of psychotropic medication in the Harada et al. (2011b) paper. Gaetz et al. (2014) and Rojas et al. (2014) excluded children with epilepsy from the ASD group. In the Rojas et al. (2014) study five ASD children used psychotropic medication, four SSRI and one an atypical antipsychotic drug. In the Gaetz et al. (2014) study four ASD children used psychotropic medication, two a SSRI, one a "medication to treat mood disorder" and one atypical antipsychotics. Regarding the effect of medication on GABA+/Cr ratio, Rojas et al. (2008), Gaetz et al. (2014) and our own study are comparable.

Most of the children in the Harada et al. (2011b) study were sedated with triclofos sodium, a GABA agonist, which might complicate the interpretation of their results. Reduced Glu and increased GABA have been observed bilaterally in all cortical regions examined following sedation with propofol (Zhang et al., 2009), which is another GABA agonist. It may be that an effect of sedation has influenced the MRS measurements and contributed to their results.

Beyond any real underlying physiological variations, our GABA+ measurements may also be affected by the different MRI acquisition parameters used—in particular, the number of averages. We chose to average over 128 pairs in order to keep the scan time down and thereby minimize any errors due to head movement. Lying completely still in the scanner is challenging for children, and even more so for children with ASD. Both the Harada et al. (2011b) and the Gaetz et al. (2014) paper used 128 averaged pairs in their MR protocols, while in the paper by Rojas they averaged 256 pairs (Rojas et al., 2014), giving twice as many spectral acquisitions and correspondingly improved signal-to-noise, but also increasing the scan time significantly (to about 20 min with their TR of 2500 ms). A recent study from our group (Craven abstract ISMRM 2014) suggests that an average of 256 pairs at TR = 1500 ms is more optimal for measuring GABA+ using MEGA-PRESS.

Applying the MEGA-PRESS GABA-editing MRS sequence for measurement of GABA+ in the human brain has become increasingly of interest during the last couple of years, especially when evaluating neurochemical underpinnings of psychiatric disorders. Even so, the most stable denominator for normalized GABA+ measures, like basic sequence parameters and specific method of spectral quantification, still remains an active area of investigation. Thus, it is a possibility that there exists a difference in GABA+ levels between groups, but that we were not able to measure it. MR parameters, like number of averages, and voxel placement are probably more a factor than the MEGA-PRESS method itself, and with further optimization a more subtle difference may become apparent.

As mentioned in the introduction, ASD patients represent a very heterogeneous group, probably reflecting variability within the autism spectrum with different neurobiological substrates. It is hardly likely that we will find one causal model for ASD. Using different methods, e.g., questionnaires and standardized interviews, to subgroup the ASD population and understand the mechanisms involved may prove vital in future ASD studies, including MRS studies.

## Conclusion

We found a negative correlation between autism severity, as measured by the ASSQ and GABA+/Cr in the ASD group, but no GABA+ or GABA+/Cr level differences between ASD and TD groups. The intricate balance between GABA and glutamate in the brain in ASD is not completely understood. Recent evidence even supports a deviant function of the GABA neurotransmitter in children with ASD with an excitatory rather than an inhibitory effect indicating that absolute levels of GABA and glutamate may

not reflect the E/I imbalance in the same way in ASD as in other patient groups (Tyzio et al., 2014).

The present study clearly shows that autism severity must be taken into account to elucidate the E/I hypothesis in autism. Future studies with larger cohorts of ASD subjects in different ages, severity and gender, and in different brain regions are required to reveal the levels of GABA and glutamate and the importance of the balance between them in relation to ASD symptoms.

## Author Contributions

Substantial contribution to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; MKB, LE, KH, RG, MBP, ÅH, ARC, RN, CJE, HBW, TM, MKB (Beyer).

Drafting the work or revising it critically for important intellectual content; MKB, LE, KH, RG, MBP, ÅH, ARC, RN, CJE, HBW, TM, MKB (Beyer).

Final approval of the version to be published; MKB, LE, KH, RG, MBP, ÅH, ARC, RN, CJE, HBW, TM, MKB (Beyer).

Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved: MKB, LE, KH, RG, MBP, ÅH, ARC, RN, CJE, HBW, TM, MKB (Beyer).

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**Conflict of Interest Statement**: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

# II

# Within- and Between-Session Reproducibility of GABA Measurements With MR Spectroscopy

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**Purpose:** The reproducibility of the MEGA-PRESS (MEshcher-GArwood Point RESolved Spectroscopy) MR spectroscopy sequence for the measurement of gamma- aminobutyric acid (GABA) is addressed, focusing on optimizing the number of repetitions at two voxel locations in the human brain and associated possibilities in analysis tools.
**Materials and Methods:** Two 20-min MEGA-PRESS acquisitions were run (echo time = 68 ms, repetition time = 1800 ms, repetitions = 328): one from a 21 mL volume in the anterior cingulate cortex (ACC) and one from a 22 mL volume in the left Broca's area in 21 healthy male volunteers (age 32 years ± 6[SD]). Subjects were scanned twice with identical protocols, 1 week apart. Data were acquired on a 3 Tesla GE Discovery 750 scanner using a 32-channel head coil. Spectroscopy data were partitioned into shorter epochs, numerically equivalent to scans of progressively increasing duration, and compared both within and between sessions. Three different analysis schemes were applied: (1) Vendor prototype preprocessor, with quantification by LCModel. (2) Pure Gannet pipeline. (3) Preprocessing with Gannet, and quantification with LCModel. The coefficient of variation (CV) were calculated as a measure of reproducibility.
**Results:** Increasing the number of repetitions showed improvements for within- and between-session reproducibility up to around 218 repetitions. (CV ranging from 4 to 14%). Gannet combined with LCModel approach proved the best method. (CV = 4–5%). Measurements from the ACC area had higher CVs than the Broca area. (CV = 6–14% versus 4–7%).
**Conclusion:** Measurement in the Broca area yields better reproducibility than the ACC. With appropriate acquisition times and preprocessing tools, measurements from the ACC area are also reliable.
**Level of Evidence:** 1.

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Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the brain and is present in more than 30–40% of all synapses; only glutamate, the main excitatory neurotransmitter, is more widely distributed.[1] There are few, if any, areas in the brain that are not affected by the action of glutamate and GABA,[1] and the widespread presence and usage of these transmitters suggests that they are critically involved in all major functions of the central nervous system (CNS), as well as in various diseases and disorders of the CNS.[2]

MR spectroscopy (MRS) is a noninvasive method used to measure the concentration of brain metabolites in health and disease.[3] By selectively exciting a volume of interest, a voxel, in a specific brain region, metabolite concentrations can be estimated by measuring characteristic resonances of the various metabolites, at frequencies differing slightly from that of water.

The concentration of GABA in the brain is relatively low, ranging from 0.5 to 1.4 mmol/cm$^3$ in healthy adults

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and is heterogeneously distributed throughout the brain.[4] GABA is difficult to measure using standard MRS sequences due to the spectral overlap with other metabolites with higher concentration. One solution is to apply special spectral editing techniques, such as the MEGA-PRESS (MEshcher-GArwood Point RESolved Spectroscopy) sequence.[5]

In the MEGA-PRESS sequence, a pair of frequency selective inversion pulses are applied within a standard PRESS sequence, allowing discrimination between overlapping coupled and uncoupled spins. For GABA-editing, the editing pulses are applied to the C-3 protons of GABA at 1.9 parts per million (ppm). Due to the spin–spin coupling, the C-4 protons of GABA at 3.02 ppm are affected while other metabolite peaks like the strong creatinine (Cr) peak remains unaffected. Subtracting the spectrum from a second acquisition with an editing pulse applied symmetric to the water signal, at 7.5 ppm, will give a difference spectrum without the strong singlet signal from Cr, allowing quantification of the GABA peak at 3.02 ppm.

Conventional GABA editing sequences face challenges due to co-editing of a signal arising from macromolecules at 3.0 ppm (J-coupled with a resonance at 1.7 ppm), which superimposes on the GABA doublet and may contribute to as much as 60% of the quantified value of GABA.[6] Various schemes have been proposed to minimize this factor, e.g,[6–8] however, many of these suffer limitations, and none as yet has been broadly adopted. For present purposes, we retain the conventional sequence (with no additional macromolecule suppression) and denote our concentrations GABA+, i.e., GABA plus an additional macromolecule contribution.

Both short-term[9–11] and long-term[12] between-session and within-session[13,14] variability studies indicates that individual GABA levels are generally fairly stable in healthy subjects, but also that the reproducibility of estimates varies in different brain regions. In addition, studies e.g.,[15] have shown that the reproducibility of estimates from a given dataset differ between software packages. Optimal parameters for reliable GABA estimation with the MEGA-PRESS sequence and subsequent processing of data is therefore, important for reliable GABA measurements.

In an attempt to isolate any processing-specific issues affecting reproducibility of the final quantification, different combinations of preprocessor implementation and quantification methods were investigated. Preprocessing was performed with a vendor prototype pipeline,[16] and using the preprocessor component of the freely-available Gannet software[17]. Preprocessed data were then fitted and quantified using the ubiquitous LCModel[18] and the standard fitting component from Gannet 17.

Both preprocessors implemented coil combination, zero-filling and line broadening before phase adjustment and alignment of frames in the frequency domain. In general, line broadening before modeling in LCModel (http://s-provencher.com/pub/LCModel/manual/manual.pdf) is discouraged, and there could be at least two reasons for this. The first is that it would broaden the peaks and make it more difficult to separate nearby metabolites based on fine details; for GABA+ this is a nonissue because only one broad multiplet is being modeling. The other reason is that it invalidates the signal-to-noise ratio (SNR) and the CRLB estimates (line broadening will reduce noise). As a result of this, these estimates are not reported in this manuscript. The argument for retaining it is that it mitigates some subtraction/phasing artifacts.

The Gannet preprocessor additionally performs outlier rejection, to exclude frames exhibiting excessive frequency shift (possibly indicative of subject motion or other transient signal quality issues).

While LCModel attempts to model a broad section of the acquired spectrum as a linear combination of basis sets, The GannetFit component uses a comparatively simple nonlinear least squares approach to model the water signal and the GABA peak at 3 ppm.

These current analyses enable us to measure the variability in GABA and use these estimates to calculate required sample size in future GABA MRS experiments involving correlations between GABA levels and behavior. The time-windowing technique also allows us to do the same required sample size calculations at different scan lengths.

The purpose of this study addresses ongoing questions of between- and within-session reproducibility of the MEGA-PRESS sequence, focusing on optimizing the scan lengths for two specific voxel placements in the human brain.

## Materials and Methods

### Participants

Twenty-one healthy male volunteers (age $32 \pm 6$[SD] years) were recruited in accordance with approval by the local data protection office and after obtaining written informed consent. Participants were instructed to abstain from caffeine, alcohol, and nicotine, exercise, and sex for the 12 h before each MR examination due to possible influences on GABA levels.

### MRI Acquisition

In vivo MEGA-PRESS data were acquired on a 3 Tesla (T) GE Discover 750 scanner system (Milwaukee, WI) with the 32-channel GE head coil, using echo time (TE) = 68 ms and repetition time (TR) = 1800 ms for 328 paired repetitions (~20 min), acquiring 4096 datapoints at 5 kHz spectral width. The data were acquired with eight-way phase cycling; averaging across the frame was not performed at the time of acquisition (noadd option = 1). A 16-ms sinc weighted Gaussian editing pulse was applied at 1.9 ppm (ON) and 7.46 ppm (OFF) in interleaved scans. One acquisition was performed from a 21 mL volume of interest in the

mid anterior cingulate cortex (ACC) and the other from a 22 mL volume in the left Broca's area. The ACC voxel was positioned in the midline of the brain, angled in the sagittal plane aligned to the foremost slope of the corpus callosum (CC). The rostral edges of the voxel was aligned to the foremost border of the CC. The Broca voxel was positioned in the left superior temporal lobe, centered anterior to the anterior central gyrus. The voxel was angled in the sagittal plan and aligned to the Sylvian fissure so that the bottom four corners of the voxel were "resting" on the Sylvian fissure. See Figure 1 for voxel placement. The order of acquiring the two areas was randomized (balanced), to avoid any bias.

Rather than selecting regions known to be particularly suited to the MEGA-PRESS acquisition, e.g., the occipital lobe,[19] the voxel placements were chosen for their relevance in basic neurophysiological and psychiatric research with critical involvement in the default mode network, auditory processing and brain connectivity in general.

The MR scanning protocol also included a three-dimensional (3D) FSPGR T1 weighted anatomical scan (number of slices = 188, slice thickness = 1.0 mm, TR = 7.8 ms, TE = 2.95 ms, inversion time (TI) = 450 ms, field of view = $256 \times 256\,mm^2$ flip angle = 12 degrees, matrix = $256 \times 256$) for anatomical imaging and positioning of the MRS voxel.

All subjects were scanned twice with identical protocols, at the same time of the day, 1 week apart. The subjects were instructed to lie completely still during the acquisitions, although no additional restraints were used; this mimics a standard MR-session.

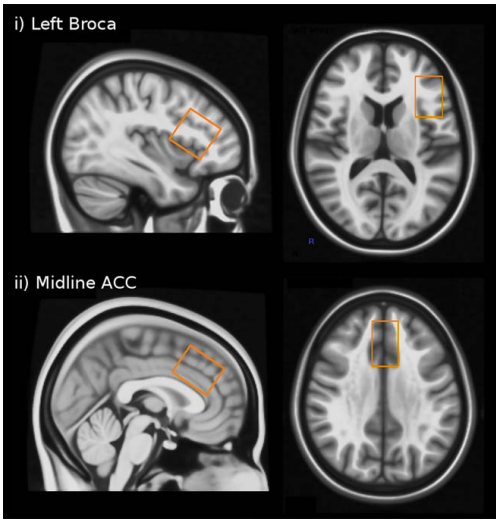


**FIGURE 1: Voxel placement.**

## *MRS Data Analysis*

*THE TIME WINDOW APPROACH.* Long acquisitions (20 min) from each of the two regions were subdivided to produce datasets equivalent to various shorter scan lengths (between 82 and 328 repetitions of edit on and off). The subdivision was performed after phase adjustment, coil combination, and realignment, just before averaging across frames for subtraction of edited and unedited spectra. For any given acquisition, the same 16 water reference frames were used with all shorter subsets.

The time-windowing approach is illustrated in Figure 2. To assess between-session reproducibility for acquisitions of N repetitions, the first N repetitions from the session one acquisition for the chosen region were quantified, and compared with the first N repetitions from the session two data for the same region.

To assess within-session reproducibility for acquisitions of N repetitions, contiguous segments of N repetitions (beginning at defined, regular positions) were extracted from individual acquisitions and quantified; different segments from the same acquisition were compared, as previously, using the same water reference

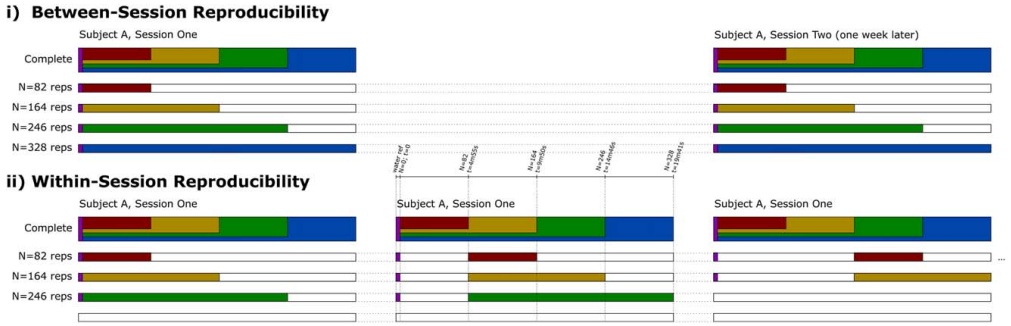


**FIGURE 2: Application of the windowing method for between- and within-session reproducibility. Block sizes are expressed internally as rational fractions of the total scan length; eight such subdivitions are examined (ref Table 1). The figures show a representative subset of comparisons. (i) Between-session reproducibility. For each location, in each subject, the first N = 82 repetitions in session one are compared with the first 82 repetitions in session two (red); the first N = 164 repetitions in session one are compared with the first 164 repetitions in session two, and so forth for various lengths up to N = 328 repetitions (blue). The same 16 water reference frames (purple) are used for each length N. (ii) Within-session reproducibility. For each location, subject and session, the entire scan is subdivided into evenly distributed regions of length N, for various values of N from 82 (shown in red) through to 246 (green). The figure shows four conveniently aligned sections for clarity, it is not that way for every length.**

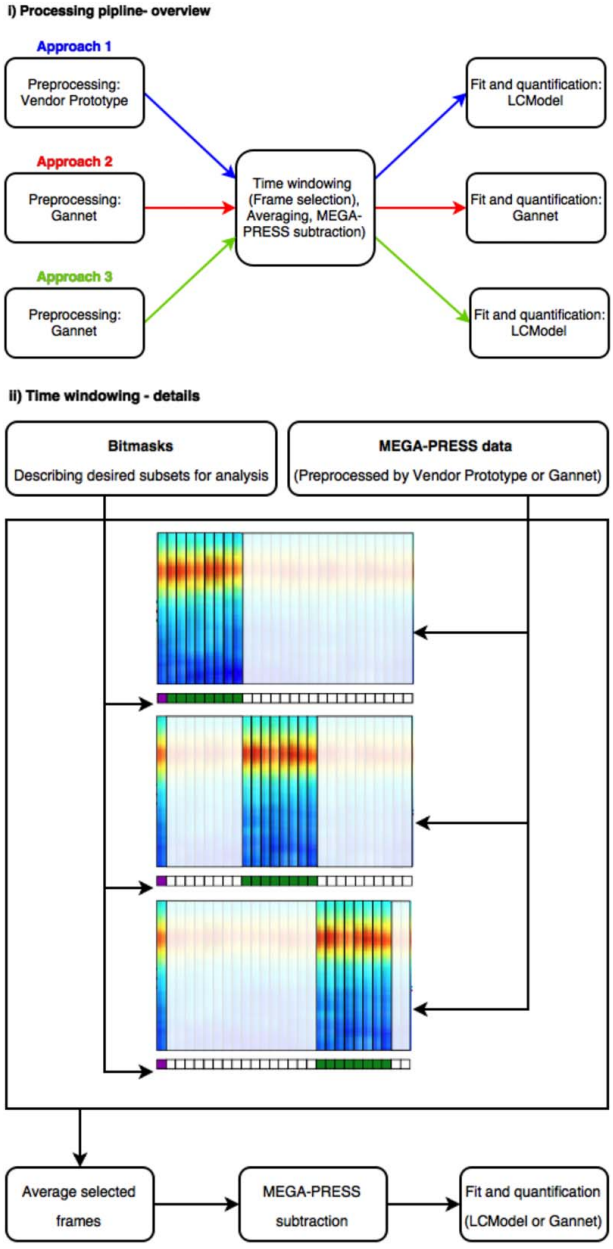


**FIGURE 3: Processing pipeline. (i) Processing pipline, overview. Processing pipeline used for windowed spectroscopy data analysis; The vendor prototype and Gannet preprocessing Mathlab based tools were modified to add a masking and selection function immediately before averaging for MEGA-PRESS subtraction and quantification. The three different analyzing approaches are depicted in the figure. (ii) Time windowing, details. Bitmasks describing the selected subsets for analysis are applied to the MEGA-PRESS data. The same water reference (magenta) is used for all subsets. The selected frames are averaged, and the MEGA-PRESS difference spectra is generated by subtracting average ON frames from average OFF frames. Last, the difference spectra is fitted and quantification is performed in either LCModel or Gannet.**

frames. For this case, data from session one and session two of each subject were pooled.

*ANALYSIS TOOLS.* Three different tools were used, to evaluate different combinations of preprocessor and fitting algorithm and isolate any processor-specific sources of variation, see Figure 3 for details. The Gannet and LCModel quantification methods use very different approaches to modeling, both of which have demonstrated good reliability for GABA data 15. Both preprocessor implementations, GannetLoad and the vendor prototype, written in Matlab (https://se.mathworks.com/products/matlab/ - version 2014b), were amenable to local modifications to implement the time-windowing necessary for the current analysis.

*WATER SCALING AND TISSUE CORRECTION.* Values reported herein are water scaled and adjusted for tissue content. In the case of Gannet, this was performed using the Harris implementation[20]; correction factors for macromolecule content (in vivo only) and editing efficiency were also incorporated. For the LCModel case, in vivo data were analyzed using LCModel's default water concentrations value (WCONC) = 35880, while phantom data were analyzed with WCONC = 55556; in both cases, estimates were subsequently scaled to take into account water and metabolite relaxation times in the different tissue classes and to account for partial volume effects, according to tissue content estimates. Metabolite relaxation times for GABA were taken as T1 = 1310/2770 ms (in vivo/phantom),[21] T2 = 88/276 (in vivo/phantom).[22]

Tissue content was derived from tissue priors and voxel masks, based on the individual, nominal voxel placement; both implementations used SPM (http://www.fil.ion.ucl.ac.uk/spm/software/spm8/) for segmentation, and a local script for mask generation. We note that such adjustments have limited impact on reproducibility metrics in the present context: for within-subject, within-session comparison the placement (hence, tissue content) is identical; between sessions the placement is nominally the same, although slight variations inevitably occur; the average variation in estimated tissue content between sessions was 4%.

A multi-factor quality assurance step was performed on the fitted data, incorporating standard signal quality metrics from the preprocessed spectra (SNR and linewidth, full-width half maximum (FWHM)) standard deviation (CRLB) of metabolite estimate for the fitted spectrum, and comparison of the fit and residual spectra against a group average spectrum for the region to identify aberrations in shape of the spectra or features in the residuals indicative of signal artifacts or improper fit. This process guided regular visual inspection of the spectra. Finally, strong outliers (corresponding with biologically impossible estimates) were rejected; in total, two acquisitions from the ACC placements were rejected on quality grounds.

### Statistical Methods

Coefficients of variation (CVs) analysis were performed with Excel (Microsoft Office 2010), and effect size and power analyses where acquired with G*Power 3.1. All significance levels were set to 0.05.[23]

### In Vitro Validation

As a benchmark for performance under ideal conditions, in vitro phantom studies were included. The phantom MEGA-PRESS acquisitions were performed using the same sequence parameters as for human subjects, with a voxel size 22 mL.

The metabolite solution was based on the GE "braino" MRS-HD-Sphere spectroscopy phantom used in Schirmer and Auer,[24] with the following metabolite concentrations: 10 mM NAA, 10 mM Creatine, 3 mM Choline, 7.5 mM Myo-Inositol, 10 mM Glutamate, 5 mM Lactate, with the addition of GABA at a concentration of 2.85 mM.

Data were quantified using the same processing pipeline and time-windowing approach as described for the in vivo experiment, with the exception of tissue correction.

## Results

### MRS

*WITHIN-SESSION REPRODUCIBILITY.* In both brain regions, increasing the number of repetitions showed notable improvements in within-session reproducibility up to around 218 repetitions. (i.e., approximately 13 min). Further increases resulted in only modest gains. The Gannet combined with LCModel approach proved to be the best method in both regions. In addition, measurements from the ACC region had higher CV than the Broca region in all three analyses approaches.

With both Gannet and LCModel quantification (and either preprocessor), GABA estimates from the phantom proved very stable across a range of scan durations, with reproducibility improving gradually with the number of averages. CVs of less than 5% were seen for a medium to high number of averages. See Figure 4 for details.

*BETWEEN-SESSION REPRODUCIBILITY.* Again, in both brain regions, increasing the number of repetitions showed notable improvements in between-session reproducibility up to around 218 repetitions. (i.e., approximately 13 min). Further increases resulted in only modest gains, and actually decreased reliability beyond 262 repetitions in the ACC region. The Gannet combined with LCModel approach proved to be the better method in both regions. In addition, measurements from the ACC region had higher CVs than the Broca region in all three analyses approaches. See Figure 5 for details.

*GABA+ QUANTIFICATION RESULTS.* GABA+ quantification results for Broca and ACC and pure GABA quantification in the phantom for all three quantification approaches are presented in Table 1, with representative spectra shown in Figure 6. While good quality spectra could be obtained in both regions, the ACC data were somewhat more prone to artifacts. Phantom data shows a better-defined doublet around 3 ppm, which is masked by line broadening and macromolecule contamination in the in vivo cases.

*ESTIMATING POWER AND REQUIRED SAMPLE SIZE.* When plotting the mean GABA + and SDs at different number of repetitions for the ACC and the Broca area, with

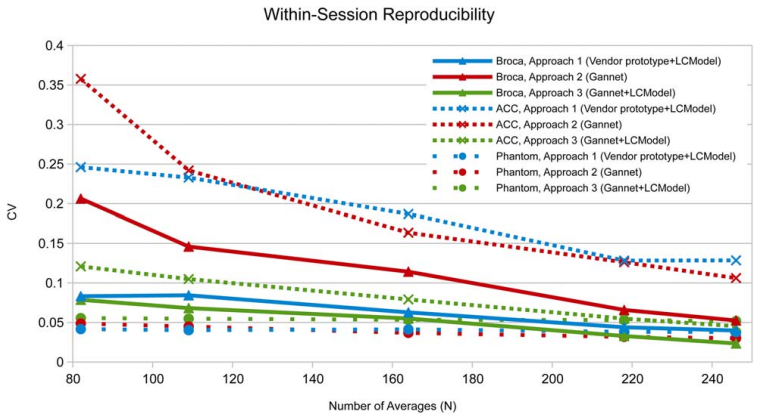


**FIGURE 4: Within-scan reproducibility for three different software options. The figure shows CV at different number of averages for the in vivo Broca and ACC area and the phantom acquisition.**

a power of 0.80, total required sample sizes can be calculated for different effects sizes, see Figure 7 (GABA + values are from the tissue corrected LCModel + Gannet analyses). For group experiments, more than 12 participants per group are required to detect an effect size of 15% or greater in the Broca area with 246 and 328 number of repetitions or in the ACC area with 246 number of repetitions. The number increases to 15 in both areas at 164 number of repetitions and 21 at 82 number of repetitions. Of interest, the number of participants required to achieve an effect size of 15% increases to 23 in the ACC area when the number of repetitions are 328.

## Discussion

Results from the phantom study indicate that the GABA+ MEGA-PRESS measuring method is reliable under optimal conditions. Given the current sequence parameter settings, we show that optimal acquisition time for in vivo studies with voxel size 21–22 mL is around 13 min (218 repetitions), but can be shorter if measuring in the Broca area. The SNR increases in proportion to voxel volume but only by the square root of the scan time. Therefore, one would expect that the optimal number of repetitions would depend on the voxel size. Measurements from the Broca area are more consistent than from the ACC region, still our study shows that data acquired from the ACC area can be reliable if the appropriate acquisition times and the most optimal software is chosen for the analyses. Gannet combined with LCModel gave the best results in our data-analyses, and had the best within- and between-subject reproducibility.

Although quantification of phantom data with Gannet was quite accurate, the %SD and between-session variability were comparatively high. Visual inspection shows the spectral peaks to be significantly better defined in the phantom case than the in vivo case, with a visible doublet around

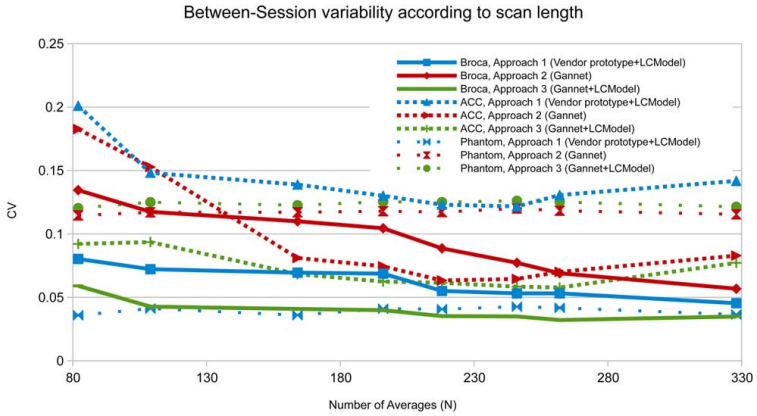


**FIGURE 5: Between-scan reproducibility for three different software options. The figure shows CV at different number of averages for the in vivo Broca and ACC area and the phantom acquisition.**

**TABLE 1. Representative GABA+ Quantification Values From In Vivo and Phantom Measurements (in mmol/L)[a]**

| | Approach 1 (vendor prototype+LCModel) | | | | | |
|---|---|---|---|---|---|---|
| **No. of repetitions** | **Broca** | **%SD** | **ACC** | **%SD** | **Phantom** | **%SD** |
| | | | | | 2.85[b] | |
| 82 | 2.17 | 5.00 | 1.65 | 7.25 | 2.19 | 6.00 |
| 108 | 2.26 | 4.33 | 1.66 | 6.58 | 2.17 | 6.50 |
| 164 | 2.26 | 4.13 | 1.69 | 5.88 | 2.17 | 6.50 |
| 197 | 2.24 | 4.17 | 1.73 | 5.67 | 2.17 | 6.50 |
| 220 | 2.27 | 4.00 | 1.67 | 5.63 | 2.16 | 6.50 |
| 246 | 2.29 | 4.00 | 1.69 | 5.63 | 2.15 | 6.50 |
| 328 | 2.48 | 3.67 | 1.66 | 5.50 | 2.17 | 6.00 |
| | **Approach 2 (Gannet)** | | | | | |
| **No. of repetitions** | **Broca** | **%SD** | **ACC** | **%SD** | **Phantom** | **%SD** |
| | | | | | 2.85[b] | |
| 82 | 1.88 | 8.53 | 1.95 | 11.05 | 2.84 | 8.71 |
| 108 | 1.88 | 8.19 | 2.01 | 9.55 | 2.81 | 8.84 |
| 164 | 1.86 | 7.01 | 1.83 | 9.68 | 2.80 | 8.93 |
| 197 | 1.85 | 6.92 | 1.81 | 8.09 | 2.76 | 9.16 |
| 220 | 1.83 | 6.36 | 1.80 | 8.05 | 2.76 | 9.03 |
| 246 | 1.84 | 6.09 | 1.76 | 7.33 | 2.77 | 9.04 |
| 328 | 1.85 | 5.69 | 1.77 | 6.64 | 2.78 | 9.05 |
| | **Approach 3 (Gannet+LCModel)** | | | | | |
| **No. of repetitions** | **Broca** | **%SD** | **ACC** | **%SD** | **Phantom** | **%SD** |
| | | | | | 2.85[b] | |
| 82 | 2.26 | 3.88 | 1.98 | 4.44 | 2.25 | 5.00 |
| 108 | 2.27 | 3.67 | 1.97 | 4.33 | 2.21 | 5.00 |
| 164 | 2.27 | 3.50 | 2.01 | 4.13 | 2.20 | 5.00 |
| 197 | 2.27 | 3.50 | 2.00 | 4.25 | 2.22 | 5.00 |
| 220 | 2.30 | 3.50 | 2.00 | 3.88 | 2.20 | 5.00 |
| 246 | 2.27 | 3.50 | 2.01 | 3.75 | 2.21 | 5.00 |
| 328 | 2.28 | 3.50 | 1.98 | 3.75 | 2.21 | 5.00 |

[a]The %SD is measured across the group and represents inter-subject variability.
[b]2.85 is the nominal GABA + concentration in the phantom.

3.0 ppm (in vivo, the doublet peaks tend to merge due to added MM contamination and increased line width). Consequently, the curve fitting in Gannet (being tuned to model in vivo data optimally) is less well suited to the phantom spectra; this may well account for the higher %SD in phantom measurements. While performance gains could be expected by adjusting the model to better fit the phantom data, in this instance we retained the standard model for a more direct comparison.

In line with our results, a previous study from our group[25] found that the optimal scan length was 256 repetitions in a 27-mL voxel placed in the occipital lobe. Also, the most recent studies measuring GABA+ with MEGA-PRESS uses between 256 and 288[26–28] repetitions.

We found the within-session in vivo variability of GABA + levels at 218 repetitions to be 2.5–6% for Broca and 5–13% for ACC and the between-session in vivo variability of GABA + levels at 218 repetitions to be 4–7% for

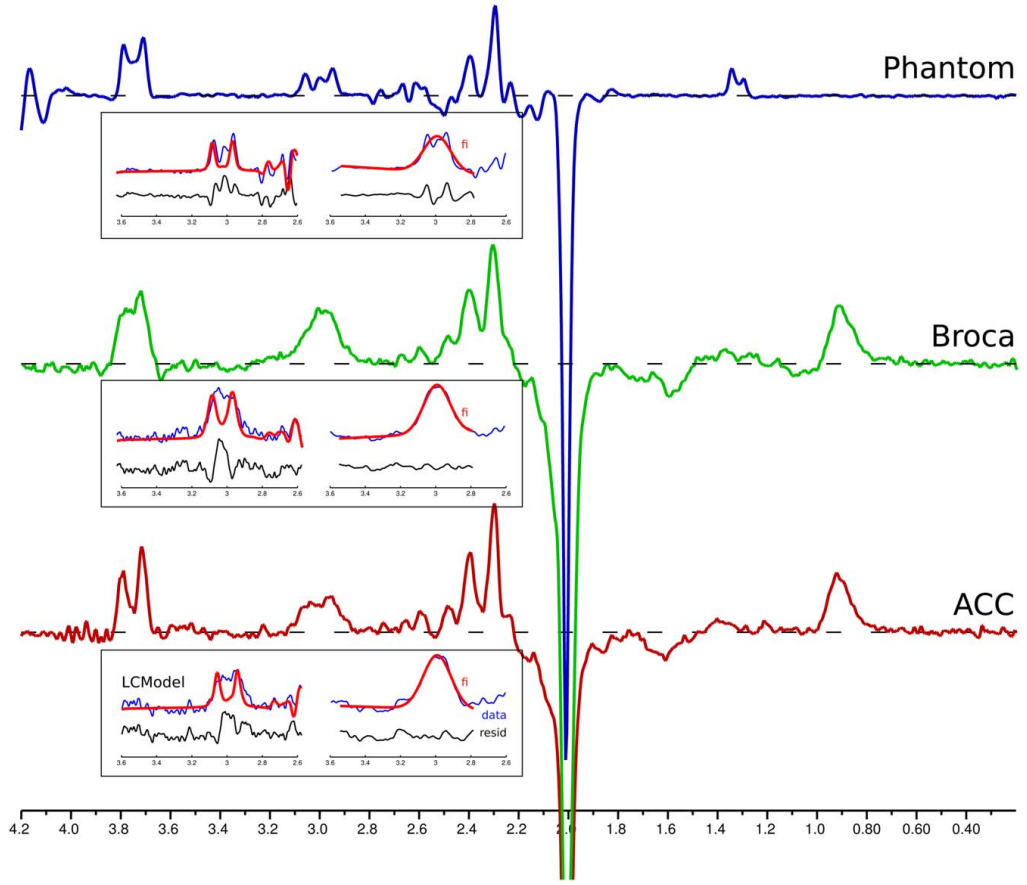


**FIGURE 6: Example spectra. Example spectra from the phantom acquisition and the in vivo acquisitions from the Broca and ACC area. Also included are spectralfits from LCModel and Gannet.**

Broca and 6–13% for ACC. This is in line with previous studies at 3T where CVs within session were found to be 13–18%[13] in the occipital lobe and between session for ACC were CV = 21,[10] sensorimotor area CV = 12,[9] occipital CV = 4.3–12.[9,12]

Only one system autoprescan is performed in the beginning of each 20 min scan, and only one set of unsuppressed water lines are acquired meaning that the reproducibility results for the within session are not quite comparable to other studies where this is collected for each scan. Whether this will improve the reproducibility compared with performing prescan and acquiring water lines for each individual scan for the within-session results will depend on how the prescan and water lines will effect, e.g., center frequency calculation, frequency drift, or editing efficiency. Bogner et al's[13] within-session reproducibility numbers from the occipital lobe were derived after repeated acquisitions with additional prescans and additional water reference lines. The CV from their study (13–18%) were higher than in this current study from an area that usually has better B0 homogeneity compared with, e.g., the ACC area, which might be due to these scan differences and could therefore account for the difference in results between the two studies. This may give better reproducibility and, hence, could account for the differences in results between the two studies.

The ACC has earlier proven to be a more difficult area for GABA+ measurements compared with, e.g., the occipital lobe and the sensory motor cortex.[19] However, the ACC has traditionally been thought as a more interesting area for neuropsychiatric research. Our data suggests that the most reliable GABA+ measurements can be achieved in the ACC area when using 220–260 repetitions, and analyzing the data with Gannet preprocessing combined with

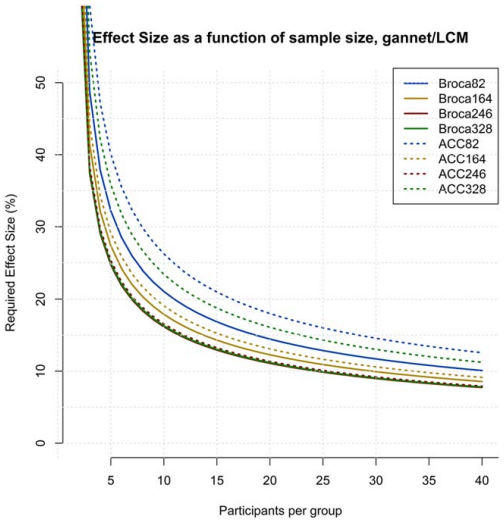


**FIGURE 7: Sample size estimation. The y-axis represents effect size and the x-axis represents sample size. A power of 0.80 is used in the analyses. GABA+ values are from the tissue corrected LCModel + Gannet analyses. Broca82 represents results from a voxel placed in the Broca area with a scan length of 82 repetitions, and so forth.**

LCmodel. Of interest, further increasing the scan length beyond a certain time can actually decrease reliability; this is particularly evident in the ACC placement, perhaps due to artifacts associated with regions of cerebrospinal fluid in the proximity to that placement. There are many possible factors contributing to the degraded performance on very long scans; the most likely is the cumulative effects of subject motion toward the end of the long scan. While both preprocessing methods can correct for associated changes in the peak frequency by simply re-aligning the spectra, either to a particular reference peak or to achieve maximum correlation between the spectra, the efficiency of the editing pulse is also affected by frequency shifts.

While the editing pulse is applied at a constant frequency throughout the scan, the frequency of the editing target may drift relative to this pulse resulting in distinct variations in the efficiency of editing for GABA and any macromolecule contamination.[29] Variations in editing efficiency are difficult to correct for retrospectively; effective handling of this issue (perhaps incorporating real-time frequency adjustment) is an area of active research.[30] In many settings, gradient-induced frequency drifts will also be a significant factor; sequences of high gradient duty cycle (such as EPI) may cause heating of various components (in particular, passive shim elements); thermal drift in the center frequency may be observed as they subsequently cool. This is seen as a gradual, somewhat linear change in center frequency throughout the course of an MRS acquisition. The presence and severity of this artifact will very much depend on the scanner hardware, and the sequences run before MRS acquisition. In the present study, gradient-induced frequency drift is not a significant factor: our particular hardware has demonstrated relatively low vulnerability to this problem; no gradient-heavy sequences were run immediately before MRS data acquisition; no symptomatic linear drift was observed in the center frequency of the scans during processing. SD of the observed water frequency was below 0.5 Hz, with a drift throughout the scan of less than 2Hz.

As written in the introduction, the concentration of GABA in the brain ranges from 0.5 to 1.4 mmol/cm$^3$ in healthy adults. Our quantification results lie above this, which most likely is due to the fact that we measure GABA + MM, and the coediting MM contributes to approximately 60%[6,31] of the measured signal.

This study does have limitations. The results in the current study probably only apply for the two areas examined and were obtained in young male healthy subjects only.

A strength is the prospective longitudinal study design. Because all participants were males, any gender effects were excluded.[15] In addition, the participants abstained from caffeine, alcohol and nicotine, exercise and sex for the 12 h before each MR examination, due to possible influencing the GABA+ levels.[32–34] To minimize any circadian effects on measured GABA+ levels,[35] all participants were scanned on the exact same time of the day, 1 week apart.

In conclusion, reliable meaningful GABA measurements can be achieved in both the ACC area and the Broca area when using appropriate scan parameters and corresponding experimental design.

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