

# Regional Myo-Inositol, Creatine, and Choline Levels Are Higher at Older Age and Scale Negatively with Visuospatial Working Memory: A Cross-Sectional Proton MR Spectroscopy Study at 7 Tesla on Normal Cognitive Ageing

Anna Lind,<sup>1</sup> Carl-Johan Boraxbekk,<sup>1,2,3</sup> Esben Thade Petersen,<sup>1,4</sup> Olaf Bjarne Paulson,<sup>5,6</sup> Hartwig Roman Siebner,<sup>1,6,7</sup> and Anouk Marsman<sup>1</sup>

<sup>1</sup>Danish Research Centre for Magnetic Resonance, Centre for Functional and Diagnostic Imaging and Research, Copenhagen University Hospital Hvidovre, Hvidovre, 2650, Denmark, <sup>2</sup>Department of Radiation Sciences, Umeå University, Umeå, 90187, Sweden, <sup>3</sup>Institute of Sports Medicine Copenhagen, Copenhagen University Hospital Bispebjerg, Copenhagen, 2400, Denmark, <sup>4</sup>Center for Magnetic Resonance, Department of Health Technology, Technical University of Denmark, Kongens Lyngby, 2800, Denmark, <sup>5</sup>Neurobiology Research Unit, Department of Neurology, Copenhagen University Hospital Rigshospitalet, Copenhagen, 2100, Denmark, <sup>6</sup>Department of Clinical Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, 2200, Denmark, and <sup>7</sup>Department of Neurology, Copenhagen University Hospital Bispebjerg, Copenhagen, 2400, Denmark

Proton MR spectroscopy (<sup>1</sup>H-MRS) has been used to assess regional neurochemical brain changes during normal ageing, but results have varied. Exploiting the increased sensitivity at ultra-high field, we performed <sup>1</sup>H-MRS in 60 healthy human volunteers to assess age-related differences in metabolite levels and their relation to cognitive ageing. Sex was balanced, and participants were assigned to a younger, middle, and older group according to their age, ranging from 18 to 79 years. They underwent 7T <sup>1</sup>H-MRS of the ACC, DLPFC, hippocampus, and thalamus and performed a visuospatial working memory task outside the scanner. A multivariate ANCOVA revealed a significant overall effect of age group on metabolite levels in all regions. Higher levels in the middle than the younger group were observed for myo-inositol (mIns) in DLPFC and hippocampus and total choline (tCho) in ACC. Higher levels in the older than the younger group were observed for mIns in hippocampus and thalamus, total creatine (tCr) and tCho in ACC and hippocampus; lower levels of glutamate (Glu) were observed in DLPFC. Higher levels in the older than the middle group were observed for mIns in hippocampus, tCr in ACC and hippocampus, tCho in hippocampus, and total *N*-acetyl aspartate (tNAA) in hippocampus. Working memory performance correlated negatively with tCr and tCho levels in ACC and mIns levels in hippocampus and thalamus, but not with tNAA or glutamate levels. As NAA and Glu are commonly regarded to reflect neuronal health and function and concentrations of mIns, tCr, and tCho are higher in glia than neurons, the findings of this study suggest a potential *in vivo* connection between cognitive ageing and higher regional levels of glia-related metabolites.

**Key words:** ACC; ageing; cognition; hippocampus; neurochemistry; thalamus

## Significance Statement

Neurochemical ageing is an integral component of age-related cognitive decline. Proton MR spectroscopy (<sup>1</sup>H-MRS) studies of *in vivo* neurochemical changes across the lifespan have, however, yielded inconclusive results. <sup>1</sup>H-MRS at ultra-high field strength can potentially improve the consistency of findings. Using 7T <sup>1</sup>H-MRS, we assessed levels of mIns, tCr, and tCho (glia-related metabolites) and tNAA and Glu (neuron-related metabolites) in ACC, DLPFC, hippocampus, and thalamus. We found higher levels of glia-related metabolites in all brain regions in older individuals. Working memory performance correlated negatively with regional levels of glia-related metabolites. This study is the first to investigate normal ageing in these brain regions using 7T <sup>1</sup>H-MRS and findings indicate that glia-related metabolites could be valuable in cognitive ageing studies.

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Correspondence should be addressed to Anouk Marsman at [anoukm@drbcmr.dk](mailto:anoukm@drbcmr.dk).

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## Introduction

Describing normal human ageing is vital for understanding what distinguishes both successful and pathologic ageing. Normal ageing involves cognitive decline related to widespread neurochemical alterations (Driscoll et al., 2003). These alterations have been studied noninvasively and *in vivo* using proton MR spectroscopy ( $^1\text{H-MRS}$ ); however, the results have been highly variable (for review, see Haga et al., 2009; Cichocka and Bereś, 2018; Cleeland et al., 2019).

The most commonly investigated brain metabolites in  $^1\text{H-MRS}$  ageing studies are myo-inositol (mIns), total creatine (tCr), total choline (tCho), total N-acetylaspartate (tNAA), and glutamate (Glu) (Cichocka and Bereś, 2018). Although results from  $^1\text{H-MRS}$  ageing studies have varied, the most consistent results indicate that mIns, tCr, and tCho levels increase with age, whereas tNAA and Glu levels decrease (Cleeland et al., 2019). Because of the variation in results, this ageing pattern is, however, not conclusively established.

One reason for the previous variation in results could be that the neurochemical changes during ageing are highly region-dependent (Eyler et al., 2016). In ageing-sensitive regions, such as PFC and hippocampus, age effects on metabolite levels are frequently observed, although the specific metabolites and the direction of the effect vary (Schubert et al., 2004; Chiu et al., 2014; Ding et al., 2016; Sporn et al., 2019). In other regions, such as thalamus, age-related effects on metabolite levels are rarely observed (Gruber et al., 2008; Yang et al., 2015; Eyler et al., 2016). These previous results may also depend on tissue composition in the ROI, as the effect of ageing on metabolite levels has been observed to differ depending on relative gray matter (GM) and white matter (WM) fractions (Ding et al., 2016). In the present study, we included one ROI in the anterior cingulate cortex (ACC), which primarily contained GM, one ROI in the DLPFC, which primarily contained WM, and one ROI in the hippocampus. Additionally, an ROI in the thalamus was chosen as this region is highly connected to the PFC and the hippocampus, while not being considered particularly ageing-sensitive.

There are a number of additional potential reasons for the variability of the results, such as the included age spans and sample sizes (Cichocka and Bereś, 2018). Further, many studies quantify metabolite levels by calculating the ratio to tCr. As tCr is indicated to change with age, this complicates interpretation of the results (Cleeland et al., 2019). Moreover, the distribution of GM, WM, and CSF within a given  $^1\text{H-MRS}$  voxel depends on each individual's anatomy, and the extent to which this is corrected for may influence the results. Last, variability of results could arise from differences in field strength (Tkáč et al., 2001; Terpstra et al., 2016) and parameters, such as TR and TE (Sporn et al., 2019). Developments in  $^1\text{H-MRS}$  hardware and best practice could, thus, increase the consistency and interpretability of the results.

The overall aim of this study was to investigate regional brain metabolite differences across three age groups of normal individuals using 7T  $^1\text{H-MRS}$  and to relate the differences in metabolite levels to cognitive ageing. The primary hypothesis was that the metabolite levels would differ across age groups in a region-dependent manner. More specifically, it was predicted that, in the regions proposed to be more ageing-sensitive, namely, ACC, DLPFC, and hippocampus, older age groups would show higher levels of mIns, tCr, and tCho and lower levels of tNAA and Glu. In thalamus, no differences were expected. The secondary hypothesis was that ageing-sensitive metabolites would be associated with cognitive ageing. Visuospatial working memory

**Table 1. Demographics<sup>a</sup>**

Variable	Younger	Middle	Older
No. of participants (women)	20 (10)	20 (10)	19 (9)
Age (yr)	22.6 ± 2.37**	44.1 ± 3.64**	72.5 ± 2.78**
BMI	22.6 ± 1.72**	26.6 ± 4.28*	26.6 ± 3.45*
Education (yr)	15.2 ± 2.03	16.0 ± 2.82	15.1 ± 2.64

<sup>a</sup>Data are mean ± SD.

Age groups that are pairwise significantly different ( $p < 0.05$ ): \*different from one other group; \*\*different from both other groups.

(vsWM) was chosen to exemplify cognitive ageing based on the literature showing how this function is sensitive to ageing and depends on the ROIs in this study (Owen et al., 1996; Brockmole and Logie, 2013; Goldstone et al., 2018).

## Materials and Methods

### Participants

Participants were recruited through online advertisements on a national Danish participant recruitment page ([www.forsoegsperson.dk](http://www.forsoegsperson.dk)) and through an advertisement in a local newspaper. Sixty participants in total were included, 20 in each of the three age groups: 18–26 years old (younger), 39–50 years old (middle), and 69–79 years old (older). The three age groups were chosen to sample brain metabolite levels at early, middle, and later points of adult life with a reasonable range of ages within each group. Inclusion happened in parallel for all three groups. Exclusion criteria were as follows: MR contraindications, major psychiatric or neurologic history, history of drug or alcohol abuse, participation in medical drug testing within 6 months of the experiment, smoking within 3 months of the experiment, infectious disease within 3 weeks of the experiment, morbid obesity, pregnancy, and insufficient understanding of Danish. One participant from the older group was excluded because of an unexpected pathologic finding (Table 1).

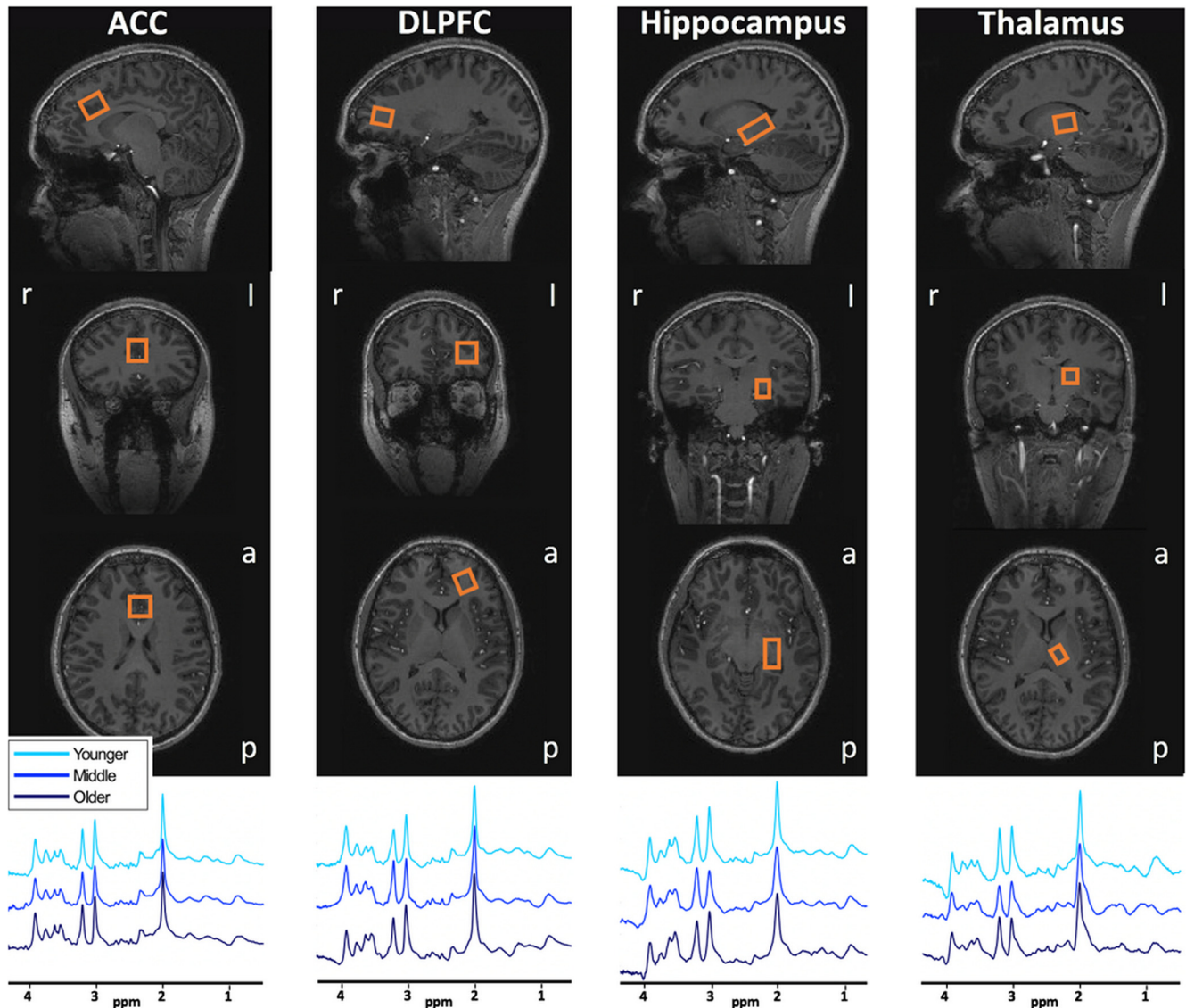
Participants fasted but were allowed to drink water from 22:00 h the day before the experiment and until the experiment was over. Although it is not clear whether having breakfast is beneficial for working memory performance compared with not having breakfast, breakfast composition may affect working memory performance (Galioto and Spitznagel, 2016). To avoid any confounding effect of different nutritional intake across participants, we required the participants to fast overnight. All participants underwent MR scanning between 9:00 h and 11:00 h immediately followed by cognitive testing. Participants were informed orally and in writing about the experiment. They provided written consent before the experiment. After the experiment, all participants were reimbursed for their time spent participating. The study was approved by the Regional Committee on Health Research Ethics from the Capital Region in Denmark and was performed in accordance with the declaration of Helsinki (amendment of Fortaleza, 2013).

### MR acquisition

A Philips 7T whole body MR scanner (Philips) was used in combination with a dual transmit coil and a 32-channel receive head coil (Nova Medical). The MR sequences described in this study were part of a larger scan protocol; total scan time per participant was 90 min at maximum.

A  $T_1$ -weighted MPRAGE sequence (slices = 380, slice thickness = 0.5 mm, TR = 8.0 ms, TE = 3.2 ms, flip angle = 7 degrees, FOV = 256 × 256 × 190, voxel size = 1 × 1 × 0.5 mm) was acquired for anatomic reference and tissue classification.

A sLASER sequence (Boer et al., 2011; Arteaga de Castro et al., 2013) (TR/TE = 3700/32 ms, bandwidth = 4 kHz, data points = 2048) was used in the medial ACC (20 × 20 × 20 mm<sup>3</sup>, 16 acquisitions), left DLPFC (12 × 20 × 20 mm<sup>3</sup>, 32 acquisitions), left hippocampus (30 × 15 × 15 mm<sup>3</sup>, 64 acquisitions), and left thalamus (16 × 12 × 16 mm<sup>3</sup>, 64 acquisitions) (Fig. 1). Because of time restrictions, only  $^1\text{H-MR}$  spectra from the left DLPFC, hippocampus, and thalamus were acquired as the left hemisphere is dominant in the majority of the population. VAPOR water suppression was applied (Tkáč et al., 2001). A non-water-suppressed spectrum was obtained at the beginning of each sequence. Second-



**Figure 1.** Voxel placements and representative spectra before processing and baseline subtraction. Orange box represents the MRS voxel. r, Right; l, left; a, anterior; p, posterior.

order  $B_0$  shimming was applied using the FASTMAP algorithm (Gruetter and Boesch, 1992; Gruetter, 1993) for all voxels separately using a shim box centered on the voxel and 15 mm longer than the voxel in each direction. Fractions of GM, WM, and CSF in each voxel were calculated with voxel-based morphometry using CAT12 (Gaser and Dahnke, 2016) implemented in SPM12 (Statistical Parametric Mapping, Wellcome Department of Cognitive Neurology) (Table 2).

#### Spectral fitting and quantification

Spectral quality assessment was performed based on Kreis (2004). First, visual inspection was performed, and poor-quality spectra and spectra with artifacts were excluded. After fitting with LCModel, spectra were excluded if line width (FWHM) exceeded 0.1 ppm. Metabolite values with a Cramèr-Rao lower bound (CRLB) of  $\geq 20$  were excluded from the analyses (for exploratory analyses, other CRLB criteria were used; see section Exploratory metabolite analysis).

After visual inspection, 9 of 236 spectra were excluded from the analyses because of poor quality and/or artifacts: one from ACC (younger), three from DLPFC (one younger, two older), two from hippocampus (one younger, one older), and three from thalamus (one younger, one middle, one older). The remaining spectra were fitted with LCModel (Provencher, 2001) using a custom basis set with 20 metabolites (alanine,

**Table 2. Voxel tissue fractions<sup>a</sup>**

Variable	Younger	Middle	Older
ACC CSF %	11.9 ± 2.90*	14.6 ± 6.13*	25.4 ± 7.45**
ACC GM/WM	2.76 ± 0.55*	2.29 ± 0.47*	2.51 ± 0.49
DLPFC CSF %	4.26 ± 1.73*	4.18 ± 2.86*	7.78 ± 3.73**
DLPFC GM/WM	0.44 ± 0.13	0.41 ± 0.13	0.50 ± 0.14
Hippocampus CSF %	4.23 ± 1.60*	6.61 ± 5.76*	12.5 ± 4.35**
Hippocampus GM/WM	1.11 ± 0.17	1.02 ± 0.23	1.11 ± 0.11
Thalamus CSF %	<0.00 ± <0.00	<0.00 ± <0.00	<0.00 ± <0.00
Thalamus GM/WM	2.06 ± 0.86*	1.74 ± 0.79*	1.13 ± 0.45**

<sup>a</sup>Data are mean ± SD.

Age groups that are pairwise significantly different ( $p < 0.05$ ): \*different from one other group; \*\*different from both other groups. For CSF fraction in thalamus, the concentrations were too low to perform statistical tests.

ascorbate, aspartate, creatine, GABA, glutamine [Gln], Glu, glycine, glycerophosphocholine, glutathione, mIns, lactate [Lac], N-acetylaspartate [NAA], N-acetylaspartylglutamate [NAAG], phosphorylcholine, phosphocreatine, phosphorylethanolamine, scyllo-inositol [sIns], serine [Ser], and taurine [Tau]), including an *in vivo* measured macromolecular baseline (van de Bank et al., 2015). Levels of mIns, tCr (creatine + phosphocreatine), tCho (glycerophosphocholine + phosphorylcholine), tNAA



**Table 3.** Quality variables for the <sup>1</sup>H-MRS measurements<sup>a</sup>

Region, age group	SNR	FWHM (Hz)	CRLB				
			mIns	tCr	tCho	tNAA	Glu
<b>ACC</b>							
Younger	44.6 ± 10.5**	7.87 ± 1.12*	2.68 ± 0.57*	1.21 ± 0.41**	3.11 ± 0.45*	1.05 ± 0.22**	2.11 ± 0.32**
Middle	36.4 ± 10.9*	8.61 ± 0.91	3.40 ± 1.82	1.80 ± 0.77*	3.60 ± 1.23	1.40 ± 0.75**	2.80 ± 1.06*
Older	30.3 ± 7.43*	8.88 ± 0.98*	3.52 ± 0.77*	1.84 ± 0.50*	3.89 ± 0.88*	1.84 ± 0.37**	3.16 ± 0.50*
<b>DLPFC</b>							
Younger	43.8 ± 13.8*	11.7 ± 2.36*	3.95 ± 1.88	1.63 ± 0.74	4.63 ± 1.35	1.32 ± 0.65	3.47 ± 1.35
Middle	35.3 ± 9.82	14.0 ± 3.40*	3.35 ± 1.14	1.85 ± 0.49	4.40 ± 1.64	1.50 ± 0.51	3.65 ± 0.93
Older	31.6 ± 9.89*	13.4 ± 1.77	4.00 ± 1.41	1.94 ± 0.52	4.29 ± 1.09	1.59 ± 0.48	4.06 ± 0.97
<b>Hippocampus</b>							
Younger	27.4 ± 8.38**	12.1 ± 1.76**	3.58 ± 0.75*	2.26 ± 0.44*	4.47 ± 1.04	1.53 ± 0.50**	3.68 ± 0.57*
Middle	22.4 ± 5.14*	14.5 ± 2.28*	3.79 ± 1.69	2.58 ± 0.61	4.60 ± 1.53	1.90 ± 0.45**	4.20 ± 0.77*
Older	17.7 ± 4.36*	15.8 ± 3.07*	4.50 ± 1.12*	2.83 ± 0.60*	5.00 ± 1.80	2.22 ± 0.42**	5.50 ± 1.17**
<b>Thalamus</b>							
Younger	22.4 ± 3.32*	11.4 ± 1.47*	5.68 ± 1.81	2.53 ± 0.50*	4.63 ± 0.93	1.53 ± 0.50	5.42 ± 1.18*
Middle	20.6 ± 3.90	12.1 ± 1.53	4.86 ± 1.43	2.75 ± 0.55	4.59 ± 1.23	1.69 ± 0.47	5.70 ± 1.03*
Older	19.2 ± 4.78*	13.2 ± 1.70*	5.17 ± 1.54	3.06 ± 0.40*	4.83 ± 1.50	1.83 ± 0.50	7.06 ± 2.61**

<sup>a</sup>Data are mean ± SD. SNR, Signal-to-noise ratio.

Age groups that are pairwise significantly different ( $p < 0.05$ ): \*different from one other group; \*\*different from both other groups.

(NAA+NAAG), and Glu were used for the main analyses. The spectra were fitted between 0.2 and 4 ppm with a knot spacing of 0.2. Spectral quality measures were calculated for all spectra, including CRLB, FWHM, and signal-to-noise ratios (SNR). Spectral quality data are summarized in Table 3. There were no spectra with FWHM exceeding 0.1 ppm and no metabolite values with a CRLB of  $\geq 20$ . Typical spectral fitting with LCModel is shown in Figure 2.

Metabolite quantification was performed through water referencing. Metabolite levels were corrected for tissue fractions in the voxel (Gasparovic et al., 2009; Quadrelli et al., 2016), including tissue-specific attenuation factors for  $T_1$  (Rooney et al., 2007) and  $T_2$  (Bartha et al., 2002) relaxation times by correcting the water concentration in the voxel ( $WaterConc_{corr}$ ) as follows:

$$WaterConc_{corr} = \frac{[H_2O] * (f_{GM} * R_{H_2O,GM} + f_{WM} * R_{H_2O,WM} + f_{CSF} * R_{H_2O,CSF})}{1 - f_{CSF}}$$

where the water fraction in tissue  $x$ ,  $f_x$ , is as follows:

$$f_x = \frac{f_{x,vol} * con_x}{f_{GM,vol} * con_{GM} + f_{WM,vol} * con_{WM} + f_{CSF,vol} * con_{CSF}}$$

and the tissue specific attenuation factors  $R_{H_2O,x}$  is as follows:

$$R_{H_2O,x} = e^{-\frac{TE}{T_2x}} * (1 - e^{-\frac{TR}{T_1x}})$$

where  $[H_2O]$  is the concentration of pure water,  $f_{x,vol}$  is the fractional volume of tissue  $x$  within the voxel,  $con_x$  is the water content in tissue  $x$  as a fraction of pure water, and  $T_{1x}$  and  $T_{2x}$  are the  $T_1$  and  $T_2$  relaxation times of water in tissue  $x$ .  $con_x$  was assumed 0.97 in CSF (Ernst et al., 1993), 0.80 in GM and 0.71 in WM (Abbas et al., 2014). The  $T_1$  relaxation time was assumed 4425 ms in CSF, 2130 ms in GM, and 1220 ms in WM (Rooney et al., 2007). The  $T_2$  relaxation time was assumed 141 ms in CSF, 50 ms in GM, and 55 ms in WM (Bartha et al., 2002).

#### Cognitive tests

Cognitive testing was performed on a tablet using a custom-composed CANTAB (Cambridge Cognition) (Sahakian and Owen, 1992). For the present study, two tasks targeting vsWM were included: the paired associates learning (PAL) and the spatial working memory (SWM) task. Both tasks have been shown to be reliable and sensitive to age-related cognitive decline (Robbins et al., 1994; Gonçalves et al., 2016).

**PAL.** In PAL, boxes were displayed in a circle. The boxes were opened and closed one by one in a randomized order. Some boxes had a pattern hidden inside. Next, the patterns were displayed one by one in the middle of the screen, and the participant had to click the box where the same pattern had previously been displayed. If the correct box for one or more patterns was not chosen, the task was repeated. The number of times a participant selected the wrong box was used as outcome score. Participants had to place all patterns correctly in maximally four attempts to reach the next level with a higher number of patterns. The levels were four, six, or eight patterns. If a participant did not reach all levels, the score was adjusted for the levels not reached.

**SWM.** In SWM, boxes were displayed in an asymmetrical pattern. The participant had to find a hidden token in as few clicks as possible by opening the boxes one by one. When the token was found, a new token was hidden in one of the other boxes. The outcome score was the number of times a participant selected a box in which a token had previously been found. When tokens had been found in all boxes, the next level was reached. The levels were four, six, or eight boxes.

The PAL and SWM scores were correlated ( $\rho = 0.478$ ,  $p < 0.001$ ). They were, therefore,  $z$ -scored and summed into a vsWM composite score. The vsWM score was inverted so that a higher score represented better performance.

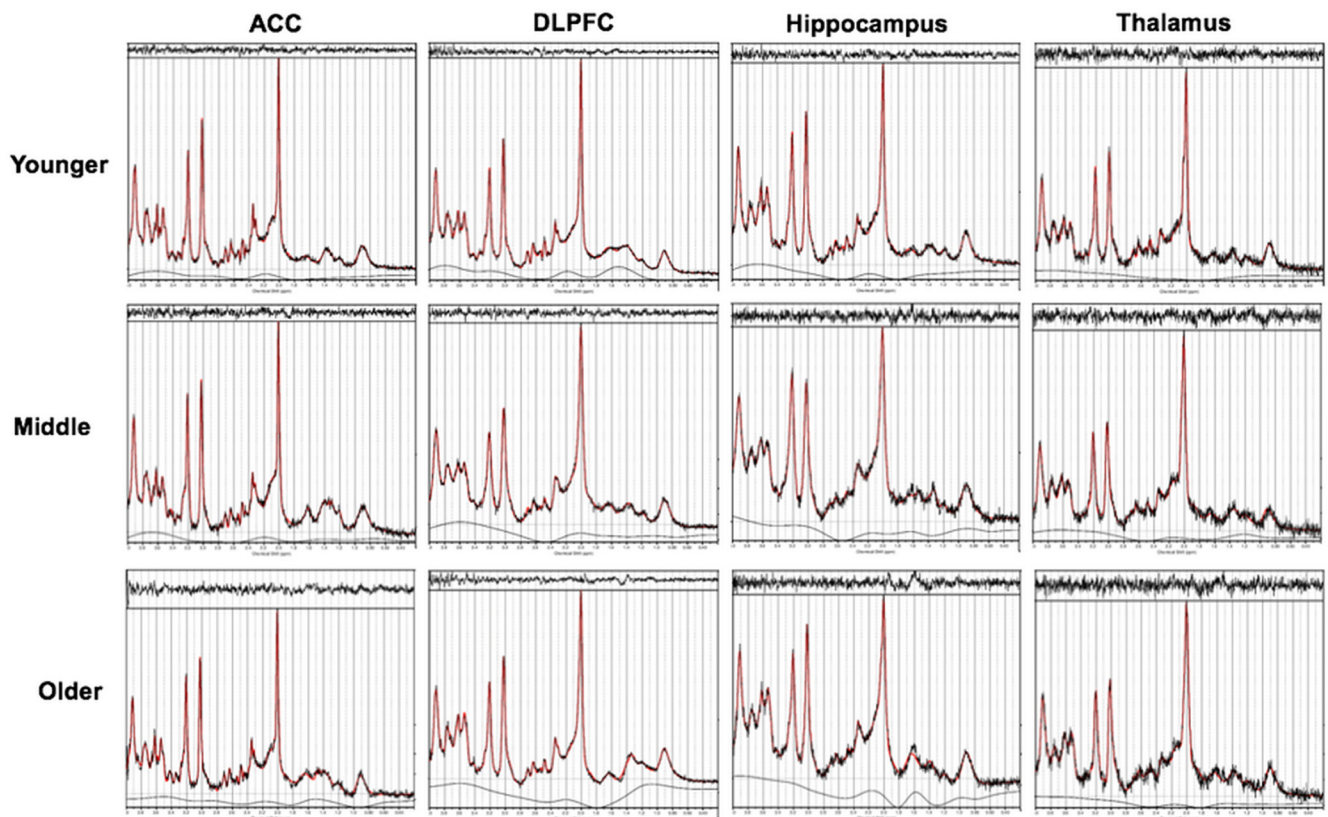
#### Experimental design and statistical analysis

To minimize data loss, spectra that were excluded based on poor quality were not considered missing values. Instead, for each metabolite, the group mean in that region was imputed. Outliers were defined as metabolite levels  $> 3$  SDs from the group mean in a given brain region. Outlier detection led to exclusion of one hippocampal tCr and one thalamus mIns value, both from participants in the middle group. Outliers were excluded from further analyses and not imputed by group means.

SPSS 25 was used for statistical analyses. Threshold of significance was set to  $p < 0.05$ , after correction for multiple comparisons where applicable. One-way ANOVA testing for the effect of age group followed by *post hoc* pairwise comparisons was applied to compare continuous demographic variables, spectral quality measures, tissue distributions, and scores from cognitive testing.

**Overall age-related metabolite differences.** A multivariate ANCOVA using Pillai's trace with mIns, tCr, tCho, tNAA, and Glu levels as dependent variables was performed to test the primary hypothesis that metabolite levels overall differed across age groups and that there was an interaction between age group and brain region. GM/WM ratio for each voxel was added as covariate.

**Age-related metabolite differences.** Next, to investigate the brain region-specific differences across age groups, a multivariate ANCOVA



**Figure 2.** Typical spectral fitting with LCModel.

using Pillai's trace for each brain region separately was performed, including mIns, tCr, tCho, tNAA, and Glu levels as dependent variables. GM/WM ratio in each voxel was added as covariate. Significant main effects were further qualified by *post hoc* ANCOVA and Bonferroni-corrected pairwise comparisons.

**Metabolite correlations with cognition.** The secondary hypothesis that age-related differences in metabolites were associated with differences in vsWM was investigated with Bonferroni-corrected (11 comparisons) partial Pearson's correlations with GM/WM ratio as covariate. Correlations were tested between vsWM score and metabolites that differed across age groups within a brain region. The tests were one-tailed, testing for a negative correlation between vsWM score and mIns, tCr, and tCho levels and a positive correlation between vsWM score and tNAA and Glu levels.

**Exploratory metabolite analysis.** Several metabolites not included in the main hypotheses were also acquired with  $^1\text{H}$ -MRS. Exploratory statistical analyses of age-related differences for these metabolites were performed per brain region with ANCOVA controlling for GM/WM ratio followed by pairwise comparisons. These exploratory analyses were not Bonferroni-corrected. CRLBs were used to filter out meaningless metabolite level estimates in an approach based on Tkáč et al. (2009). First, all metabolite levels with a CRLB higher than 100% were excluded. Metabolite level estimates were only included in the analysis if >50% of the estimates in a specific age group and brain region remained and if the average CRLB for each group was <50%.

## Results

### tCho levels

There was a main effect of age group for tCho in ACC, DLPFC, and hippocampus. In ACC, tCho levels were higher in the older than the younger group ( $p < 0.001$ ) and in the middle than the younger group ( $p = 0.033$ ). In hippocampus, tCho levels were higher in the older than the younger group ( $p < 0.001$ ) and the older than the middle group ( $p = 0.010$ ).

### Overall age-related metabolite differences

When including all metabolites in all brain regions, there was a main effect of age group ( $F_{(10,436)} = 12.49$ ,  $p < 0.001$ ) and an age group  $\times$  brain region interaction ( $F_{(30,1105)} = 1.87$ ,  $p = 0.003$ ). Thus, metabolite levels differ across age groups in a brain region-dependent way, and each brain region was therefore next studied separately.

### Age-related metabolite differences

There was a main effect of age group in all separate brain regions, ACC ( $F_{(10,104)} = 4.06$ ,  $p < 0.001$ ), DLPFC ( $F_{(10,104)} = 4.20$ ,  $p < 0.001$ ), hippocampus ( $F_{(10,102)} = 6.75$ ,  $p < 0.001$ ), and thalamus ( $F_{(10,102)} = 2.35$ ,  $p = 0.015$ ). These differences are further qualified with *post hoc* testing for each metabolite below. See Table 4 for metabolite levels and statistics and Figure 3 for visualization.

### mIns levels

There was a main effect of age group for mIns in all brain regions. In DLPFC, mIns levels were higher in the middle than the younger group ( $p < 0.001$ ). In hippocampus, mIns levels were higher in the older than the younger group ( $p < 0.001$ ), in the older than the middle group ( $p = 0.002$ ), and in the middle than the younger group ( $p = 0.028$ ). In thalamus, mIns levels were higher in the older than the younger group ( $p = 0.010$ ).

### tCr levels

There was a main effect of age group for tCr in ACC and hippocampus. In ACC, tCr levels were higher in the older than the younger group ( $p = 0.005$ ) and the older than the middle group ( $p = 0.037$ ). In hippocampus, tCr levels were higher in the older

**Table 4. Brain region-specific analysis testing for effect of age group<sup>a</sup>**

Metabolite, age group	Brain region ( <i>N</i> younger, middle, older)			
	ACC <i>N</i> = 20, 20, 19	DLPFC <i>N</i> = 20, 20, 19	Hippocampus <i>N</i> = 20, 19, 19	Thalamus <i>N</i> = 20, 19, 19
<b>mIns</b>				
Younger	5.67 ± 0.41	5.16 ± 0.56*	5.72 ± 0.61**	4.39 ± 0.70*
Middle	6.34 ± 0.97	6.25 ± 0.78*	6.55 ± 0.82**	4.73 ± 0.47
Older	6.31 ± 0.69	5.67 ± 0.74	7.45 ± 0.94**	5.25 ± 1.10*
	$p = 0.032, F_{(2,55)} = 3.65$	$p < 0.001, F_{(2,55)} = 11.46$	$p < 0.001, F_{(2,54)} = 23.06$	$p = 0.013, F_{(2,54)} = 4.73$
<b>tCr</b>				
Younger	6.96 ± 0.34*	6.79 ± 0.50	6.29 ± 0.51*	6.13 ± 0.67
Middle	7.20 ± 0.71*	6.99 ± 0.49	6.12 ± 0.51*	5.84 ± 0.52
Older	7.60 ± 0.50**	7.07 ± 0.48	6.99 ± 0.74**	6.13 ± 0.90
	$p = 0.004, F_{(2,55)} = 6.15$	$p = 0.242, F_{(2,55)} = 3.29$	$p < 0.001, F_{(2,54)} = 13.41$	$p = 0.390, F_{(2,54)} = 0.96$
<b>tCho</b>				
Younger	1.44 ± 0.16**	1.36 ± 0.20	1.52 ± 0.20*	1.54 ± 0.23
Middle	1.63 ± 0.22*	1.51 ± 0.23	1.63 ± 0.23*	1.54 ± 0.24
Older	1.71 ± 0.19*	1.49 ± 0.17	1.83 ± 0.24**	1.60 ± 0.27
	$p = 0.001, F_{(2,55)} = 8.49$	$p = 0.045, F_{(2,55)} = 3.29$	$p < 0.001, F_{(2,54)} = 10.19$	$p = 0.907, F_{(2,54)} = 0.10$
<b>tNAA</b>				
Younger	9.71 ± 0.57	11.07 ± 0.72	9.71 ± 0.84	12.53 ± 1.12
Middle	9.98 ± 0.80	10.87 ± 1.07	9.49 ± 1.11*	11.86 ± 1.12
Older	9.82 ± 0.75	10.63 ± 0.92	10.24 ± 0.94*	12.39 ± 1.47
	$p = 0.876, F_{(2,55)} = 0.13$	$p = 0.309, F_{(2,55)} = 1.20$	$p = 0.020, F_{(2,54)} = 4.19$	$p = 0.255, F_{(2,54)} = 1.53$
<b>Glu</b>				
Younger	9.62 ± 0.36	7.07 ± 0.55*	6.68 ± 0.52	6.04 ± 0.70
Middle	9.59 ± 1.02	6.55 ± 0.88	6.50 ± 0.66	5.51 ± 0.84
Older	9.20 ± 0.60	6.39 ± 0.79*	6.68 ± 0.83	5.09 ± 0.99
	$p = 0.139, F_{(2,55)} = 2.05$	$p = 0.003, F_{(2,55)} = 6.64$	$p = 0.631, F_{(2,54)} = 0.46$	$p = 0.093, F_{(2,54)} = 2.49$

<sup>a</sup>Data are mean ± SD.

Age groups that are pairwise significantly different ( $p < 0.05$ ): \*different from one other group; \*\*different from both other groups.

than the younger group ( $p < 0.001$ ) and the older than the middle group ( $p < 0.001$ ).

### tNAA levels

There was a main effect of age group for tNAA in hippocampus. In the hippocampus, the tNAA levels were higher in the older than the middle group ( $p = 0.018$ ).

### Glu levels

There was a main effect of age group for Glu in DLPFC. In DLPFC, Glu levels were lower in the older than the younger group ( $p = 0.002$ ).

All analyses were also performed controlling for sex; this did not change the significance of the results. Registering excluded poor-quality spectra as missing values rather than imputing them with group means did not change the significance of the results, except for tCho levels in DLPFC, which were no longer different across groups.

### Metabolite correlations with cognitive ageing

The vsWM, PAL, and SWM scores all differed across age groups (for statistics, see Table 5). vsWM score correlated negatively with mIns in the hippocampus ( $\rho = -0.471, p = 0.001$ ) and thalamus ( $\rho = -0.355, p = 0.037$ ) and with tCr ( $\rho = -0.364, p = 0.027$ ) and tCho ( $\rho = -0.486, p < 0.001$ ) in the ACC (for plots, see Fig. 4). These findings remained when inserting missing values instead of imputed data. Before Bonferroni correction, significant correlations were also observed for mIns in ACC ( $\rho = -0.244, p$  uncorrected = 0.032), tCr in hippocampus ( $\rho = -0.263, p$  uncorrected = 0.0424), and Glu in DLPFC ( $\rho = 0.269, p$  uncorrected = 0.021).

### Exploratory metabolite analysis

Gln, glutathione, NAA, NAAG, sIns, and Tau could be included in the analysis within the defined limits in all regions. Additionally, aspartate and GABA could be quantified in ACC, DLPFC, and thalamus and Lac and Ser could be quantified in ACC, DLPFC, and hippocampus. The exploratory analysis revealed a main effect of age group in the ACC for Lac, in the DLPFC for NAA, and in the hippocampus for Gln, NAAG, sIns, Ser, and Tau. Except for NAA, levels were higher in older age groups for all metabolites. See Table 6 for metabolite levels and statistics.

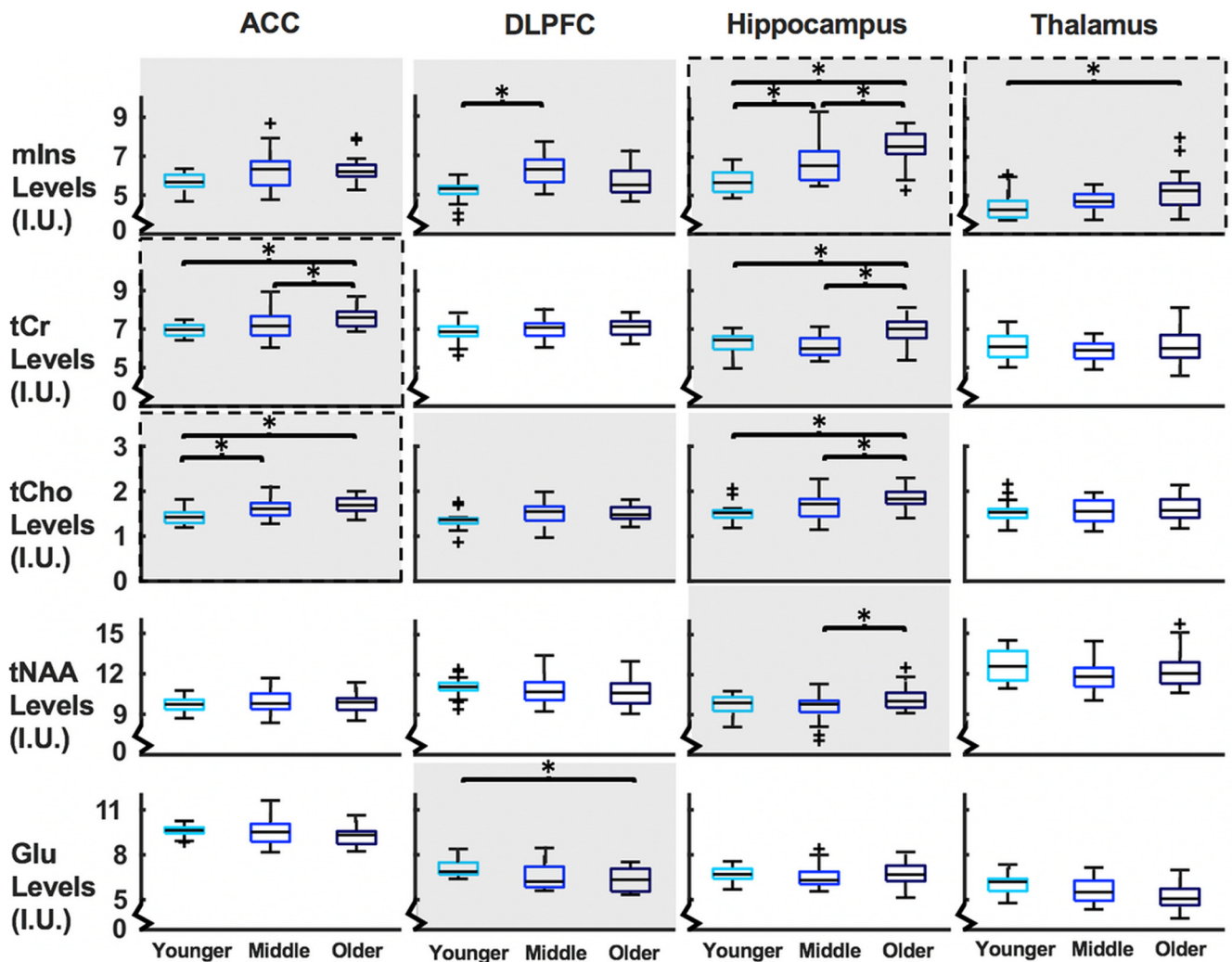
### Discussion

Using 7T <sup>1</sup>H-MRS, we found differences across age groups in mIns levels in DLPFC, hippocampus, and thalamus, and tCr and tCho levels in ACC and hippocampus with the most common difference being higher levels in the older than the younger group. Additionally, mIns levels in hippocampus and thalamus and tCr and tCho levels in ACC correlated negatively with cognitive ageing as reflected by vsWM performance. Levels of tNAA in hippocampus were higher in the older than the middle group, and Glu levels in DLPFC were lower in the older than the younger group. No correlations between tNAA or Glu and vsWM performance were observed.

### mIns, tCr, tCho, and cognitive ageing

As hypothesized, regional levels of mIns, tCr, and tCho were generally higher in older age groups in the ACC and hippocampus. This corresponds with some previous studies (Chang et al., 1996; Sporn et al., 2019), although other studies observed unaltered levels (Hädel et al., 2013; Ding et al.,





**Figure 3.** Boxplots of metabolite levels (*y* axes) separated by region and age group (*x* axes). Metabolites with a significant main effect of age group at ANCOVA level have a gray background. \*Significant pairwise differences between groups. Metabolites that significantly correlate with vsWM score are enclosed in a dashed line. I.U., Institutional units.

**Table 5. Cognitive scores<sup>a</sup>**

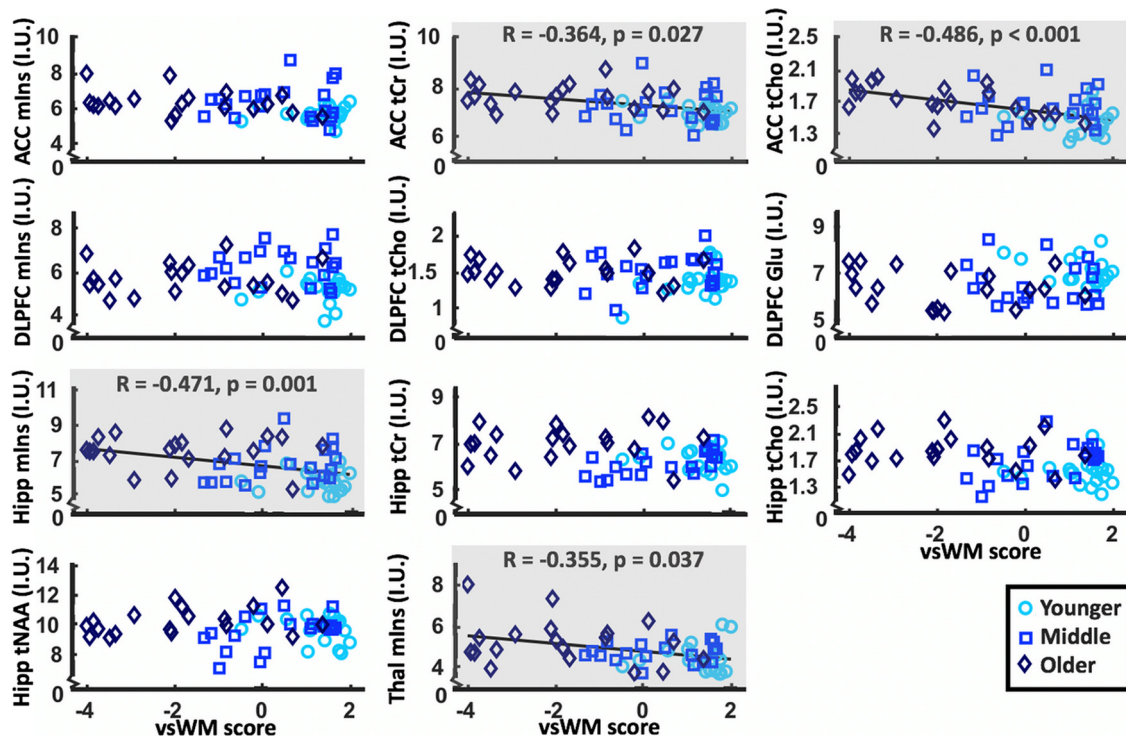
Variable	Younger	Middle	Older	Significance test
vsWM score	1.30 ± 0.15**	0.43 ± 0.24**	−1.81 ± 0.39**	$p < 0.001$ , $F_{(2,56)} = 33.80$
PAL errors	4.75 ± 0.91*	12.0 ± 2.15*	29.6 ± 3.93**	$p < 0.001$ , $F_{(2,56)} = 24.20$
SWM errors	3.20 ± 0.98*	6.65 ± 1.48*	16.1 ± 2.03**	$p < 0.001$ , $F_{(2,56)} = 18.41$

<sup>a</sup>Data are mean ± SD.

Age groups that are pairwise significantly different ( $p < 0.05$ ): \*different from one other group; \*\*different from both other groups.

2016). Some of the previous negative findings could, however, have arisen from not including participants older than 70 years, low field strengths, the selected scan parameters, or insufficient correction for tissue fractions (Cleland et al., 2019; Sporn et al., 2019). In the DLPFC, levels of mIns, tCr, and tCho were not higher in the older than the younger group. As the DLPFC voxel contained more WM than GM, this corresponds with a previous study showing that changes in prefrontal mIns, creatine, and Cho levels during ageing are primarily related to GM (Chang et al., 1996). Overall, the findings of differences in mIns, tCr, and tCho across age groups in ACC and hippocampus are consistent with the common notion that these regions are especially sensitive to ageing (Hedden and Gabrieli, 2004).

Thalamus also had higher mIns levels in the older than the younger group. This has not been observed before; however, very few <sup>1</sup>H-MRS ageing studies have included the thalamus. To our knowledge, this is the first <sup>1</sup>H-MRS study of normal ageing in thalamus at 7T and the first to include an age group >70 years. Accordingly, other MR techniques suggest that the thalamus may be a central node in ageing and could thus be of special interest in future ageing studies (Sullivan et al., 2004; Goldstone et al., 2018). Overall, the results indicate that levels of glia-related metabolites are elevated during ageing in ACC, hippocampus, and thalamus. vsWM was negatively correlated with tCho and tCr in ACC and mIns in hippocampus and thalamus. This study is the first to show these correlations during normal ageing; however, only few studies of metabolites and cognitive ageing exist (for review, see Cleland et al., 2019). In PFC, the studies have mainly focused on WM (Ross et al., 2005, 2006; Kochunov et al., 2010). In hippocampus, an association between tNAA/tCr and vsWM has been observed; however, the ratio complicates interpretation (Driscoll et al., 2003). None of the studies included thalamus; however, our results correspond to findings from other types of MR studies suggesting that neurobiological ageing effects on thalamus could be a critical part of cognitive ageing (Grieve et al., 2007; Goldstone et al., 2018).



**Figure 4.** Scatterplots of metabolite levels that differ between age groups (y axes) and vsWM score (x axes). Gray background represents significant correlations after Bonferroni correction between metabolite levels and vsWM score. Hipp, Hippocampus; I.U., institutional units; Thal, thalamic.

**Table 6.** Exploratory analysis testing for effect of age group<sup>a</sup>

Region, metabolite	N, younger, middle, older	Metabolite levels (I.U.)			Significance test
		Younger	Middle	Older	
ACC					
Lac	19, 19, 16	0.66 ± 0.23*	1.10 ± 1.08	1.63 ± 1.17*	p = 0.007, F <sub>(2,50)</sub> = 5.46
DLPFC					
NAA	20, 20, 19	10.16 ± 0.69*	9.82 ± 0.79	9.41 ± 0.81*	p = 0.015, F <sub>(2,55)</sub> = 4.51
Hippocampus					
Gln	20, 20, 19	1.96 ± 0.49*	2.10 ± 0.67	2.47 ± 0.63*	p = 0.027, F <sub>(2,55)</sub> = 3.85
NAAG	20, 20, 19	1.84 ± 0.48**	2.26 ± 0.61*	2.28 ± 0.68*	p = 0.041, F <sub>(2,55)</sub> = 3.39
sIns	18, 18, 18	0.23 ± 0.09*	0.25 ± 0.11*	0.34 ± 0.16**	p = 0.004, F <sub>(2,50)</sub> = 6.23
Ser	15, 17, 15	0.99 ± 0.40**	1.58 ± 0.58*	1.65 ± 1.03*	p = 0.028, F <sub>(2,43)</sub> = 3.90
Tau	20, 20, 19	1.57 ± 0.32*	1.67 ± 0.59*	2.09 ± 0.65**	p = 0.013, F <sub>(2,55)</sub> = 4.70

<sup>a</sup>Data are mean ± SD. I.U., Institutional units.

Age groups that are pairwise significantly different (p < 0.05): \*different from one other group; \*\*different from both other groups. Only significant findings are reported.

Although mIns, tCr, and tCho can be found in both glial cells and neurons, their concentrations are significantly higher in glial cells than neurons, and an increase in their concentrations therefore potentially reflects gliosis and neuroinflammation (Glanville et al., 1989; Urenjak et al., 1993). Moreover, a concomitant increase in mIns and tCho indicates glial proliferation (Bitsch et al., 1999). This is consistent with stereological findings that the number of glial cells increase with age in the frontal and temporal cortex (Terry et al., 1987). With age, glial cells, including astrocytes and microglia, change phenotype into a more proinflammatory state (Perry et al., 2007; Cohen and Torres, 2019). This increase in neuroinflammation may negatively impact cognitive function (Sartori et al., 2012). As elevations in mIns, tCho, and tCr are associated with neuroinflammation, this could be the underlying mechanism for the relationship between age group, metabolites, and cognitive ageing (Chang et al., 2013). Additionally, ageing could specifically impact the synthesis and

breakdown of mIns, tCr, and tCho. This would imply that ageing is associated with imbalances in cell signaling, energy homeostasis, and membrane turnover, respectively (Chang et al., 1996, 2013; Rae, 2014).

In summary, our study is the first to indicate a possible relationship between cognitive ageing of vsWM and higher levels of glia-related <sup>1</sup>H-MRS metabolites in ACC, hippocampus, and thalamus. Overall, these results add to the literature, suggesting a central role for glial cells in normal cognitive ageing potentially via neuroinflammation.

#### NAA and Glu

Contrary to mIns, tCr, and tCho, tNAA and Glu are both commonly regarded to reflect neuronal health and function, suggesting that the often observed decrease in their levels is linked to compromised neuronal integrity (Clark, 1998; Demougeot et al., 2001; Rae, 2014). In this study, regional levels of tNAA



and Glu were not lower in older age groups, except for Glu levels in DLPFC. This corresponds to some studies (Harada et al., 2001; Chang et al., 2009; Haga et al., 2009; Reingoudt et al., 2012), whereas other studies report lower levels with age (Brooks et al., 2001; Schubert et al., 2004; Ding et al., 2016). Studies reporting lower levels have, however, often quantified the metabolite by calculating the ratio to tCr, which could just as well reflect higher tCr levels (Haga et al., 2009). The lower levels of Glu observed only in the DLPFC in this study could suggest that neuronal health or function is altered in this region. That tNAA remains unaltered in PFC during normal ageing is consistent with stereological observations that frontal neuronal density does not decline with age (Terry et al., 1987). In hippocampus, tNAA levels were even higher rather than lower between the middle and the older group. When previously observed, higher tNAA levels with older age were proposed to arise from the hippocampal neurons' retained ability for growth in later life and might thus represent a beneficial effect (Lie et al., 2004; Bettio et al., 2017; Sporn et al., 2019). On the contrary, lower tNAA levels have been associated with age-related neurodegenerative diseases (Kantarci et al., 2013). Inadvertent recruitment of participants in preclinical stages of disease in studies of normal ageing could thus have resulted in an apparent tNAA decrease with age. Lower tNAA might thus be a trait of pathologic rather than normal ageing (Harada et al., 2001; Murray et al., 2014; Wang et al., 2015).

In our exploratory analysis, we used the increased spectral resolution of 7T <sup>1</sup>H-MRS to distinguish between NAA and NAAG. Our exploratory analysis indicated that NAA levels were lower in older age groups in DLPFC, which was not the case for tNAA. In hippocampus, the results suggest that the higher tNAA levels in the older than middle group could arise from higher NAAG rather than higher NAA. NAAG is a derivative of NAA which might be involved in neuron to glia signaling (Baslow, 2000). It has previously been observed to be associated with ageing in parietal and occipital cortex (Marjańska et al., 2017). The results in this study were only exploratory; however, they support that there could be valuable information in separating NAA and NAAG in future ageing studies.

### Other metabolites

In ACC, Lac levels were higher in the older group compared with the younger group. Together with the differences across age group in mIns and tCho levels, this further indicates neuroinflammation (Bitsch et al., 1999). In hippocampus, Gln, sIns, Ser, and Tau levels were higher in older age groups. For Gln and sIns, levels have also previously been observed to be higher with age, albeit not necessarily in the same brain regions as used in this study (Kaiser et al., 2005; Hädel et al., 2013). Together with the effect on tCr, this could indicate age-related differences in energy metabolism (Rae, 2014). Higher levels of the amino acids Tau and Ser have, to our knowledge, not been observed before. Overall, the results suggest that several different processes are changing with age in the hippocampus. This analysis was, however, exploratory and complicated by low metabolite concentrations and overlapping <sup>1</sup>H-MRS signals (Kaiser et al., 2005). Thus, more studies are needed to clarify the association between these metabolites and ageing.

### Limitations

The study is cross-sectional rather than longitudinal; thus, claims of causality and temporal dynamics (i.e., true decline in

metabolites or cognitive performance) cannot be made. Participant recruitment was based on participants responding to an advertisement, which might have caused a recruitment bias. Further, only one cognitive domain was used to exemplify cognitive ageing and correlations might thus be specific for vsWM function rather than relating to cognitive ageing in general. Last, participants underwent cognitive testing while in a fasting state, which could have affected cognitive functioning (Galioto and Spitznagel, 2016).

Regarding <sup>1</sup>H-MRS, age and tissue type may affect T<sub>2</sub> relaxation time constants of metabolites differently; however, these attenuation factors are unknown for 7T (Kirov et al., 2008). In relation to tissue type, the setup of the <sup>1</sup>H-MRS voxel allowed us to control for tissue type across participants but not for distinguishing whether metabolite differences related specifically to one tissue type. The same macromolecular baseline was applied for all age groups and brain regions. Macromolecular content may change across age groups and brain regions (Marjańska et al., 2018); however, the effect of this on measured metabolite levels is not clear. Furthermore, the quality of the <sup>1</sup>H-MRS spectra was generally better for the younger than the older group.

Because of time restrictions, only <sup>1</sup>H-MR spectra from the left DLPFC, hippocampus, and thalamus were acquired. vsWM has been suggested to be primarily a right lateralized function (Smith and Jonides, 1997); however, recent evidence shows that vsWM is probably supported by both the right and the left hemisphere (Paulraj et al., 2018). Moreover, lateralization of spatial WM function may change in older populations (Reuter-Lorenz et al., 2000).

In conclusion, this study provides 7T <sup>1</sup>H-MRS evidence that, in ACC, hippocampus, and thalamus, older age groups have higher levels of the glia-related metabolites mIns, tCr, and tCho, scaling negatively with vsWM performance. These findings emphasize the role for glial cells across multiple brain regions in normal cognitive ageing and suggest that 7T <sup>1</sup>H-MRS measurements of glia-related metabolites in key regions of normal brain ageing might be useful in future studies. We have thus identified a set of 7T <sup>1</sup>H-MRS biomarkers that may help describe cognitive ageing. Understanding normal cognitive ageing is a vital step toward identifying the distinguishing features of successful and pathologic ageing.

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