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A 1H magnetic resonance spectroscopy study at 7 tesla

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Do glia provide the link between low-grade systemic inflammation and normal cognitive ageing? A \textsuperscript{1}H magnetic resonance spectroscopy study at 7 tesla

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Conflict of interest
Hartwig R. Siebner has received honoraria as speaker from Sanofi Genzyme, Denmark and Novartis, Denmark, as consultant from Sanofi Genzyme, Denmark, Lophora, Denmark, and Lundbeck A/S, Denmark, and as editor-in-chief (NeuroImage: Clinical) and senior editor (NeuroImage) from Elsevier Publishers, Amsterdam, The Netherlands. He has received royalties as book editor from Springer Publishers, Stuttgart, Germany, and from Gyldendal Publishers, Copenhagen, Denmark. All other authors declare no conflict of interest.

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Data statement
Due to Danish data handling legislation data will only be available upon request and only after finalizing a formal data sharing agreement.

Keywords
Working memory
C-reactive protein
Interleukin 8
Myo-inositol
Choline
Creatine

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Introduction: 682
Materials and methods: 1493
Results: 437
Discussion: 1309
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-MRS</td>
<td>proton magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>ACC</td>
<td>anterior cingulate cortex</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>CANTAB</td>
<td>Cambridge Neuropsychological Test Automated Battery</td>
</tr>
<tr>
<td>CAT</td>
<td>Computational Anatomy Toolbox</td>
</tr>
<tr>
<td>CRLB</td>
<td>Cramèr-Rao lower bound</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FASTMAP</td>
<td>fast automatic shimming technique by mapping along projections</td>
</tr>
<tr>
<td>FWHM</td>
<td>full-width at half-maximum</td>
</tr>
<tr>
<td>GM</td>
<td>grey matter</td>
</tr>
<tr>
<td>Hipp</td>
<td>hippocampus</td>
</tr>
<tr>
<td>IL-8</td>
<td>interleukin 8</td>
</tr>
<tr>
<td>mIns</td>
<td>myo-inositol</td>
</tr>
<tr>
<td>MPRAGE</td>
<td>magnetization prepared rapid gradient echo</td>
</tr>
<tr>
<td>MR</td>
<td>magnetic resonance</td>
</tr>
<tr>
<td>sLASER</td>
<td>semi-localised by adiabatic selective refocusing</td>
</tr>
<tr>
<td>tCho</td>
<td>total choline</td>
</tr>
<tr>
<td>tCr</td>
<td>total creatine</td>
</tr>
<tr>
<td>SPM</td>
<td>Statistical Parametric Mapping</td>
</tr>
<tr>
<td>TE</td>
<td>echo time</td>
</tr>
<tr>
<td>Thal</td>
<td>thalamus</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor α</td>
</tr>
<tr>
<td>TR</td>
<td>repetition time</td>
</tr>
<tr>
<td>VAPOR</td>
<td>variable pulse power and optimized relaxation delay</td>
</tr>
<tr>
<td>vsWM</td>
<td>visuo-spatial working memory</td>
</tr>
<tr>
<td>WM</td>
<td>white matter</td>
</tr>
</tbody>
</table>
Abstract

Low-grade systemic inflammation contributes to ageing-related cognitive decline, possibly by triggering a neuroinflammatory response through glial activation. Using proton magnetic resonance spectroscopy (1H-MRS) at 7T in normal human individuals from 18 to 79 years in a cross-sectional study, we previously observed higher regional levels of myo-inositol (mIns), total creatine (tCr) and total choline (tCho) in older than younger age groups. Moreover, visuo-spatial working memory (vsWM) correlated negatively with tCr and tCho in anterior cingulate cortex (ACC) and mIns in hippocampus and thalamus. As mIns, tCr and tCho are higher in glia than neurons, this suggest a potential in vivo connection between cognitive ageing and higher regional levels of glia-related metabolites. In the present study, we tested whether these metabolic differences may be related to low-grade systemic inflammation. In the same individuals, plasma concentrations of the proinflammatory markers C-reactive protein (CRP), interleukin 8 (IL-8) and tumor necrosis factor α (TNF-α) were measured on the same day as 1H-MRS assessments. We tested whether CRP, IL-8, and TNF-α concentrations correlated with the levels of glia-related metabolites. CRP and IL-8, but not TNF-α, were higher in older (69-79 years) than younger (18-26 years) individuals. CRP correlated positively with thalamic mIns and negatively with vsWM. IL-8 correlated positively with ACC tCho and hippocampal mIns, but not with vsWM. Mediation analysis revealed an indirect effect of IL-8 on vsWM via ACC tCho. Together, these findings corroborate the role of glial cells, perhaps via their role in neuroinflammation, as part of the neurobiological link between systemic inflammation and cognitive ageing.
1. Introduction

Inflammation is one of the central pillars driving ageing with concomitant negative effects on the brain and cognitive functions (Kennedy et al., 2014; Rosano et al., 2012). With age, increasing levels of chronic low-grade sterile inflammation, termed inflammaging, leads to higher levels of systemic inflammation (Franceschi et al., 2000). The resulting increase in proinflammatory markers in the blood has repeatedly been linked to cognitive ageing (Baune et al., 2008; Laurin et al., 2009; Noble et al., 2010; Yaffe et al., 2003). The neurobiological link between systemic inflammation and cognitive ageing is, however, unknown.

One hypothesis for this neurobiological link is that increased systemic inflammation reflects levels of neuroinflammation in the brain. This hypothesis is strengthened by neuroinflammation being a landmark of brain ageing with links to cognitive decline, dementia and neurodegenerative disease (Chen et al., 2016; Lupo et al., 2019; Lynch, 2010; Ransohoff, 2016). The neurobiological mechanism underlying age-related neuroinflammation is intimately connected to changes in glial cells (DiSabato et al., 2016). Both astrocytes and microglia increase their secretion of pro-inflammatory cytokines, and astrocytes undergo senescence whereas microglia become primed and more responsive to inflammatory stimuli (Cohen and Torres, 2019; Perry et al., 2007). Recently, we used proton magnetic resonance spectroscopy ($^1$H-MRS) to show that in vivo brain metabolites primarily found in glial cells, i.e. myo-inositol (mIns), total creatine (tCr) and total choline (tCho) are elevated with normal ageing in multiple brain regions (Lind et al., 2020). This elevation is consistent with what would be expected during neuroinflammation (Chang et al., 2013). The elevation in tCr and tCho levels in anterior cingulate cortex (ACC) and mIns levels in hippocampus and thalamus were, furthermore, negatively associated with visuo-spatial working memory (vsWM) performance. This supports the notion that neuroinflammation mediated by glial cells plays a role in cognitive ageing.

The hypothesis that elevated systemic inflammation during ageing is linked to neuroinflammation is also supported by the extensive cross-talk between the systemic immune system and the brain (Irwin and Cole, 2011). Proinflammatory markers in the blood can impact the brain both directly, through active transportation, and indirectly through interactions with afferent nerves, endothelial cells and macrophages (Dantzer et al., 2008; Perry, 2004). In this way, systemic proinflammatory markers can lead to activation of microglia and astrocytes and impact brain structure, function, behaviour and mood (Baune et al., 2009; Marsland et al., 2015; Miller and Raison, 2016; Reichenberg et al., 2001; Rosano et al., 2012). Likewise, the brain can affect
the level of systemic inflammation e.g. via spill-over of centrally produced cytokines or through HPA-axis and sympathetic nervous system stimulation (Irwin and Cole, 2011; Nybo et al., 2002). In accordance, levels of immunological cells in blood and brain are strongly correlated (Kanegawa et al., 2016). The relationship between the brain and the immune system changes with age and the ageing brain’s neuroinflammatory response to systemic inflammation is exaggerated with more glial proliferation and a higher impact on cognition (Chen et al., 2008; Godbout et al., 2005; Haroon et al., 2015). Systemic inflammation and neuroinflammation can, thus, affect each other in an age-dependent manner and it may be through this interaction that systemic inflammation is linked to cognitive ageing.

The primary aim of this paper was to investigate systemic inflammation as the link between age-related brain differences and cognitive ageing. We did this by linking systemic inflammation to our previous findings of elevated levels of potential neuroinflammatory brain metabolites related to cognitive ageing (Lind et al., 2020). We used the same sample of sixty normal human individuals of both sexes in three age groups. Concentrations of C-reactive protein (CRP), tumor necrosis factor α (TNF-α) and interleukin 8 (IL-8) were used as markers of systemic inflammation, levels of tCho and tCr in ACC and mIns in hippocampus and thalamus as putative brain metabolite markers of neuroinflammation, and vsWM performance as a proxy for cognitive ageing. We hypothesised that concentrations of systemic proinflammatory markers would be higher in older age groups and would correlate positively with levels of brain metabolites and negatively with vsWM performance. For significant correlations between systemic proinflammatory markers and brain metabolites, we followed up with a mediation analysis.

2. Materials and methods

2.1 Participants

In total, sixty human individuals were recruited with 20 (10 females) in each of three age groups: younger (18-26 years), middle (39-50 years) and older (69-79 years) as described in (Lind et al., 2020). Participants were recruited through online advertisements on a national Danish participant recruitment page (www.forsoegsperson.dk) and through an advertisement in a local newspaper. Inclusion happened in parallel for all three groups. No randomisation was performed to allocate participants in the study. Participants were allocated to groups according to age. Blinding to
participants’ age was not possible for obvious reasons. Inclusion criteria were: healthy, and an age within the age range of one of the groups (~18-25 years, ~40-50 years, or ~70-80 years). Exclusion criteria were: magnetic resonance (MR) contraindications, major psychiatric or neurological history, history of drug or alcohol abuse, participation in medical drug testing within six months of the experiment, smoking within three months of the experiment, infectious disease within three weeks of the experiment, morbid obesity, pregnancy, and insufficient understanding of Danish. No sample size calculations were performed, as previous studies these could be based on are sparse and largely inappropriate due to differences in methodologies (Cichocka and Bereś, 2018). Nevertheless, based on previous reports we deemed a sample size of 20 participants per group appropriate (Marsman et al., 2017a, Marsman et al., 2017b).

All measures were collected on the same day (Figure 1). Participants fasted but were allowed to drink water from 22:00h the day before the experiment and the whole duration of the experiment. Each experiment started at 9:00h with an MR session, followed at 11:00h by cognitive assessment and ended with blood sampling from the cubital fossa between 11:50h and 14:00h (mean±SD = 12:30±21 min). Participants were reimbursed for participating. The study was approved by the Regional Committee on Health Research Ethics from the Capital Region in Denmark (protocol number H-17003772) and was performed in accordance with the declaration of Helsinki (amendment of Fortaleza, 2013). Written informed consent was obtained from all participants. The study was not preregistered and exploratory in nature.

Followingly, one participant from the older group was excluded due to an incidental MR finding and two participants were excluded due to values of CRP above 10 mg/L which might indicate acute infection (Eklund, 2009). The final population sample included 19 in the younger, 19 in the middle and 19 in the older group (Figure 1; Table 1).
Figure 1. Flow chart of the experimental procedure. CRP: C-reactive protein.

Table 1. Demographics (mean±SD).

<table>
<thead>
<tr>
<th></th>
<th>Younger</th>
<th>Middle</th>
<th>Older</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (females)</td>
<td>19 (9)</td>
<td>19 (9)</td>
<td>19 (9)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>22.58±2.43**</td>
<td>43.89±3.62**</td>
<td>72.47±2.78**</td>
</tr>
<tr>
<td>BMI</td>
<td>22.53±1.71**</td>
<td>26.03±3.65*</td>
<td>26.60±3.45*</td>
</tr>
<tr>
<td>Education (years)</td>
<td>15.21±2.07</td>
<td>15.84±2.29</td>
<td>15.11±2.64</td>
</tr>
</tbody>
</table>

Note: Age groups that are pairwise significantly different (p<0.05) are marked with ** if different from both other groups and * if different from one other group. BMI: body mass index.

2.2 Systemic proinflammatory markers

Ethylenediaminetetraacetic acid (EDTA)-plasma was separated by centrifugation, aliquoted and stored within two hours of the blood sampling at -80 °C for maximally 1.5 years. High-sensitivity CRP was determined in duplicates using a quantitative sandwich immunoassay technique (R&D Systems, Minneapolis, MN, cat.no. DCRP00). Average inter- and intra-assay CV were <10 %.

Plasma levels of IL-8, TNF-α, interleukin 1β, interleukin 6 and interleukin 10 were measured in duplicate analyses using the Luminex Human High Sensitivity Cytokine Premixed Kit A (R&D Systems, Minneapolis, MN, cat.no. FCSTM09). As more than 90% of the values of interleukin 1β, interleukin 6 and interleukin 10 were below the threshold for detection, these systemic proinflammatory markers were not included in further analyses. For IL-8, two participants from the younger group had concentrations below the detectable threshold and these were considered
missing values. The average inter- and intra-assay coefficients of variation (CV) were <20% for IL-8 and TNF-α. All assays were run in accordance with the manufacturer’s instructions.

2.3 Neuropsychological assessment

The neuropsychological assessment is explained in full in (Lind et al., 2020). Briefly, two tests targeting vsWM were selected from a custom composed Cambridge Neuropsychological Test Automated Battery (CANTAB) (Cambridge Cognition Ltd, Cambridge, United Kingdom) (Sahakian and Owen, 1992). First, participants underwent a paired associates learning task in which one has to memorise the spatial position of random patterns. Second, participants underwent a spatial working memory task in which one searches for a hidden token amongst a defined number of locations. When found, the token will be hidden in a new location, but never in the same location twice and, therefore, participants must continually update their search strategy. In both tasks, the number of errors were used as outcome score and, as these correlated (rho=0.473, p<0.001), they were z-scored and summed into a composite vsWM score. The vsWM score was inverted so that higher scores indicated better performance.

2.4 MR acquisition

MR acquisition is described in full in (Lind et al., 2020). Briefly, semi-localised by adiabatic selective refocusing (sLASER) 1H-MRS data (Arteaga de Castro et al., 2013; Boer et al., 2011) (repetition time (TR)/echo time (TE)=3700/32 ms, bandwidth=4 kHz, data points=2048) was collected with a Phillips 7T whole body MR scanner (Philips, Best, Netherlands) equipped with a dual transmit coil and a 32-channel receive head coil (Nova Medical, Wilmington, MA, USA). SmartExam Brain (Philips, Best, Netherlands) was used to guide voxel placement. At the beginning of each scan, a non-water suppressed spectrum was acquired and afterwards variable pulse power and optimized relaxation delay (VAPOR) water suppression (Tkac et al., 2001) was applied. The fast automatic shimming technique by mapping along projections (FASTMAP) algorithm was used for second order B₀ shimming for each voxel separately with a voxel-centered shim box 15 mm larger than the voxel in each direction (Gruetter, 1993; Gruetter and Boesch, 1992). For the present study, three voxels were selected for analysis; medial ACC (20x20x20 mm³, 16 acquisitions), left hippocampus (30x15x15 mm³, 64 acquisitions) and left thalamus (16x12x16 mm³, 64 acquisitions) (Figure 2).
T₁-weighted magnetization prepared rapid gradient echo (MPRAGE) structural MR imaging data (slices=380, slice thickness=0.5, TR=8.0 ms, TE=3.2 ms, flip angle=7 degrees, field of view=256x256x190, acquisition matrix=64x64x380) was acquired for anatomical reference and tissue classification. The tissue classification was done with CAT 12 (Computational Anatomy Toolbox, The Structural Brain Mapping Group, University of Jena, Jena, Germany) implemented in SPM 12 (Statistical Parametric Mapping, Institute of Neurology, London, UK). For voxel tissue fractions per age group, we refer to Table 2 in (Lind et al., 2020).

![Image of brain slices with labeled areas](image)

**Figure 2.** Voxel placement and representative spectra before processing and baseline subtraction. The orange box represents the MRS voxel. ACC: anterior cingulate cortex, a: anterior, p: posterior. Figure is adapted from (Lind et al., 2020).

### 2.5 Quantification of ¹H-MRS data

Spectra were visually inspected and 4% were excluded from further analysis due to artefacts or poor quality based on (Kreis, 2004). LCModel (Provencher, 2001) was applied to fit included spectra using a custom basis set including 20 metabolites and a measured macromolecular baseline (for detailed information, see Lind et al. 2020). Spectra were fitted in the 0.2 to 4 ppm range using a knot spacing of 0.2 and the estimated metabolite levels were corrected for fractions of grey matter (GM), white matter (WM) and cerebrospinal fluid (CSF) in the voxel (Gasparovic et al., 2009; Quadrelli et al., 2016) using tissue specific attenuation factors (Bartha et al., 2002; Rooney...
(et al., 2007) by correcting the water concentration in the voxel (WaterConcorr) in the following manner:

$$\text{WaterConcorr} = \frac{[H2O] \ast (f_{GM} \ast R_{H2O,GM} + f_{WM} \ast R_{H2O,WM} + f_{CSF} \ast R_{H2O,CSF})}{1 - f_{CSF}}$$

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

$$f_x = \frac{f_{x,vol} \ast \text{con}_x}{f_{GM,vol} \ast \text{con}_{GM} + f_{WM,vol} \ast \text{con}_{WM} + f_{CSF,vol} \ast \text{con}_{CSF}}$$

and the tissue specific attenuation factors $R_{H2O,x}$ is:

$$R_{H2O,x} = e^{-\frac{TE}{T2_x} \ast (1 - e^{-\frac{TR}{T1_x}})}$$

where $[H2O]$ is the concentration of pure water, $f_{x,vol}$ is the fractional volume of tissue x within the voxel, $\text{con}_x$ is the water content in tissue x as a fraction of pure water, and $T1_x$ and $T2_x$ are the $T_1$ and $T_2$ relaxation times of water in tissue x. $\text{Con}_x$ 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).

After fitting, spectra were excluded if linewidth at full-width half-maximum (FWHM) exceeded 0.1 ppm. Metabolite values with a Cramèr-Rao lower bound (CRLB) of 20 or higher were excluded from the analyses. No spectra were excluded based on FWHM and no metabolite values were excluded based on CRLB, and a sufficient signal-to-noise ratio was verified for all spectra (see Table 3 in Lind et al., 2020). CRLB’s for tCr and tCho in ACC and mIns in hippocampus and thalamus were respectively <4, <7, <9 and <12.

To minimize data loss, spectra that were excluded by visual inspection were not considered missing values. Instead, the missing values were replaced by the group mean of the metabolite in that region.
2.5 Data analysis

Statistical analysis and visualisation were performed with Matlab R2019b (The Mathworks, Natick, MA, USA) and SPSS 25 (Statistical Package for the Social Sciences, Chicago, IL, USA) and threshold of significance was set to $p<0.05$ after correction for multiple comparisons with Bonferroni correction. Outliers more than three standard deviations away from the group mean were identified and removed. This led to removal of one value for IL-8 and one value for mIns in thalamus, both from individuals in the middle group.

Differences across age groups for demographic variables were tested with one-way ANOVA and post-hoc pairwise comparisons. The differences between age groups were tested for systemic proinflammatory marker concentrations, vsWM score and brain metabolite levels with an ANOVA followed by post-hoc pairwise comparisons. This to confirm that similar results were obtained in comparison with (Lind et al., 2020), in which data from the same participants were tested with ANOVA. For this study, the participant group differed slightly from (Lind et al., 2020) due to CRP values being higher than 10 mg/L in two participants and an outlying IL-8 value in one participant.

Spearman’s rank correlations tested the associations between systemic proinflammatory marker concentrations and vsWM score and brain metabolite levels. Spearman’s rather than Pearson’s correlations were used as it was chosen not to transform the inflammatory marker data and therefore linearity, normality and homoscedasticity could not be assumed in all cases. The significance tests were one-tailed testing for negative correlations between systemic proinflammatory marker concentrations and vsWM score and positive correlations between systemic proinflammatory marker concentrations and brain metabolite levels. Correlation analysis was also tested within group. In cases where concentrations of a systemic proinflammatory marker correlated with levels of a brain metabolite, mediation analysis was performed using the PROCESS v3.3 macro implemented in SPSS (Preacher and Hayes, 2008). Variables were centred and bootstrapping (5000 iterations) was applied to calculate 95 % confidence intervals for the indirect effect. The model tested whether the effect of the systemic proinflammatory marker concentration on vsWM score was mediated by the levels of the brain metabolite.

To ensure correction for different GM/WM ratios across participants was performed cohesively across the different types of data analysis, the correction was implemented on the $^1$H-MRS data before analysis. Correction was done by calculating the residuals from a linear fit.
between metabolites and GM/WM fraction and adding it to the mean. This was done for each age group and metabolite separately.

Based on (Lind et al., 2020), four brain metabolites of interest were included in the current analysis; tCr and tCho in ACC and mIns in hippocampus and thalamus. As the final sample was smaller than in (Lind et al., 2020), it was tested if the brain metabolites still correlated with vsWM and this was true for all metabolites; tCr in ACC (R=-0.380, p=0.002), tCho in ACC (R=-0.462, p<0.001), mIns in hippocampus (R=-0.367, p=0.002) and mIns in thalamus (R=-0.308, p=0.010).

3. Results
Systemic proinflammatory marker concentrations, brain metabolite levels and vsWM scores are summarized in Table 2.

Table 2. Measures of systemic proinflammatory markers, cognition, and brain metabolites (mean±SD).

<table>
<thead>
<tr>
<th></th>
<th>Younger</th>
<th>Middle</th>
<th>Older</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systemic proinflammatory marker concentrations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>0.75±0.60*</td>
<td>1.73±1.88</td>
<td>2.08±1.83*</td>
</tr>
<tr>
<td>IL-8 (ng/L)</td>
<td>2.83±1.11*</td>
<td>3.14±1.12</td>
<td>4.21±2.11*</td>
</tr>
<tr>
<td>TNF-α (ng/L)</td>
<td>7.36±2.39</td>
<td>8.11±3.02</td>
<td>8.85±2.75</td>
</tr>
<tr>
<td>Cognitive measure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vsWM score</td>
<td>1.28±0.66*</td>
<td>0.51±1.02*</td>
<td>-1.80±1.71**</td>
</tr>
<tr>
<td>Brain metabolite levels (I.U.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tCr in ACC</td>
<td>6.97±0.33*</td>
<td>7.17±0.70*</td>
<td>7.60±0.50**</td>
</tr>
<tr>
<td>tCho in ACC</td>
<td>1.44±0.16**</td>
<td>1.63±0.23*</td>
<td>1.71±0.19*</td>
</tr>
<tr>
<td>mIns in Hipp</td>
<td>5.68±0.60**</td>
<td>6.69±1.02**</td>
<td>7.45±0.94**</td>
</tr>
<tr>
<td>mIns in Thal</td>
<td>4.43±0.69*</td>
<td>4.74±0.48</td>
<td>5.25±1.10*</td>
</tr>
</tbody>
</table>

Note: Age groups that are pairwise significantly different (p<0.05) are marked with ** if different from both other groups and * if different from one other group. Brain metabolite levels were corrected for differences in within-voxel GM/WM ratio. Brain metabolite levels and vsWM score for the full sample before exclusion of participants due to high CRP concentrations and correction of metabolite levels for GM/WM differences can be found in (Lind et al., 2020). ACC: anterior cingulate cortex, CRP: C-reactive protein, GM: grey matter, Hipp: hippocampus, IL-8:

3.1 Systemic proinflammatory markers across age groups

There was a main effect of age group for concentrations of CRP (F_{2,54}=3.688, p=0.032) and IL-8 (F_{2,51}=4.078, p=0.023) but not for TNF-α (F_{2,54}=1.434, p=0.247) (Figure 3). The older group had higher concentrations than the younger group for CRP (p=0.034) and IL-8 (p=0.028). Before Bonferroni correction, IL-8 concentrations were higher in the older than the middle group (p=0.039 uncorrected). Controlling for sex did not change the significance of the results, but no results remained significant when controlling for age.

Additionally, it was tested whether the systemic inflammatory markers correlated with each other. This was not observed to be the case, however, before Bonferroni correction, IL-8 correlated with TNF-α (rho=0.267, p=0.026 uncorrected).

**Figure 3.** Boxplots of systemic proinflammatory markers by age group. Age groups that are pairwise significantly different from each other (p<0.05) after Bonferroni correction are marked with *. For CRP, N=19 in each group. For IL-8, N=17 in the younger group, N=18 in the middle group, and N=19 in the older group. For TNF-α, N=19 in each group. CRP: C-reactive protein, IL-8: Interleukin 8, TNF-α: Tumor necrosis factor α.
3.2 Systemic proinflammatory markers, cognitive performance and brain metabolites

3.2.1 Correlation analysis

As age-related differences were observed for CRP and IL-8 concentrations, correlations with vsWM score and brain metabolite levels were calculated. Exact statistics for all correlations can be found in Table 3 and significant findings are visualised in Figure 4. CRP concentrations correlated negatively with vsWM score and positively with mIns levels in thalamus. IL-8 concentrations correlated positively with tCho levels in ACC and mIns levels in hippocampus. All findings remained significant when controlling for sex, but none remained significant when controlling for age. Furthermore, all findings remained significant when excluding participants with CRP concentrations above 4 mg/L and even when excluding participants with concentrations above 2 mg/L. Significant findings also remained when removing outliers that were more than two standard deviations from the mean rather than three. The correlations were, thus, not driven by a few participants with high values.

When analysing within groups, no correlations were found. Before Bonferroni correction, a correlation was found between CRP concentrations and mIns levels in thalamus in the younger group (rho=0.420, p=0.042 uncorrected) and IL-8 concentrations and tCr levels in ACC for the middle group (rho=0.463, p=0.031 uncorrected).

![Figure 4](image-url) Scatterplots of significant associations between systemic proinflammatory marker concentrations and vsWM score or brain metabolite levels. Brain metabolite levels were corrected.

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for within-voxel GM/WM ratio. Top left: N=19 in each group; lower left: N=19 in the younger and older group, N=18 in the middle group; top and lower right: N=17 in the younger group, N=18 in the middle group, N=19 in the older group. ACC: anterior cingulate cortex, CRP: C-reactive protein, GM: grey matter, Hipp: hippocampus, IL-8: Interleukin 8, I.U.: institutional units, mIns: myo-inositol, tCho: total choline, tCr: total creatine, Thal: thalamus, vsWM: visuospatial working memory, WM: white matter.

Table 3. Correlations between concentrations of systemic proinflammatory markers and vsWM score and brain metabolite levels.

<table>
<thead>
<tr>
<th>Systemic proinflammatory marker concentrations</th>
<th>CRP</th>
<th>IL-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cognitive measure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>vsWM score</td>
<td>rho=-0.322*, p=0.015</td>
<td>rho=-0.209, p=0.129</td>
</tr>
<tr>
<td>Brain metabolite levels</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tCr in ACC</td>
<td>rho=0.077, p=0.569</td>
<td>rho=0.267, p=0.051</td>
</tr>
<tr>
<td>tCho in ACC</td>
<td>rho=0.176, p=0.190</td>
<td>rho=0.337*, p=0.013</td>
</tr>
<tr>
<td>mIns in hippocampus</td>
<td>rho=0.172, p=0.200</td>
<td>rho=0.297*, p=0.029</td>
</tr>
<tr>
<td>mIns in thalamus</td>
<td>rho=0.426*, p=0.001</td>
<td>rho=0.055, p=0.695</td>
</tr>
</tbody>
</table>

Note: Significant findings are marked with *. Brain metabolite levels were corrected for within voxel GM/WM ratio.

3.2.2 Mediation analysis

Mediation models and exact statistics are visualised in Figure 5. It was tested whether the effect of CRP concentrations on vsWM score was mediated by thalamic mIns levels and whether the effect of IL-8 concentrations on vsWM score was mediated by ACC tCho levels or hippocampal mIns levels. There was an indirect effect of IL-8 concentrations on vsWM score via ACC tCho levels. No indirect effects were observed in the two other mediation analyses. Controlling for sex did not change the results, but when controlling for age there was no longer a significant indirect effect for IL-8 concentrations on the vsWM score via tCho levels.
Figure 5. Mediation analysis. Point estimates and 95 % confidence intervals for the indirect effects of the three models testing A) whether the effect of CRP concentrations on vsWM score was mediated by thalamic mIns levels (mIns Thal) (N=19 in the younger and older group, N=18 in the middle group), B) whether the effect of IL-8 concentrations on vsWM score was mediated by ACC tCho levels (tCho ACC) (N=17 in the younger group, N=18 in the middle group, N=19 in the older group), and C) whether the effect of IL-8 concentrations on vsWM score was mediated by hippocampal mIns levels (mIns Hipp) (N=17 in the younger group, N=18 in the middle group, N=19 in the older group). Only model B had significant indirect effects. Dashed lines denote direct relationships that are not significant (p<0.05). Coefficients are denoted by $\beta$ and (standard error). ACC: anterior cingulate cortex, CRP: C-reactive protein, Hipp: hippocampus, IL-8: interleukin 8, mIns: myo-inositol, tCho: total choline, Thal: thalamus, vsWM: visuospatial working memory.

4. Discussion
In this paper, we explored the associations between systemic inflammation, brain metabolites mIns, tCr and tCho, and vsWM across different age groups. Concentrations of the systemic proinflammatory markers CRP and IL-8 were higher in the older than the younger group, indicative of inflammaging. The TNF-$\alpha$ concentrations were not different across age groups. CRP
concentrations correlated negatively with vsWM score and positively with mIns levels in thalamus, however, the effect of CRP concentration on vsWM score was not mediated by mIns levels. In contrast, IL-8 concentrations correlated positively with both tCho levels in ACC and mIns levels in hippocampus. IL-8 concentrations did not correlate with the vsWM score, but the mediation analysis showed an indirect effect for IL-8 concentrations on vsWM score via tCho levels in ACC. As mIns and tCho are primarily found in glial cells, our results thus indicate associations between systemic inflammation, glia-related brain metabolites and vsWM. This is consistent with the hypothesis of the neurobiological link between systemic inflammaging and cognitive ageing involving glial cells potentially through their role in neuroinflammation.

CRP concentrations were higher in older individuals compared to younger individuals and were associated with higher mIns levels in thalamus. The latter has not been observed before in thalamus across age groups, although CRP has previously been related to mIns levels in occipitoparietal cortex in a middle-aged population and to mIns levels in the hippocampus in patients with cognitive impairment (Eagan et al., 2012; Ge et al., 2013). Although structural MR studies do not indicate thalamus to be a particularly ageing sensitive brain region, the concentration of activated microglia have been found to increase in thalamus during ageing (Jernigan et al., 2001; Schuitemaker et al., 2012). Furthermore, the thalamus is a critical node in several networks underlying ageing sensitive cognitive functions including vsWM (Fama and Sullivan, 2015; Goldstone et al., 2018).

In the present study, CRP concentrations also correlated with vsWM score. This corresponds with previous literature describing CRP concentrations to increase with age and link with cognitive ageing (Ferrucci et al., 2005; Yaffe et al., 2003). The effect of CRP concentrations on vsWM score was not mediated by mIns levels in thalamus. However, the sample size was limited and might be too low to detect a potential mediation effect with sufficient sensitivity. Furthermore, CRP is a non-specific marker of inflammation. It functions in the immune system as an acute phase protein that mediates clearance by binding to ligands, e.g. on pathogens or distressed cells and its production is induced by various proinflammatory cytokines (Eklund, 2009). The inflammaging-related elevation in CRP during ageing might, thus, arise from many different processes. The lack of mediation in spite of correlation could arise from the link between the variables being formed by different indirect processes. Overall, the results of the present study provide supporting evidence for a link between inflammaging of CRP, thalamic glial cells and lower vsWM performance, although this link may be formed by indirect connections.
Like CRP, IL-8 concentrations were also higher in the older than the younger age group which is consistent with previous findings (Koelman et al., 2019; Verschoor et al., 2017). IL-8 concentrations correlated with tCho levels in ACC and mIns levels in hippocampus. The ACC, as part of the prefrontal cortex, and the hippocampus are considered the two most ageing-sensitive brain regions (Hedden and Gabrieli, 2004). IL-8 concentrations were, thus, associated with glial metabolites in the most ageing sensitive brain regions. IL-8 can be released by almost all cell types including glial cells and it functions as a chemokine which lingers for a long time after the initial inflammatory response (Mukaida et al., 1998; Remick, 2005; Walker et al., 2001). The observed association between glia-related metabolite levels and IL-8 concentration could, thus, be caused by brain IL-8 production spilling over into the blood stream, by systemic IL-8 stimulating glial cells or by indirect mechanisms connecting the systemic immune system and the brain. This study promotes the role of systemic IL-8 as a potential marker of neurobiological ageing, however, further studies are needed to clarify the origin of the systemic IL-8 elevation.

In relation to cognitive ageing, IL-8 concentrations did not correlate with vsWM score. Previous studies have linked IL-8 concentrations in older individuals to poorer memory, but not to vsWM in particular (Baune et al., 2008). Mediation analysis, however, revealed an indirect effect relating IL-8 concentrations to vsWM score via tCho levels in ACC. Traditional mediation methods would not recommend doing a mediation analysis in this case (Baron and Kenny, 1986), as IL-8 concentrations and vsWM score were not significantly correlated. However, more recent theories of mediation consider this an unnecessary prerequisite (Hayes, 2017). In the present study, the mediation analysis suggested that, even though IL-8 concentrations did not correlate directly with vsWM score, there still might be an indirect link between these variables through tCho levels in the ACC.

TNF-α concentrations were not higher in older age groups. Although this has previously been observed, an increase with age has also been observed (Bruunsgaard et al., 1999; Ferrucci et al., 2005). Previous evidence suggests that the increase in TNF-α concentrations may primarily be detected in the oldest old, i.e. centenarians, where it relates to frailty and mortality (Bruunsgaard et al., 2003; Krabbe et al., 2004). In this study, we did not detect a difference between age groups in TNF-α concentrations, but we cannot rule out that it might have appeared, had we extended our oldest age group to include a wider age range.

This study adds in vivo neurochemical evidence to the literature linking inflammaging of systemic proinflammatory markers and cognitive ageing to brain changes (for
review see: (Frodl and Amico, 2014)) and it extends it by indicating glial cells as part of the neurobiological link. Although a relationship between systemic inflammation and brain metabolite markers has been demonstrated in diseases such as mild cognitive impairment (Ge et al., 2013), no previous studies have, to our knowledge, investigated the association between in vivo brain metabolites and systemic inflammation during normal ageing. Studies of normal ageing using other MR modalities have shown that systemic inflammation is associated with brain atrophy, WM microstructural disintegration and hyperintensities, lower regional cerebral blood flow and altered functional connectivity (Dev et al., 2017; Satizabal et al., 2012; Warren et al., 2018; Wersching et al., 2010). $^1$H-MRS could prove a valuable tool to describe the cellular and chemical underpinnings of the previously established MR changes. Multimodal studies including $^1$H-MRS, thus, have the potential to unravel the role of glial cells in the multitude of brain changes occurring during ageing in association with systemic inflammation.

4.1 Limitations

The findings of this study should be interpreted in the light of several limitations. Firstly, the study is cross-sectional rather than longitudinal. For this reason, causal inferences cannot be based on this data and some of the findings may represent cohort effects or independent processes changing with age. Furthermore, the number of individuals in each group was relatively low and the findings thus require replication in a larger study. Lastly, with regards to $^1$H-MRS, the metabolite specific attenuation factors could be affected by age and tissue type, however, as these are not all known for 7T it was not possible to take this into account (Kirov et al., 2008).

4.2 Conclusion

In conclusion, concentrations of CRP and IL-8 were higher in older age groups which is consistent with inflammaging. These systemic proinflammatory markers were associated with elevated gliar-related metabolite levels in older individuals, possibly reflecting neuroinflammation. The CRP concentrations, furthermore, correlated with vsWM score, and IL-8 concentrations were indirectly linked to vsWM score via tCho levels in ACC. As vsWM score was used as a proxy for cognitive ageing, these results are consistent with the notion that systemic inflammaging is linked to cognitive ageing through neuroinflammation. However, future longitudinal studies are needed to clarify the causal mechanisms linking these ageing sensitive measures.
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