In vivo characterization of downfield peaks at 9.4 T: $T_2$ relaxation times, quantification, pH estimation, and assignments

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Purpose: Relaxation times are a valuable asset when determining spectral assignments. In this study, apparent $T_2$ relaxation times ($T_{2}^{app}$) of downfield peaks are reported in the human brain at 9.4 T and are used to guide spectral assignments of some downfield metabolite peaks.

Methods: Echo time series of downfield metabolite spectra were acquired at 9.4 T using a metabolite-cycled semi-LASER sequence. Metabolite spectral fitting was performed using LCModel V6.3-1L while fitting a pH sweep to estimate the pH of the homocarnosine (hCs) imidazole ring. $T_{2}^{app}$ were calculated by fitting the resulting relative amplitudes of the peaks to a mono-exponential decay across the TE series. Furthermore, estimated tissue concentrations of molecules were calculated using the relaxation times and internal water as a reference.

Results: $T_{2}^{app}$ of downfield metabolites are reported within a range from 16 to 32 ms except for homocarnosine with $T_{2}^{app}$ of 50 ms. Correcting $T_{2}^{app}$ for exchange rates ($T_{2}^{corr}$) resulted in relaxation times between 20 and 33 ms. The estimated pH values based on hCs imidazole range from 7.07 to 7.12 between subjects. Furthermore, analyzing the linewidths of the downfield peaks and their $T_{2}^{app}$ contribution led to possible peak assignments.

Conclusion: $T_{2}^{app}$ relaxation times were longer for the assigned metabolite peaks compared to the unassigned peaks. Tissue pH estimation in vivo with proton MRS and simultaneous quantification of amide protons at 8.30 ± 0.15 ppm is likely possible. Based on concentration, linewidth, and exchange rates measurements, tentative peak assignments are discussed for adenosine triphosphate (ATP), N-acetylaspartylglutamate (NAAG), and urea.
INTRODUCTION

Proton magnetic resonance spectroscopy (1H-MRS) allows for non-invasive detection and quantification of metabolites. The upfield 1H spectrum (between 0.0 and 4.7 ppm) is well characterized and understood. However, the downfield 1H spectrum (between 4.7 and 10.0 ppm) poses a significant challenge to the MRS community because most resonances in this ppm range are low in signal-to-noise ratio (SNR), severely overlapped, and/or fast exchanging. Therefore, it remains less explored, and most peaks remain unassigned.

Increased SNR and better peak separation are prime advantages of ultra-high field strengths and could help tackle the challenges posed by downfield spectra. Non-water saturation techniques such as metabolite cycling are crucial to handle the challenge posed by chemical compounds, which exchange protons with water. Utilizing these 2 complementary approaches, exploring downfield spectra was feasible, and the spectral assignment of the downfield peaks, which is of interest, was achievable.

T1 and T2 relaxation times and exchange rates at different field strengths play a pivotal role in guiding spectral assignment of the downfield peaks. Downfield resonances have shorter T2 relaxation times at 7 T compared to the more widely studied upfield metabolites. At 9.4 T, they are expected to have even shorter T2 relaxation times due to the B0 dependence of T2 relaxation times. Because T2 relaxation times are measured by observing the exponential decay in echo time (TE) series spectra, a sequence that allows short TEs was chosen in this work. A previous study used metabolite-cycled (MC) stimulated echo acquisition mode (STEAM). However, due to the improved SNR of semi-localized by adiabatic selective refocusing (semiLASER) compared to STEAM, the MC-semiLASER was preferred in the current study design.

A previous study at 9.4 T characterized the T1 relaxation times and the chemical exchange rates of downfield metabolite resonances and reported several peaks that reflect a chemical exchange of protons with water in the downfield metabolite spectrum. Chemical exchange saturation transfer (CEST) experiments also exploit the mechanism of chemical exchange, and the major exchange effects in Z-spectra are observed in the downfield proton spectral range. There are many exchangeable amide protons with resonances between 5.0 and 10.0 ppm. The CEST amide proton transfer signal has shown improved contrast between healthy and diseased tissue in diseases such as cancer or stroke. The CEST amide signal has, however, a strong dependence on both the concentration of mobile amide protons and pH, as demonstrated by simulations and on rat brain metastasis. Although a recent study on human breast cancer found that "the concentration of mobile amide protons is the main contributor to the observed amide proton transfer signal," compared to pH, the contributions of the amide-CEST contrast require further investigation.

CEST shows a significant increase in contrast at ultra-high field. Recent CEST research is moving toward a more quantitative analysis, such as CEST MR-fingerprinting or Bloch fitting, for which knowledge about amide T2 relaxation times and pH values is crucial. Hence, the characterization of downfield spectra through 1H-MRS, including the amide resonances and assessing the achievable pH sensitivity at ultra-high field, will also benefit the CEST community.

In 1H-MRS, only homocarnosine (hCs) and histidine (hist) are reported to have pH-sensitive chemical shifts for their downfield imidazole rings: for hCs, the hCsIm-C4 at ~7.08 ppm and hCsIm-C2 at ~8.08 ppm; and for hist, the histIm-C4 at ~7.06 ppm and histIm-C2 at ~7.79 ppm. Measuring pH variations is useful because diseases such as glioblastoma not only change the concentration of a few metabolites in the brain but also cause variations in the tissue pH, as shown in phosphorus-31 spectroscopy (31P-MRS). Therefore, assessing the pH sensitivity in vivo with 1H-MRS is of interest.

The primary goal of this study was to measure the apparent T1 relaxation times (T2app) of the downfield resonances at 9.4 T. Although T2 relaxation times have been reported in a previous study at 7 T for resonances that do not undergo chemical exchange, this study aims at quantifying T2app also for the downfield resonances with protons exchangeable with water (15 peaks in total). These T2app relaxation times were further corrected for the previously reported exchange rates. Additionally, estimated tissue concentrations of molecules resonating downfield are reported after applying relaxation corrections. The linewidth (Δν1/2), after accounting for T2app relaxation times and micro- and macro-susceptibility effects of the downfield peaks, might indicate J-coupling or overlapping resonances. This quantitative linewidth characterization helped analyze the degree of spectral overlap, which along with the concentrations aided spectral assignments.

METHODS

2.1 Technical description and subjects

Measurements were performed on 11 healthy volunteers (8 males, 3 females, age: 26 ± 3 years) on a 9.4 T Magnetom whole-body MRI scanner (Siemens Healthineers, Erlangen,
Germany) using a home-built proton coil with 8 transmit and 16 receive channels. Single voxel MRS data were acquired in a gray-matter (GM)–rich region within the occipital lobe. The coil was driven in surface mode by driving the bottom 3 channels of the coil using an unbalanced 3-way Wilkinson splitter as previously described for 2D FLASH imaging and spectroscopy acquisitions. The local ethics board approved the study, and all subjects gave written informed consent before the examination.

2.2 | Data acquisition

Spectroscopy voxels of $2 \times 2 \times 2$ cm³ were positioned in the occipital lobe in a GM-rich region, facilitated by gradient-echo images acquired using a 2D FLASH sequence (in-plane resolution: $0.7 \times 0.7$ mm², 20 slices, 3 mm slice thickness, flip angle: $25°$, TE = 9 ms, TR = 378 ms, acquisition time: 2:03 min, field-of-view: $197 \times 197$ mm) along axial and sagittal orientations. FAST (EST) MAP² was used for $B_0$ shimming and $T_2$ measurements of the downfield resonances were performed by acquiring a TE series using MC semi-LASER² (excitation pulse duration: 1.2 ms, refocusing pulse duration: 3.5 ms, TR: 6 s, number of excitations: 96). The TEs were nonlinearly spaced (TE: 24, 32, 40, 52, 60 ms). The increase in TE was spread evenly across all timings (between the excitation pulse, refocusing pulses, and the start of the data sampling), whereas the timing between the second and third refocusing pulse was kept at the minimum system specified ramp time. The 8 kHz bandwidth of both the excitation pulse and the adiabatic full-passage pulse resulted in a chemical shift displacement error of $5\%$ per ppm. The transmit reference frequency ($v_{ref}$) was set at 7.0 ppm, which led to a chemical shift displacement error of $-7.5\%$ for the 8.50 ppm peak. A 16-step phase-cycling was implemented to avoid contributions from other unwanted coherence pathways.

In order to use the internal water as a reference for estimated tissue concentration calculations of molecules, water reference signals were acquired with the same semi-LASER sequence (TE: 24 ms, $v_{ref}$: 4.7 ppm) without metabolite cycling. This was done in order to avoid any influence of MC pulses on the water signal due to the asymmetry of the MC pulse and localization differences due to the difference of $v_{ref}$ of 2.4 ppm in the metabolite spectral measurements and the water frequency.

The data used in the current study were also used for the Murali-Manohar²⁷ up-field metabolite $T_2$ study. High-resolution MP2RAGE²⁸ images ($0.6 \times 0.6 \times 0.6$ mm³) were acquired using the same coil by driving radio-frequency power to all 8 channels for 5 out of the 11 volunteers. These images were then segmented using SPM12²⁹ into GM, white matter (WM), and cerebrospinal fluid (CSF) tissue probability maps for use in quantification of the estimated tissue concentrations of molecules.

2.3 | Data preprocessing

Raw data were analyzed with in-house written software in MatLab (version 2016a, MathWorks, Natick, MA). The metabolite MRS data were processed as described previously. The following steps were used in the processing of the raw data: 1) truncation of free induction decays at 250 ms to get a better SNR for subsequent processing steps, 2) frequency and phase alignment, 3) metabolite-cycling subtraction, 4) averaging, 5) zero-order phase and eddy current correction using the phase information from the MC water signal, 6) coil channel combination using a singular value decomposition method, 7) peak alignment in the frequency domain to 3.028 ppm, 8) residual water removal using a Hankel singular value decomposition method, 9) reversing in the frequency domain the downfield spectra to positive magnetization while keeping the upfield spectra also positive, 10) residual water removal using an Hankel singular value decomposition method for the potential water residuals not handled in step 8, and 11) truncation of free induction decays at 200 ms because the signal decays by that time.

The SNR of the N-acetylaspartate (NAA) downfield resonance was calculated using the real part of the spectrum, dividing the peak amplitude by the standard deviation (SD) of the noise between $-4.0$ and $-1.0$ ppm.

2.4 | Fitting

The metabolite basis set was simulated in Vespa (v0.9.3) with full quantum mechanical density matrix calculations for the semi-LASER sequence, including the actual excitation and adiabatic RF pulse shapes for all the TEs specified. The upfield metabolites were included in the basis set as described in Murali-Manohar et al, while simulating the $v_{ref}$ at 7.0 ppm. The simulated basis set consisted of following downfield (DF) peaks: NAA, hCs, and nicotinamide adenine dinucleotide (NAD⁺). The simulated Voigt lines (CHSIMU) included: DF 5.75 (5.75 ppm), DF 5.97 (5.97 ppm), DF 6.12 (6.12 ppm), DF 6.83 (6.83 ppm), DF 7.04 (7.04 ppm), DF 7.30 (7.30 ppm), DF 7.48 (7.48 ppm), DF 8.18 (8.18 ppm), DF 8.24 (8.24 ppm), DF 8.37 (8.37 ppm), NAA broad component (7.86 ppm), and DF 8.49 (8.49 ppm). The chemical shifts were chosen based on the previous work by Fichtner et al. These were further tailored by finding the maximum peak amplitudes in subject-wise summed spectra and matching the linewidths of LCMonitor V6.3-1L to best describe the data of the TE series. After finding these settings from the across subjects summed spectra, these settings were used to fit all the per subject TE series. Because most of the downfield
peaks are unassigned, J-evolution effects were not considered. DKNTMN was set to 0.5 to enforce a flat spline baseline by LCModel. The fitting range was from 0.6 to 9.5 ppm to fit upfield and downfield metabolites simultaneously, and an LCModel ppm gap between 4.1 and 5.5 ppm was used to avoid baseline effects from the water residual. The upfield portion of spectra was used to constrain the LCModel software by providing metabolite lineshapes. None of the upfield and downfield components of the same metabolites were linked; they all were fitted independently. For instance the NAA aspartyl moiety was split into its upfield and downfield components due to very different $T_2$ relaxation times and exchange effects. The chemical shifts, linewidth settings, and all other LCModel parameters are described in Supporting Information Annex A.

Experimentally acquired TE-specific upfield macromolecular spectra were included in the basis set, but the downfield part of the spectra was set to 0.

### 2.5 pH estimation

The chemical shift of most peaks in metabolite spectra are sensitive to pH changes; however, in $^1$H-MRS only hCs and hist have been shown to have measurable sensitivities for physiological pH variations. The effects of pH in MRS are described by the derived form of the Henderson-Hasselbalch equation for fast exchanging acid/base systems, for which the observed chemical shift ($\delta_{\text{obs}}$) is a weighted average of the conjugated acid ($\delta_{\text{acid}}$) and base ($\delta_{\text{base}}$) endpoints, and $pK_a$ is the logarithm of the acid dissociation constant:

$$\text{pH} = pK_a + \log \frac{\delta_{\text{obs}} - \delta_{\text{acid}}}{\delta_{\text{base}} - \delta_{\text{obs}}}.$$  \hspace{1cm} (1)

Rewriting the equation, the chemical shift can be calculated for any given pH by:

$$\delta_{\text{obs}} = \frac{10^{pK_a-pH} \times \delta_{\text{base}} + \delta_{\text{acid}}}{1 + 10^{pK_a-pH}}. $$ \hspace{1cm} (2)

The coefficients of these equations for hCs and hist are summarized in Table 1. Basis sets of hCs were created using Equation (2) for a pH sweep varying from 6.90 to 7.15 in steps of 0.01 (Supporting Information Figure S1). The previously described upfield and downfield metabolites, including the hCs imidazole peaks (ignoring the upfield GABA moiety of hCs), were used to create a combined upfield and downfield basis set specific to each pH value and TE.

Because pH differences between subjects are possible, each subject’s set of spectra was fitted with all basis sets of the pH sweep. LCModel was constrained to not shift the hCs peaks using the following parameters: $nsdsh = 1$; $chsdsh(1) = \text{hCs}$; $alsdsh(1) = 0.0005$. After this fitting iteration, the pH value for each subject (pHy) was estimated by finding the pH value, for which the concentration of hCs [$\text{conc}_{\text{hCs}}(y, pH)$] was maximal:

$$pH_y = \arg\max_{pH=6.90,6.91,\ldots,7.15} \left[ \text{conc}_{\text{hCs}}(y, pH) \right], \text{ with } y = 1..11, $$ \hspace{1cm} (3)

where $y$ represents the individual subject.

The hCs imidazole resonance at 7.08 ppm (hCs$_{\text{Im-C4}}$) has an overlap with a fast decaying component, named DF$_{7.04}$, observable both visually in spectra (Figures 1 and 2) and reported previously. To avoid the influence of this DF$_{7.04}$ peak in the estimation of the pH, the peak was excluded from the basis set in the pH estimation step. TE = 40 ms was chosen for the estimation of the pH$_y$ following visual analysis, observing the longer relaxation times of hCs compared to other peaks, as also reported previously but also considering the loss in SNR at later TEs.

After this pH estimation step, the entire TE series for each subject was refitted with the basis set using the calculated subject-specific pH$_y$ while also including the previously excluded DF$_{7.04}$ peak.

### 2.6 $T_2$ relaxation

The LCModel-fitted concentrations of the molecules resonating downfield were fit to a mono-exponential decay across the TE series to measure the $T_2^{\text{app}}$. The goodness of the exponential fits was evaluated by the mean coefficient of determination ($R^2$). Relaxation time estimates with $R^2 < 0.50$ were discarded. Because some peaks exchange with water at an

| hCs$^{18}$ | hist$^{19}$ |
|-----------|-----------|
| **Im C4 7.08 Resonance** | **Im C2 7.08 Resonance** | **Im C4 7.06 Resonance** | **Im C2 7.79 Resonance** |
| $pK_a$ | 6.86 | 6.86 | 6.127 | 6.114 |
| $\delta_{\text{acid}}$ (ppm) | 7.27 | 8.58 | 7.39 | 8.651 |
| $\delta_{\text{base}}$ (ppm) | 6.92 | 7.68 | 7.029 | 7.719 |

**Table 1**: Coefficients of the derived form of the Henderson-Hasselbalch equation for the 2 metabolites in $^1$H-MRS sensitive to pH in the physiological range.

C2/4, carbon two and four in the imidazole ring; hCs, homocarnosine; hist, histidine; Im, imidazole.
exchange rate $k$, the $T_2^{pp}$ were corrected to get the exchange rate-corrected $T_2$ relaxation time ($T_2^{cor}$) \(^{17}\) such that:

$$\frac{1}{T_2^{cor}} = \frac{1}{T_2^{pp}} - k.$$  \hspace{1cm} (4)

### 2.7 Linewidth calculations

The full-width-half-maxima ($\Delta v_{1/2}$) were measured by extracting the fitted lineshapes of the peaks from the .coord files of the LCModel quantification. Contribution of $T_2$ relaxation and exchange to $\Delta v_{1/2}$ were calculated according to \(\pi T_2^{pp} \) \(^{-1}\), using the calculated $T_2^{pp}$ values.

The residual linewidth was defined as $\Delta v_{\text{residual}} = \Delta v_{1/2} - (\pi T_2^{pp})^{-1} - \Delta v_{\text{micro,macro}}$. The $B_0$ component was calculated from the total creatine $\text{CH}_3$ resonance [7Cr($\text{CH}_3$)] as: $\Delta v_{\text{micro,macro}} = \Delta v_{1/2} - (\pi T_2^{pp})^{-1} \approx \Delta v_{\text{micro}} + \Delta v_{\text{macro}}$, respectively. $\Delta v_{\text{micro}}$ and $\Delta v_{\text{macro}}$ represent the micro- and macro-susceptibility, respectively. Please find a more comprehensive discussion in Supporting Information Annex B.

### 2.8 Concentrations

Quantification of the fitted concentrations was done as described in Supporting Information Annex C.

### 3 RESULTS

Downfield spectra from all subjects displayed good spectral quality with the NAA downfield SNR = 59 ± 14 for $TE = 24$ ms; SNR = 12 ± 4 for $TE = 60$ ms; shimming achieved a linewidth of unsuppressed water of 17.9 ± 1.5 Hz.

Figure 1 shows the TE series of downfield spectra. The shaded area represents the SD across all subjects indicating the reproducibility. No data sets were excluded from the study.

#### 3.1 Fitting

Sweeping the pH from 6.90 to 7.15 yielded chemical shifts for hCs\(_{\text{Im-C4}}\) ranging from 7.087 to 7.039 ppm, and hCs\(_{\text{Im-C2}}\) ranging from 8.109 to 7.985 ppm (Supporting Information Figure S1).

Adjusting the pH value of hCs subject-wise resulted in improved spectral fitting (Figure 2). Visually notable differences were observed for pH changes of 0.03 and were especially pronounced on the more pH-sensitive hCs\(_{\text{Im-C2}}\). Plotting the resulting concentrations across the pH range provided parabolic curves, with each subject having a well-defined maximum, defining the calculated pH value ($pH_y$) (see hCs pH sweep results in Figure 2). The Cramer-Rao lower bounds show the opposite trend, also because the noise in LCModel was calculated from the fit residue.

The estimated $pH_y$ values are reported for each subject in Supporting Information Table S1. Whereas only the results of the TE = 40 ms were used in the further fitting, a good agreement was observed between the calculated pH value per subject $pH_y$ and the mean calculated pH value. All the estimated pH values are reported with a SD of 0.02 or lower.

Figure 3 shows a representative downfield spectrum acquired with $TE = 24$ ms from 5.5 to 9.5 ppm, with minimum fit residuals. A similar good quality fit of spectra was achieved.
for all subjects, as seen in the representative TE series spectra in Supporting Information Figure S2 but with some structured negative phase noise observed at later TEs, which could suggest some J-evolution effects. Supporting Information Figure S3 also shows the fit of the entire spectrum ranging from 0.5 to 9.5 ppm because the upfield and the downfield resonances were fit simultaneously for all datasets. The fitted singlet metabolites showed Lorentzian-like lineshapes without distortions.

Cramer-Rao lower bounds, as calculated by LCModel for the fitted metabolites, are reported in Supporting Information Table S2 and in Supporting Information Figure S4.

### 3.2 | T₂ relaxation

Figure 4 shows the exponential decay curves of the downfield metabolites. The calculated $T_{2\text{pp}}$ of the downfield resonances in decreasing order are shown in the box plots of Figure 5. $T_{2\text{pp}}$ of the downfield peaks were between ~16 and ~32 ms, except the $T_{2\text{pp}}$ of hCs was measured to be ~50 ms. Table 2 provides the value of $T_{2\text{pp}}$ with each $R^2$ (after the $R^2 < 0.50$ exclusion criterion). The $R^2$ values are all above 0.75, showing the goodness of the exponential fits to individual datasets. NAD⁺ fits were excluded due to low reliability (see also Cramer-Rao lower bounds values), whereas from the across-subjects summed spectra, the $T_{2\text{pp}}$ of NAD⁺ was estimated as 30.3 ms ($R^2 = 0.86$). The calculated $T_{2\text{err}}$ values are longer and closer to each other than $T_{2\text{pp}}$ and lie mostly between 21 and 30 ms (Table 2).

### 3.3 | Linewidth calculations

$\Delta v_{1/2}$ of the named downfield metabolites NAA and hCs were the lowest with 29.3 and 17.6 Hz, respectively (Table
Small $\Delta \nu_{1/2}$ were also measured for DF$_{8.24}$ and DF$_{8.49}$, with both being around 29.0 Hz, whereas all other resonances were found to be between approximately 40 and 80 Hz (Table 2). The $T_2^{pp}$ contributions to the linewidths $\Delta \nu_{\text{residual}}$ of hCs is shown in Figure 7. This was calculated using $\Delta \nu_{\text{micro,macro}}$ from tCr(CH$_3$)$_3$ as ~15.4 Hz.
and NAA, but also of DF\textsubscript{8.49} and DF\textsubscript{8.24}, were close to 0. Relatively small $\Delta v_{\text{residual}}$ (10.0-12.0 Hz) were measured for DF\textsubscript{7.48}, DF\textsubscript{6.12}, and DF\textsubscript{5.97}; whereas all other downfield resonances $\Delta v_{\text{residual}}$ ranged from 20 to 60 Hz.

### 3.4 | Concentrations

The estimated tissue concentrations of the downfield metabolites and the proton density of the unassigned downfield peaks in millimolal are given in Figure 8 and Table 2 with and without $T_{2}^{\text{app}}$ correction. For ease of comparison with the previous literature values, the estimated tissue concentration values in millimoles per tissue volume in a liter (mmol/L) are reported in Supporting Information Table S3.

### 4 | DISCUSSION

$T_{2}^{\text{app}}$ relaxation times and concentrations of downfield metabolites and proton density of downfield peaks are reported in the human brain at 9.4 T in this work. Also, $\Delta v_{1/2}$ of these peaks when the spectra across all subjects was averaged was: DF\textsubscript{5.97} = 37.5 ms, DF\textsubscript{6.12} = 23.9 ms.

| TABLE 2 | Results for the downfield peaks, summarizing $T_{2}^{\text{app}}$, $R^2$ fit quality, calculated linewidth $\Delta v_{1/2}$, and millimolal concentrations given both with and without metabolite specific $T_{2}^{\text{app}}$ correction. The exchange rates were taken from Fichtner et al., and the $T_{2}^{\text{corr}}$ values are reported only for the resonances, where exchange rates were measured. The concentrations were measured for only 5 out of the 11 volunteers, for whom the anatomical images for tissue segmentation were also acquired. Concentrations in (mmol/L) are reported in Supporting Information Table S2. All concentrations are corrected for tissue fractions, water relaxation times ($T_1$ and $T_2$), and downfield $T_1$ relaxation times (see Supporting Information Annex C). The concentrations for the unassigned peaks are reported as proton densities because the number of contributing protons is not known. All values are presented as mean ± SD.

| Peak | $T_{2}^{\text{app}}$ (ms) | $R^2$ | $T_{2}^{\text{corr}}$ (ms) | $k$ (s\textsuperscript{-1}) [Fichtner et al.\textsuperscript{2}] | $\Delta v_{1/2}$ (Hz) | Conc. ± SD with $T_{2}^{\text{app}}$ correction (mmol/kg) | Conc. ± SD w/o $T_{2}^{\text{app}}$ correction (mmol/kg) |
|------|-----------------|-----|------------------|-----------------------------|-----------------|-----------------------------------------------|-----------------------------------------------|
| DF\textsubscript{5.75} | 17.9 ± 6.7 | 0.85 ± 0.13 | 20.4 ± 6.7 | 6.76 ± 1.59 | 52.5 ± 4.0 | 9.83 ± 5.60 | 3.06 ± 1.74 |
| DF\textsubscript{5.97} | 19.3 ± 3.3\textsuperscript{a} | 0.83 ± 0.10 | -- | -- | 40.4 ± 0.5 | 1.67 ± 0.87 | 1.07 ± 0.56 |
| DF\textsubscript{6.12} | 20.4 ± 9.9\textsuperscript{a} | 0.90 ± 0.11 | -- | -- | 40.5 ± 0.5 | 2.36 ± 0.89 | 0.98 ± 0.37 |
| DF\textsubscript{6.83} | 21.5 ± 2.6 | 0.94 ± 0.08 | 22.7 ± 2.6 | 2.34 ± 0.44 | 69.6 ± 2.1 | 11.72 ± 2.22 | 3.90 ± 0.74 |
| DF\textsubscript{7.04} | 30.8 ± 7.1 | 0.85 ± 0.07 | -- | -- | 81.2 ± 0.7 | 6.08 ± 1.86 | 2.80 ± 0.86 |
| DF\textsubscript{7.30} | 31.9 ± 10.2 | 0.90 ± 0.05 | -- | -- | 63.5 ± 2.3 | 6.76 ± 1.30 | 3.01 ± 0.58 |
| DF\textsubscript{7.48} | 23.6 ± 7.6 | 0.84 ± 0.13 | -- | -- | 40.9 ± 0.7 | 1.24 ± 0.48 | 0.44 ± 0.17 |
| DF\textsubscript{8.18} | 18.8 ± 6.4 | 0.97 ± 0.05 | 22.8 ± 6.4 | 9.32 ± 0.91 | 61.4 ± 0.8 | 15.94 ± 5.44 | 3.88 ± 1.32 |
| DF\textsubscript{8.24} | 20.1 ± 5.7 | 0.94 ± 0.04 | 24.8 ± 5.7 | 9.32 ± 0.91 | 29.0 ± 0.3 | 4.91 ± 0.57 | 1.45 ± 0.17 |
| DF\textsubscript{8.37} | 16.3 ± 4.3 | 0.90 ± 0.12 | 21.1 ± 4.3 | 13.8 ± 0.79 | 60.3 ± 1.5 | 8.23 ± 2.87 | 1.83 ± 0.64 |
| DF\textsubscript{8.49} | 23.3 ± 6.2 | 0.81 ± 0.08 | 25.2 ± 6.2 | 3.31 ± 0.06 | 28.6 ± 0.2 | 2.43 ± 0.47 | 0.83 ± 0.16 |
| hCs | 48.9 ± 11.0\textsuperscript{b} | 0.78 ± 0.14 | -- | -- | 17.6 ± 2.0 | 0.58 ± 0.31 | 0.38 ± 0.20 |
| NAA broad | 28.4 ± 5.5 | 0.95 ± 0.07 | 29.0 ± 5.5 | 0.74 ± 0.23 | 70.9 ± 1.1 | 4.16 ± 1.45 | 1.83 ± 0.64 |
| NAA | 31.6 ± 11.0 | 0.96 ± 0.04 | 32.3 ± 11.0 | 0.74 ± 0.23 | 29.5 ± 4.7 | 7.02 ± 0.42 | 2.88 ± 0.17 |
| total NAA | 28.4 ± 2.5 | 0.98 ± 0.02 | 29.0 ± 2.5 | 0.74 ± 0.23 | -- | 17.47 ± 2.25 | 7.45 ± 0.96 |

\textsuperscript{a}Whereas for most metabolites $T_{2}^{\text{app}}$ of maximally two subjects were eliminated due to unreliable fits ($R^2 < 0.50$), measurement imprecisions were encountered for metabolites denoted with \textsuperscript{a}. The measured $T_{2}^{\text{app}}$ of these peaks when the spectra across all subjects was averaged was: DF\textsubscript{5.75} = 37.5 ms, DF\textsubscript{6.12} = 23.9 ms.

\textsuperscript{b}hCs has some degree of uncertainty in terms of combined factor of high SD in both $T_{2}^{\text{app}}$ and $R^2$. The worst 2 $R^2$ fits passing the $R^2 > 0.5$ criteria were: $T_{2}^{\text{app}} = 64.2$ ms and 52.5 ms with $R^2$ of 0.58 and 0.61, respectively.
peaks were quantitatively analyzed by calculating $T_2^{relax}$ contributions and micro- and macro- susceptibility components. Characterizing these attributes aids in the understanding of the degree of overlap between components in the downfield proton spectrum. Furthermore, concentrations of the downfield molecules in the occipital lobe are reported both in mmol/kg and mmol/L.

### 4.1 Spectral quality

A previous study at 9.4 T required a larger voxel size ($2 \times 2 \times 3 \text{ cm}^3$) for 96 averages in order to achieve good SNR because the study used MC-STEAM localization. However, the current study used an MC-semiLASER sequence, which resulted in good SNR from a smaller voxel ($2 \times 2 \times 2 \text{ cm}^3$). As expected, the SNR of the peaks in Figure 1 decreased as the signal decayed exponentially with increasing TEs. At TE $= 60 \text{ ms}$, almost all of the peak signals had completely decayed except NAA and hCs. The shaded region in Figure 1 represents the SD between all the subjects, which is larger closer to the water resonance, probably originating from water residuals. Using metabolite-cycling, which is a non-water saturation technique, made it possible to observe peaks.
such as DF$_{5.75}$, DF$_{6.83}$, DF$_{8.18}$, DF$_{8.24}$, DF$_{8.37}$, and DF$_{8.49}$ with reported exchangeable protons.$^2$

### 4.2 Fitting

For fitting purposes, the decision was taken to fit the full upfield spectrum in addition to the downfield spectrum. The LCModel software uses “for initial referencing and phasing ... major landmarks,” especially singlets in the “Preliminary Analysis.”$^{34}$ (p 140) To get this lineshape-information, only an upfield singlet was used in an initial trial; however, this information proved to be insufficient. One singlet from the upfield range was likely inadequate because all upfield singlets are overlapped to some extent with macromolecular contributions. The fitting results showed distorted lineshapes in some spectra (results not shown). Hence, the entire upfield spectrum was included to provide as much information as possible to improve LCModel quantification.

The residuals from spectral fitting were minimal, with some structured noise and negative phase appearing at later TEs, which could suggest some J-evolution effects. Acquiring more averages for later TEs would have resulted not only in better fitting, and therefore more certain $T_2^{app}$, but also would have helped confirm potential J-evolution effects. However, to have feasible scan durations, a higher number of averages were not acquired for later TEs in this study.

### 4.3 pH estimation

Generally, a neutral pH value is assumed and used among all subjects. In this work, the estimation of pH for each subject proved useful in eliminating structured noise commonly present in fit residues. The estimated pH values were observed to be consistent among the different TEs for all subjects (Supporting Information Table S1). The reported pH values (pH = 7.07 to 7.12) are comparable to the pH = 7.06 of hCs reported by Rothman et al.$^{18}$ measured in epileptic patients under vigabatrin treatment. pH measurements using $^{31}$P-MRS estimate the pH value using the inorganic phosphate peak$^{35}$ and report intracellular pH values of 6.96 to 6.98 and extracellular pH values of 7.35 to 7.45. $^{31}$P-MRS imaging measurements indicate a spatially homogeneous pH of around 7.0 throughout the brain.$^{21}$ Because the CSF and extracellular concentrations of hCs are “several orders of magnitude below the level of detection using in vivo spectroscopy,”$^{18}$ (p 927) the measured pH value of hCs primarily reflects the intracellular pH. The differences observed in the measured pH from $^{31}$P-MRS could originate from different compartments (neurons, glia) or a possibly imprecise pK$_a$ value for either of the Henderson-Hasselbalch equations.

Nevertheless, differences of the fit residual for changes of 0.03 in pH values were observable; thus, pH changes of up to 0.2 as reported for gliomas$^{21}$ could be quantifiable.

### 4.4 T$_2$ relaxation

The measured $T_2^{app}$ follow the $B_0$ dependence in comparison with the previous literature.$^6$ The $T_2^{app}$ could be reliably estimated with the exclusion of at most 2 subjects per peak due to a poor fit ($R^2 < 0.50$), except for DF$_{5.97}$, DF$_{6.12}$, NAD$^+$, and hCs. The DF$_{5.97}$ and DF$_{6.12}$ have low peak intensities, and due to the close proximity of the water peak some residual artifacts disrupt a reliable $T_2^{app}$ estimation. Hence, for both peaks the $T_2^{app}$ are also reported for the across subjects summed spectra in the Table 2 caption. The $T_2^{app}$ of NAD$^+$ estimated from the across subjects summed spectra was 30.3 ms, which is much shorter than the times reported by de Graaf and Behar$^5$ (60 ± 13 ms) in the rat brain at 11.7 T. For the hCs peak, 3 subjects were excluded from the reported $T_2^{app}$ values; however, no correlation was found between these excluded subjects and the estimated pH value or voxel GM content.

Of the measured $T_2^{app}$, the peaks reported previously to have a fast exchange with water$^2$ are also among the fastest $T_2^{app}$ decaying peaks: DF$_{5.75}$, DF$_{8.18}$, and DF$_{8.37}$. These same resonances also decayed the fastest in TE series spectra (starting at TE = 5 ms) acquired in the rat brain at 9.4 T.$^{36}$ Furthermore, Liu et al.$^{37}$ reported a $T_2$ relaxation time of 22.7 ms and 28.5 ms in WM and GM, respectively, at 7 T for the 3.5 ppm CEST peak. This 3.5 ppm CEST peak corresponds to the frequency range between 8.0 to 8.4 ppm in $^1$H-MRS considering that the CEST saturation pulse had a bandwidth of 0.4 ppm. The calculated $T_2$ values by Liu et al. match closely the $T_2^{corr}$ values of this study (21 to 30 ms) if the $B_0$ dependence is considered. $T_2^{app}$ and $T_2^{corr}$ of the unnamed downfield peaks from the current work have the same order of magnitude as the macromolecular peaks: 14 to 36 ms in the upfield spectrum.$^{27}$

The measured $T_2^{app}$ values are specific to the occipital lobe at 9.4 T and the semiLASER sequence used. Although the corrections applied for concentrations in this work are intrinsic to the semiLASER sequence, the contributions of the Carr-Purcell effect$^{38}$ to $T_2^{app}$ should be investigated in future work.

### 4.5 Linewidth calculations

$\Delta v_{1/2}$ is composed of 2 components, namely the static $B_0$ field inhomogeneity, which is composed of micro- and macro- susceptibility effects, and $T_2^{app}$. Although the $B_0$ component is the same across all metabolites in a spectrum, the $T_2^{app}$ contribution is specific to each peak depending on how
quickly or slowly it decays.\(^7\) Figure 6 shows \(\Delta \nu_{1/2}\) as well as the contribution to the linewidth from the \(T_{2,pp}^{pp} \left(\pi T_{2,pp}^{pp}\right)^{-1}\) calculated specific to each peak. \(\Delta \nu_{\text{residual}}\) for hCs and NAA are close to 0, indicating that there are either no or insignificant other components contributing to these peaks. The longer \(T_{2,pp}^{pp}\) indicates additionally that these are pure contributions from those metabolites. Similar assumptions of contributions from identical chemical shifts can also be made for DF\(_{6.12}\) and DF\(_{8.24}\), whereas the relatively small \(\Delta \nu_{\text{residual}}\) of DF\(_{7.48}\), DF\(_{6.12}\), and DF\(_{5.97}\) may indicate J-coupling effects or resonances with minor chemical shift differences. All the remaining peaks, however, have a considerable \(\Delta \nu_{\text{residual}}\) ranging from 20 to 60 Hz. These \(\Delta \nu_{\text{residual}}\) could denote the presence of multiple components, with different chemical shifts or substantial contributions from J-evolving components. These non-zero \(\Delta \nu_{\text{residual}}\) of the unassigned downfield peaks are similar to those in macromolecules as described in Murali-Manohar et al.\(^{27}\) An exchange-induced line-broadening of a few Hz will occur as described in NMR for the protons exchanging with water.\(^{39}\) This line broadening effect, however, will mostly be equivalent to the difference between \(\left(\pi T_{2,pp}^{pp}\right)^{-1}\) and \(\left(\pi T_{2,orr}^{pp}\right)^{-1}\).

### 4.6 Concentrations and peak assignments

The estimated tissue concentrations of the downfield metabolites and the proton densities of the unassigned resonances are reported in Figure 8 and Table 2 with and without \(T_{2,pp}^{pp}\) correction in mmol/kg. Supporting Information Figure S6 and Supporting Information Table S3 give concentration values in mmol/L with and without \(T_{2,pp}^{pp}\) correction for the ease of comparison with the previous literature. Because the \(T_{2,pp}^{pp}\) relaxation times of the downfield peaks are shorter compared to the upfield peaks, the \(T_{2,pp}^{pp}\) relaxation correction factor makes a significant impact on the concentrations as seen in the reported values. Concentrations reported here are within the range from previously reported values at 9.4 T and 7 T studies\(^5,6\) (Supporting Information Table S4). Specifically, the previous study at 9.4 T did not correct for \(T_{2,pp}^{pp}\) because the TE used in the study was only 10 ms. However, the current study used TE = 24 ms, and a \(T_{2,pp}^{pp}\) correction was necessary. On the other hand, the study at 7 T used a water suppression method, which resulted in a bias toward lower quantified concentrations for chemical compounds with exchangeable protons with water.

NAA has a resonance at 7.82 ppm, as reported by Govindaraju et al.\(^{40}\) Recently, de Graaf added N-acetylaspartylglutamate (NAAG) resonances at 7.95 and 8.26 ppm in the third edition of his book.\(^{41}\) Expected concentrations from upfield measurements\(^{27}\) are 12 mmol/kg and 1.4 mmol/kg for NAA and NAAG, respectively. Although the measured downfield NAA concentration (7 mmol/kg) of this study is lower than expected, the total NAA concentration (17.4 mmol/kg) is in line with literature.\(^2\) This high concentration value suggests that some other resonances are contributing to total NAA, possibly amides as seen for the DF\(_{8.18}\) and DF\(_{8.37}\). The coupling constant (6.4 Hz) of NAA reported by Govindaraju et al\(^{40}\) was used in the current study, which fit the observed spectral pattern. Although a concentration of ~10 mmol/kg could be achieved when using the coupling constant (7.9 Hz) from de Graaf,\(^{41}\) this J-splitting was broader than the splitting observed in the spectra (see also Supporting Information Annex D).

hCs concentration has been reported with concentrations from 0.3 to 1.6 mM,\(^{2,6,42}\) with higher concentrations in cortical GM, which is in line with the reported value of ~0.43 mmol/L of this work.

We would like to remark, however, that all reported concentrations from this study should be considered with care because tissue fraction-corrected concentration quantification was possible for only 5 subjects, and thus the sample size was too small to perform statistical tests.

Adenosine triphosphate (ATP) has 3 observable peaks at 6.126, 8.224, and 8.514 ppm. Initially, ATP was simulated as a basis set metabolite in this study; however, the chemical shift from Govindaraju et al\(^{33}\) did not match the peaks present in 9.4 T human brain spectra. Simulating Voigt peaks as DF\(_{6.12}\), DF\(_{8.24}\), and DF\(_{8.49}\), as shown in the final settings, resulted in proton densities of 2.36, 4.95, and 2.41 mmol/kg, respectively. De Graaf et al\(^{43}\) reported 2.8 mmol/L as the reference concentration for ATP in \(^{31}\)P-MRS. The concentrations of DF\(_{8.49}\) and DF\(_{6.12}\) are close to the previously published literature value of ATP, whereas the DF\(_{8.24}\) concentration is close to the sum of ATP and NAAG (peak at 8.260 ppm) concentrations. It should be noted that the baseline is often positive from 7.7 to 8.4 ppm, which could also introduce a deviation from the actual concentrations. Therefore, DF\(_{8.49}\) and DF\(_{6.12}\) can be potentially assigned to ATP and DF\(_{8.24}\) to a combination of ATP and NAAG. The \(\Delta \nu_{\text{residual}}\) equal almost 0 Hz (Figure 7), which also suggests that the DF\(_{8.24}\) and DF\(_{8.49}\) peaks are indeed metabolite singlets. The DF\(_{6.12}\) peak was often contaminated with water sideband artifacts in our spectra, which could be the \(^{1}\)CH resonance of the ATP ribose moiety. ATP measurements in \(^{31}\)P MRS include potentially additional triphosphate resonances. In contrast the chemical shifts of the adenosine and ribose moieties of ATP in \(^{1}\)H-MRS are similar to those of adenosine diphosphate\(^{44}\) (measurements made in pH neutral D\(_2\)O solution at 35°C). The chemical shift for DF\(_{8.49}\) peak, however, does not seem to match; therefore, the discrepancy with results from Govindaraju et al.\(^{33}\) should be investigated.

Peaks between 8.0 and 8.4 ppm have been described by the literature as fast decaying amide resonances.\(^{2,6,36,43}\) The
measured exchange times (−10−30 s⁻¹) and resonance frequency (3.5 ppm in CEST) of these peaks closely match those of the amide proton transfer resonances measured with CEST. The quantification of these broad lines simultaneously with a pH estimation could indeed complement CEST amide proton transfer measurements for tumor tissues.

Watanabe et al. assigned the DF₅.₇₅ peak to urea [CO(NH₂)₂], whereas Fichtner et al. speaks about a tentative assignment because the concentration, not corrected for relaxation times, was too low. The reported proton density of the second kind caused by the fast quadrupolar relaxation of the most abundant nitrogen isotope ¹⁴N,” as described in Stabinska et al. (p 939) and Finer et al. and we therefore would assign the DF₅.₇₅ peak to urea.

5 CONCLUSION

T²pp relaxation times of 15 downfield resonances are reported for the first time in the human brain, particularly in a GM-rich voxel in the occipital lobe at 9.4 T. They range from 30 to 50 ms for labeled metabolite peaks, which are typically longer than 16 to 30 ms for the other unassigned downfield peaks. The quantitative analysis of the contribution of T²pp relaxation times and B₀ components to the linewidth of the downfield peaks let one demarcate which peaks have a significant contribution from a single metabolite or which have significantly overlapped resonances. Furthermore, estimated tissue concentrations of molecules resonating downfield are reported with and without T²pp corrections both in mmol/kg and mmol/L. The feasibility of tissue pH estimation, simultaneously with the quantification of amide resonances at around 8.30 ± 0.15 ppm, has also been demonstrated.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

FIGURE S1 A sample of the pH sweep (steps of 0.04) of the homocarnosine (left) and histidine (right) basis sets. The plots show that the hCsIm-C2 peak at 8.08 ppm is the most sensitive to pH changes and that the imidazole peaks should be treated together accounting jointly for the pH value since the peaks are differently sensitive to pH changes.
FIGURE S2 Sample spectra with the fits and residual are shown for the echo times 32, 40, 52, 60 ms. The fitted peaks include simulated metabolites: hCs with the calculated pH value, N-acetyl aspartate (NAA), nicotinamide adenine dinucleotide (NAD\(^+\)) and simulated Voigt lines for all other peaks. The structured noise seen in the fit residuals could be from the fact that J-coupling effects were not considered for the unlabeled downfield peaks.

FIGURE S3 The sample spectrum with the fit from Figure 3 is shown together with the upfield spectral fit. All spectra were always fit together for both upfield and downfield, such that the fitting software LCModel could estimate the metabolite lineshape correctly. The upfield metabolites were chosen by splitting them into moieties, where larger differences in \(T_2^{app}\) could be expected, as described in Murali-Manohar et al.\(^{27}\) None of the upfield metabolite basis vectors have peaks in the downfield: peaks of creatine, phosphocreatine, glutamine, glutathione were assumed to have decayed. At the same time, the aspartate moiety of NAA was split into the upfield and downfield parts. Similarly, the upfield GABA moiety of hCs was not considered. Abbreviations: Macromolecular spectrum (MM Spec.), N-acetyl aspartate – acetyl moiety [NAA(CH\(_3\)]\(_3\)], N-acetyl aspartate – aspartyl moiety [NAA(CH\(_2\)]\(_2\)], N-acetyl aspartyl glutamate (NAAG), \(\gamma\)-aminobutyric acid (GABA), aspartate (Asp), total creatine – 3.9 singlet [tCr(CH\(_2\))], total creatine – 3.0 singlet [tCr(CH\(_3\))], glutamate (Glu), glutamine (Gln), glutathione (GSH), glycine (Gly), myo-inositol (mI), scylo-inositol (Scy), lactate (Lac) and combined phosphocholine, glycerophosphocholine and phosphoethanolamine molecules (tCho+).

FIGURE S4 Cramer-Rao Lower Bounds (CRLBs) as calculated by LCModel for the individual fitted metabolites at each TE. The values are given as mean ± standard deviation between the subjects. Concentrations of the downfield metabolites reported in both mmol/kg and mmol/L, but also with and without metabolite specific \(T_2^{app}\). All concentrations are corrected for tissue fractions, water relaxation times (T\(_1\) and T\(_2\)) and downfield T\(_1\) relaxation times (See Annex C). The concentrations for the unassigned peaks are reported as proton densities since the number of contributing protons is not known. Horizontal lines inside the boxes indicate median values (50% quartile), whereas the bottom and top box boundaries illustrate 25% and 75% quartiles, respectively. Plus signs (+) show outliers.

FIGURE S5 The \(T_2^{app}\) decay curves are shown for eight of the fitted metabolite peaks. The error bars show the fitted concentrations in arbitrary units for the different subjects at all TEs. The \(T_2^{app}\) were estimated by fitting the following signal equation: \(\text{Signal} \propto \exp(-TE/T_2^{app})\). The dashed lines are showing the decay curve for the estimated mean \(T_2^{app}\) value (See also Table 2). The other eight metabolite peaks are shown in Figure 4.

FIGURE S6 Box plots of peak concentrations in mmol/L of the downfield metabolites. Concentrations are given with and without correcting for metabolite \(T_2^{app}\). All concentrations are corrected for tissue fractions, water relaxation times (T\(_1\) and T\(_2\)) and downfield T\(_1\) relaxation times (See Annex C). The pH values are summarized for each subject and each TE. The chosen values for the follow-up fitting step were the values in the TE 40 ms column. The mean values across all the TEs for each subject are closely matching the chosen pH value.

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