Contribution of macromolecules to brain $^1$H MR spectra: Experts' consensus recommendations


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Abbreviations used: ADC, apparent diffusion coefficient; AMARES, Advanced Method for Accurate, Robust and Efficient spectral fitting; BPP, Bloembergen-Purcell-Pound; CEST, Chemical Exchange Saturation Transfer; COSY, Correlated Spectroscopy; DIR, double inversion recovery; DW, diffusion-weighting; FID, free induction decay; FWHM, full width at half maximum; GM, gray matter; HLSVD, Hankel-Lanczos singular value decomposition; HRMAS, high-resolution magic angle spinning; IR, inversion recovery; J-RES, J-resolved spectroscopy; LASER, localization by adiabatic selective refocusing; MEGA-PRESS, Mescher-Garwood point-resolved spectroscopy; ML, mobile lipids; MM, mobile macromolecules; MQ, multiple quantum; MR, magnetic resonance; MRI, magnetic resonance imaging; MRS, magnetic resonance spectroscopy; MRSI, magnetic resonance spectroscopic imaging; MS, multiple sclerosis; NOE, Nuclear Overhauser Effect; PRESS, point-resolved spectroscopy; QUEST, quantitation based on semi-parametric quantum estimation; RF, radiofrequency; SNR, signal-to-noise ratio; SPECIAL, spin-echo full intensity-acquired localized spectroscopy; STEAM, Stimulated Echo Acquisition Mode; SVS, single voxel spectroscopy; TE, echo time; TI, inversion time; TM, mixing time; TR, repetition time; VOI, volume of interest; WEX, Water Exchange; WM, white matter.
Proton MR spectra of the brain, especially those measured at short and intermediate echo times, contain signals from mobile macromolecules (MM). A description of the main MM is provided in this consensus paper. These broad peaks of MM underlie the narrower peaks of metabolites and often complicate their quantification but they also may have potential importance as biomarkers in specific diseases. Thus, separation of broad MM signals from low molecular weight metabolites enables accurate determination of metabolite concentrations and is of primary interest in many studies. Other studies attempt to understand the origin of the MM spectrum, to decompose it into individual spectral regions or peaks and to use the components of the MM spectrum as markers of various physiological or pathological conditions in biomedical research or clinical practice. The aim of this consensus paper is to provide an overview and some recommendations on how to handle the MM signals in different types of studies together with a list of open issues in the field, which are all summarized at the end of the paper.

**KEYWORDS**
brain macromolecules, fitting, metabolite quantification, mobile lipids, parameterization, proton magnetic resonance spectroscopy, quantification, spectral analysis

### 1 | ORIGIN OF MACROMOLECULE SIGNALS IN PROTON SPECTRA

Broad signals underlying the narrower signals of low molecular weight metabolites are observable in $^1$H MR spectra of the human and animal brain (likely present in other tissues as well) especially at short echo times (TEs) and remain detectable at intermediate TEs as well (section 3.2). These signals arise from mobile macromolecules (MM), which display shorter $T_1$ and $T_2$ relaxation times and a lower apparent diffusion coefficient (ADC) compared with metabolites. In the normal brain, MM signals arise mainly from the protons of amino acids within cytosolic proteins, primarily in regions undergoing rapid motions on the time scale of NMR. The “mobile” of MM highlights this fact, although the acronym is interchangeable with “macromolecules”, as used by many authors. With the onset of disease (e.g., tumors, multiple sclerosis [MS] and stroke) signals from mobile lipids (ML) appear in addition, overlapping with peaks of mobile proteins/peptides, making their separation difficult and thus their sum is mainly reported. Hence, the proteins and lipids detected in vivo with MRS reflect a smaller fraction of the total proteins and lipids of tissue, much of which is bound within membranes, producing extreme line broadening with loss of NMR “visibility”.

MM signals upfield of tissue water ($\sim$0.5 to 4.5 ppm) correspond to aliphatic (methyl, methylene and methine) protons, whereas peaks downfield of water ($\sim$5.5 to 9.0 ppm) reflect aromatic CH and exchangeable NH protons (amide, amine and imine). Direct transfer of magnetization between water protons and exchangeable amide or amine protons (tentative assignment based on similarity in chemical shift and exchange rate seen in protein NMR spectra) has been reported using Water EXchange (WEX) spectroscopy, as well as Chemical Exchange Saturation Transfer (CEST) imaging. The pattern of aliphatic resonance intensities in WEX spectra resembles brain MM spectra measured in vitro and in vivo, but these resonances have not been assigned specifically to MM, nor can exchangeable free amino acids and metabolites identified in the downfield region (e.g., N-acetylaspartate [NAA], glutathione [GSH], ATP, NAD(H)) be excluded. Indirect transfer of label through intramolecular relayed Nuclear Overhauser Effect (NOE) to upfield aliphatic and downfield (possibly aromatic) protons has been reported with these techniques, particularly in CEST imaging, and may contribute to the appearance of MM spectra.

Post-translational modification of chemical groups in proteins (e.g., methylation, acetylation, glycosylation, sialylation) may contribute to the signals in MM spectra. For example, sharp singlets in the acetyl region ($\sim$2.05-2.1 ppm) with relatively longer $T_2$ (similar to the NAA acetyl signal) are seen in some fractions of dialyzed brain cytosol. Many brain proteins are acetylated, including histone and non-histone nuclear proteins, cytoplasmic, mitochondrial proteins and myelin proteins, the primary target of acetylation being lysine (N\textsubscript{ε}-acetyl lysine), often with multiple acetyl lysines on a given protein. N-acetylated hexoses of glycoproteins (e.g., N-acetylgalactosamine, $\alpha$-galactose or $\alpha$-neuraminic acid containing oligosaccharides) may contribute signal at 2.05 ppm to brain MM, particularly in necrotic tissue and in cystic tumors, while methyl protons of fucosylated glycoproteins can contribute at 1.3 ppm. As glycoproteins are present mainly on the cell surface, these signals originate extracellularly. To the current understanding, MM, such as glycogen or polynucleotides (DNA/RNA), do not contribute to brain MM when isolated and measured in vitro, although potential contributions to the MM spectrum in vivo may exist.
MM in dialyzed brain cytosol display the same number and pattern of proton signals (relative intensities and chemical shifts) as seen for brain in vivo when metabolites are suppressed via \( T_1 \)- or diffusion-weighted sequences.\(^3\) The same spectral pattern is seen for certain perchloric acid-soluble polyamines (<40 kD), such as thymosin-\( \beta_4 \) and histone-H1 isolated from guinea pig cerebral cortex,\(^6\) as well as microtubule-associated proteins (55-240 kD) isolated from bovine brain.\(^14\) The broad signals from MM in perchloric acid extracts or dialyzed cytosol disappear upon treatment with strong acid and heat (boiling with 6 M HCl for 24 hours), or with proteolytic enzymes, with the appearance of various free amino acids. By contrast, normal brain tissue extracted into chloroform/methanol solutions, which solubilizes all brain lipids (including membrane phospholipids), produces peaks not seen in MM spectra of normal brain. Most significantly, cross-peaks characteristic of fatty acyl chains of lipids are not seen in 2D-CORrelated Spectroscopy (COSY) spectra of dialyzed cytosol or whole homogenate of nondiseased brain, ruling out significant lipid contributions to their spectra.

It is well known from the protein solution state NMR literature\(^18\) that sharp signals arise in proton NMR spectra. These signals are generally thought to reflect more mobile regions of polypeptide chains of rapidly tumbling proteins. In contrast, membrane bound proteins investigated by conventional solution NMR yield broad and mostly featureless spectra.\(^20\) Thus the assignment in vivo of detectable MM to amino acids in freely tumbling cytosolic proteins is consistent with the extensive multiplicity and connectivity in 2D \( J \)-RES and COSY spectra of brain MM (discussed below). The closely similar spectral intensity patterns for MM over a large molecular weight range (3.5 to >100 kD) suggest that MM signals are largely nonspecific with regard to any particular protein and further support the notion that cytosolic proteins in general contribute to MM spectra. This would explain the highly similar spectral patterns for brain MM during development, across brain regions and species.\(^21\) MM signals of dialyzed nerve terminal lysates and myelin-enriched fractions from rat brain are qualitatively similar both to MM of dialyzed brain cytosol and to spectra recorded in vivo (unpublished data of K. L. Behar\(^22\)), suggesting that MM signals may arise from cytosolic proteins/peptides in different cellular compartments, but the distribution is unknown. In principle, altered MM signal intensity, as might be observed with aging or disease, could reflect changes in total protein level or mobility.

### 1.1 Spectral characteristics of MM

Broad peaks in MM spectra are composite signals, composed of multiple overlapping and closely spaced multiplets (due to scalar couplings) that originate from different amino acids.\(^4\) Spectral patterns of the same amino acids also differ slightly with respect to their chemical shifts across different proteins.\(^23\) Thus, MM spectra in vivo most likely represent distributions of overlapping multiplets from different amino acids within different proteins,\(^25\) contributing to the apparent broad linewidths of the various peaks (Appendix 1, Table S1).

Chemical shifts, multiplicities and coupling constants of MM signals are consistent with functional groups (methyl, methylene and methine) of various amino acids in polypeptides. Coupling constants of MM signals reflect geminal (two-bond, \( J_2 \)) and vicinal (three-bond, \( J_3 \)) scalar couplings. MM signals undergo \( J \)-modulation and their appearance changes with TE. The most prominent spin–spin couplings in brain MM are between peaks at 1.70 and 3.0 ppm (\( M_{1.70} \leftrightarrow M_{3.00} \)), assigned tentatively to lysine\(^18\), between the peaks at 0.94 and 2.07 ppm (\( M_{0.94} \leftrightarrow M_{2.07} \)), tentatively assigned to branched-chain amino acids, eg, valine\(^39\) and isoleucine\(^39\) and \( \sim 1.3 \) to \( \sim 4.35 \) ppm\(^7\) (for more details on the nomenclature of the MM components, see Table 1).

MM spectra of healthy brain have shown some variations in peak intensities, for instance between gray matter (GM) and white matter (WM), mainly in humans (section 6).\(^34,37\) With the known (\( M_{1.22} + M_{1.43} \))/\( M_{0.94} \) ratio in a healthy region of brain where ML signals are absent, an increase of this ratio in the diseased brain can be assigned to the contribution of ML, without specific knowledge of the composition of each. Other uses of MM signal ratio combinations, as prior knowledge for estimation of individual MM peak intensities, must be employed with caution (sections 4 and 6). Intensities of several MM signals are highly correlated, as expected due to existing \( J \)-couplings and the underlying spectral pattern consisting of multiple resonances from the contributing amino acids. Signals from different amino acids may also be correlated when originating from the same protein/peptide; for example: \( M_{0.94} \) and \( M_{3.00} \) share no \( J \)-couplings and are ascribed to different amino acids, yet both resonances can occur in the same protein (eg, thymosin-\( \beta_4 \)). As composite signals reflecting a mix of proteins/peptides of unknown composition and density, the interpretation of variations in MM spectral components is best considered in their totality. The parametrization into individual MM components and their influence on metabolite concentrations has also been evaluated, but further studies are required for the identification of the possible soft constraints and systematic errors (sections 4 and 6).

### 1.2 Estimation of MM content

The proton concentration in vivo for the presumed methyl MM signals at 1.22 and 1.43 ppm in rat cortex was estimated by Kauppinen et al\(^7\) (using surface radiofrequency [RF] coil localization and spectral editing) with estimates of \( \sim 2 \) and \( \sim 4 \) mM for \( M_{1.22} \) and \( M_{1.43} \), respectively. In line with these results, a concentration estimate for \( M_{3.00} \) which shows the least overlap with other resonances, is \( \sim 1.7-13 \) mM\(^4,27,34\) as proton density, or 0.8-6.5 mM as lysine residues, assuming this peak to represent lysine\(^1 \) [\( \text{CH}_2 \)] only (relaxation effects were taken into account in the calculations). Since lysine constitutes \( \sim 6\% \) of total brain protein by weight,\(^36-40\) and protein is \( \sim 10\% \) of tissue weight,\(^41\) total lysine in protein is
<table>
<thead>
<tr>
<th>Recommended nomenclature</th>
<th>Previous nomenclature</th>
<th>ppm</th>
<th>Cross-peaks in 2D spectra</th>
<th>$J, \text{Hz}$</th>
<th>Tentative assignment</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0.94</td>
<td>M1</td>
<td>0.94</td>
<td>0.94, 2.05</td>
<td>7.7(d)</td>
<td>Leucine, isoleucine, valine</td>
<td>-reported in vivo at all magnetic fields</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.88</td>
<td></td>
<td></td>
<td></td>
<td>-two main peaks$^{4,27,31,33}$</td>
</tr>
<tr>
<td>M1.22</td>
<td>M2</td>
<td>1.22</td>
<td>1.24, 4.24</td>
<td>6.6(d)</td>
<td>Threonine</td>
<td>-reported in vivo starting at 3 T$^{34-36}$</td>
</tr>
<tr>
<td>M1.43</td>
<td>M3</td>
<td>1.43</td>
<td>1.43, 1.70, 1.43, 4.32</td>
<td>7.7(d)</td>
<td>Alanine</td>
<td>-reported in vivo at all magnetic fields</td>
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<tr>
<td>M1.70</td>
<td>M4</td>
<td>1.70</td>
<td>1.70, 1.43, 1.70, 3.00</td>
<td>m 7.8(t)</td>
<td>Lysine, arginine, leucine</td>
<td>-reported in vivo starting at 3 T$^{34-36}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-scalar coupling between M1.70-M3.00 is important for the detection of GABA</td>
</tr>
<tr>
<td>M1.81</td>
<td>-</td>
<td>1.81</td>
<td>-</td>
<td>-</td>
<td>To be confirmed in future studies</td>
<td>-reported in vivo in rat brain at 17.2 T$^{27}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>This peak was identified based on a parameterization of the in vivo acquired MM signal using 32 individual Gaussian functions, therefore its biological relevance needs to be confirmed</td>
</tr>
<tr>
<td>M1.9</td>
<td>-</td>
<td>1.9</td>
<td>1.89, 2.03</td>
<td>-</td>
<td>To be confirmed in future studies</td>
<td>-reported in vivo in rat brain at 9.4 T$^{31}$ and 17.2 T$^{27}$ Several peaks were identified based on a parameterization of the in vivo acquired MM signal using 15 or 32 individual Gaussian functions, therefore their biological relevance needs to be confirmed</td>
</tr>
<tr>
<td>M2.05</td>
<td>M5</td>
<td>2.05</td>
<td>2.05, 0.94, 2.03, 1.89, 2.07, 3.22</td>
<td>m</td>
<td>Glutamate, glutamine,</td>
<td>-reported in vivo at all magnetic fields</td>
</tr>
<tr>
<td></td>
<td>(M5a$^{31}$)</td>
<td>1.99</td>
<td></td>
<td></td>
<td></td>
<td>-peak reported in refs$^{4,8}$</td>
</tr>
<tr>
<td></td>
<td>(M5b$^{31}$)</td>
<td>2.04</td>
<td></td>
<td></td>
<td></td>
<td>-peak considered to belong to the MM group M5 in refs$^{4,8}$</td>
</tr>
<tr>
<td>M2.07</td>
<td>-</td>
<td>2.07</td>
<td>None</td>
<td>s</td>
<td>To be confirmed in future studies</td>
<td>-peak reported in refs$^{5,6,27,31,33}$ and considered to belong to the MM group M5 -reported in vivo starting at 9.4 T in rat brain</td>
</tr>
<tr>
<td>M2.17</td>
<td>(M5c$^{31}$)</td>
<td>2.17</td>
<td>2.16, 2.54, 2.17, 3.80</td>
<td>-</td>
<td>To be confirmed in future studies</td>
<td>-peak reported in vivo at 9.4 T$^{31}$ 16.4 T$^{31}$ and 17.2 T$^{27}$ Identified based on a parameterization of the in vivo acquired MM signal using 15, 17 or 32 individual Gaussian functions,</td>
</tr>
<tr>
<td>M2.27</td>
<td>M6</td>
<td>2.27</td>
<td>-</td>
<td>8.2(m)</td>
<td>Glutamate, glutamine</td>
<td>-reported in vivo starting at 3 T$^{34-36}$</td>
</tr>
<tr>
<td></td>
<td>(M6a$^{31}$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-reported in vivo in rat brain at 9.4 T$^{31}$ 16.4 T$^{31}$ and 17.2 T$^{27}$ Identified based on a parameterization of the in vivo acquired MM signal using 15, 17 or 32 individual Gaussian functions,</td>
</tr>
<tr>
<td>M2.37</td>
<td>(M6b$^{31}$)</td>
<td>2.37</td>
<td>-</td>
<td>-</td>
<td>To be confirmed in future studies</td>
<td>-reported in vivo in rat brain at 9.4 T$^{31}$ 16.4 T$^{31}$ and 17.2 T$^{27}$ Identified based on a parameterization of the in vivo acquired MM signal using 15, 17 or 32 individual Gaussian functions,</td>
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</tr>
<tr>
<td>M250</td>
<td>(M6c[^3])</td>
<td>2.47</td>
<td>-</td>
<td>-</td>
<td>To be confirmed in future studies</td>
<td>reported in vivo in rat brain at 9.4 T[^31] and 17.2 T[^27] Identified based on a parameterization of the in vivo acquired MM signal using 15 or 32 individual Gaussian functions, therefore their biological relevance needs to be confirmed.</td>
</tr>
<tr>
<td>M254</td>
<td>(M6d[^3])</td>
<td>2.54</td>
<td>2.54, 2.16 2.55, 2.74</td>
<td>~10.18(dd)</td>
<td>β-methylene protons of aspartyl groups</td>
<td>reported in vivo starting at 3 T[^35] and assigned to β-methylene protons of aspartyl groups at 9.4 T[^29]</td>
</tr>
<tr>
<td>M274</td>
<td>(M6e[^3])  (M8[^29])</td>
<td>2.68</td>
<td>2.74, 2.55</td>
<td>~4.18(dd)</td>
<td>β-methylene protons of aspartyl groups</td>
<td>reported in vivo starting at 9.4 T and assigned to β-methylene protons of aspartyl groups in ref[^29]</td>
</tr>
<tr>
<td>M297</td>
<td>(M7a[^3])</td>
<td>2.97</td>
<td>-</td>
<td>-</td>
<td>To be confirmed in future studies</td>
<td>reported in vivo in rat brain at 9.4 T[^31] and 17.2 T[^27] Identified based on a parameterization of the in vivo acquired MM signal using 15 or 32 individual Gaussian functions, therefore their biological relevance needs to be confirmed.</td>
</tr>
<tr>
<td>M300</td>
<td>M7 (M9[^29]) (M7a[^31])</td>
<td>3.00</td>
<td>3.00, 1.70 3.00, 3.31</td>
<td>7.6(t)</td>
<td>Lysine, (Cys)₂</td>
<td>reported in vivo at all magnetic fields</td>
</tr>
<tr>
<td>M321</td>
<td>(M8[^30]) (M8a[^31])</td>
<td>3.21</td>
<td>3.22, 2.07</td>
<td>-</td>
<td>Valine-H₄[^4] αCH protons of protein amino acids[^29]</td>
<td>reported in vivo starting at 3 T[^34-36] phosphatidyl choline methyl (singlet) may contribute</td>
</tr>
<tr>
<td>M343-395</td>
<td>(M9[^31])</td>
<td>3.43-3.95 3.54-3.95</td>
<td>-</td>
<td>To be confirmed in future studies</td>
<td>reported in vivo in rat brain at 9.4 T[^31] and 17.2 T[^27] Identified based on a parameterization of the in vivo MM signal using 15 or 32 individual Gaussian functions, therefore their biological relevance needs to be confirmed.</td>
<td></td>
</tr>
<tr>
<td>Recommended nomenclature</td>
<td>Previous nomenclature(^{6,8})</td>
<td>ppm(^a)</td>
<td>Cross-peaks in 2D spectra(^b)</td>
<td>(J), Hz(^c)</td>
<td>Tentative assignment</td>
<td>Observations</td>
</tr>
<tr>
<td>--------------------------</td>
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</tr>
<tr>
<td>M(_3.71)</td>
<td>(M11(^{2,9}))</td>
<td>3.71</td>
<td>-</td>
<td>-</td>
<td>(\alpha)CH protons of protein amino acids</td>
<td>-reported in vivo starting at 3 T(^{34-36}) with improved spectral resolution at 7 T</td>
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<td>M(_3.79)</td>
<td>(M9(^{20}))</td>
<td>3.77</td>
<td>3.80, 2.17</td>
<td>-</td>
<td>(\alpha)CH protons of protein amino acids</td>
<td>-reported in vivo starting at 3 T(^{34-36}) with improved spectral resolution at 7 T</td>
</tr>
<tr>
<td>M(_3.87)</td>
<td>(M12(^{20}))</td>
<td>3.79</td>
<td>3.80, 4.91</td>
<td>-</td>
<td>(\alpha)CH protons of protein amino acids</td>
<td>-reported in vivo starting at 7-9.4 T</td>
</tr>
<tr>
<td>M(_3.97)</td>
<td>(M13(^{20}))</td>
<td>3.87</td>
<td>-</td>
<td>-</td>
<td>(\alpha)CH protons of protein amino acids</td>
<td>-reported in vivo starting at 3 T(^{34-36}) with improved spectral resolution at 7 T</td>
</tr>
<tr>
<td>M(_4.05-4.43)</td>
<td>(M10(^{21}))</td>
<td>4.05-4.42</td>
<td>4.24, 1.24</td>
<td>4.24, 1.24</td>
<td>Threonine-(\beta)(^4,8) Threonine-(\gamma)(^4,8)</td>
<td>-reported in vivo starting at 7-9.4 T</td>
</tr>
<tr>
<td>M(_4.20)</td>
<td>(M15(^{20}))</td>
<td>4.20</td>
<td>-</td>
<td>-</td>
<td>(\alpha)CH protons of protein amino acids</td>
<td>-at 9.4 T and higher several MM peaks were reported in this range, care has to be taken regarding the water suppression in this range</td>
</tr>
</tbody>
</table>

\(^a\)Small variations in ppm (~0.01-0.04 ppm) can be found in different papers.

\(^b\)Cross-peak chemical shifts may differ by +0.02 ppm between rat\(^6\) and human\(^8\) brain studies.

\(^c\)Coupling constants \((J, \text{Hz})\) and multiplicities (s-singlet, d-doublet, t-triplet, m-multiplet, dd-double doublet) were determined from 2D \(J\)-resolved \(^1\)H NMR spectra and published in references\(^4,8\). Small variations in Hz (~0.04 Hz) can also be noticed in different papers.
~47 μmol/g brain. Thus, the intensity of M_{3.00} reflects 2%-14% of total lysine, suggesting a large fraction of the total protein is not MRS visible in vivo.

An estimate of the M_{0.94} signal assuming methyl groups was assessed using ultra-short TE STEAM data acquired from mouse and human brain. In the human brain (occipital lobe), the MM concentration contributing to M_{0.94} peak was estimated to be ~11.1 μmol CH₃/g wet tissue. The same value was assessed from 4 T (TE = 4 ms, TM = 42 ms) and 7 T (TE = 6 ms, TM = 32 ms) spectra using corrections for T₁ and T₂ relaxation.² A slightly higher concentration of ~15.7 μmol/g was quantified in the mouse hippocampus at 9.4 T (TE = 2 ms, TM = 20 ms). The unsuppressed water resonance was used as an internal reference, assuming 80% brain water content. Of note, the molar concentration of protons (¹H) forming the M_{0.94} signal is three times higher than that of the CH₃ entities. Furthermore, if M_{0.94} arises from an equivalent mix of leucine, iso-leucine and valine, which together comprise ~16% of human GM total protein mass (~70 μmol/g wet wt),³⁹ then M_{0.94} would reflect ~8%-11% ([11-16 μmol/g methyl groups] / 2 methyl groups per μmole amino acid) / 70 μmol/g wet wt) of their respective concentrations in the total protein. Another study²⁸ performed in humans at 1.5 T (TE = 20 ms) estimated the M1 area (M_{0.94}) at ~40 mmol/kg proton density, which would be equivalent to ~7 mM for combined amino acids if considering a factor of 3 for proton stoichiometry and a factor of 2 for the two methyls per residue of branched-chain amino acids.

Thus, different estimates of MM proton densities suggest that a large fraction of the total protein is not MRS visible in vivo. Further investigations are needed to address the issue of MRS visibility and the extent to which other cellular compartments (eg, mitochondria and nucleus) might contribute to the in vivo MM spectrum.

### 1.3 Recommendations on nomenclature

In the present paper we provide some recommendations on a unified nomenclature of the different MM components, which would be easily expandable to new peaks, MM signals being uniformly described by their resonant frequency in ppm (eg, M_{0.94}). More details can be found in Table 1 together with a brief description of each MM signal component.

Furthermore, a clear distinction should be made between MM and ML signals and an underlying baseline.³² The “baseline” consists of smoothly varying components and spurious signals arising through imperfections during data acquisition (for details on “baseline”, see Kreis et al).³²

### 2 B₀ DEPENDENCE OF MM SPECTRUM

#### 2.1 Changes in MM spectral pattern with B₀

The apparent “linewidth” of MM components is dictated by four main factors: T₂ relaxation, B₀ inhomogeneities (ΔB₀), multiplicity of J-coupled signals and the overlap of cytosolic protein signals with slightly different chemical shifts. In general, the spectral linewidth under in vivo conditions is determined by T₂ relaxation and by microscopic (ΔB_{micro}) and residual macroscopic (ΔB_{macro}) inhomogeneities of the B₀ (ie, FWHM = 1/(πT₂) + Δν B₀). In addition, the contribution of J-couplings has to be taken into account for the apparent MM signal linewidths since the multiplicity pattern is not directly observable (FWHM > J). According to relaxation theory (see section 2.2), the T₂ relaxation of MM has a very mild B₀ dependence.⁵ However, the line broadening resulting from microscopic ΔB₀ increases linearly with B₀.⁴ Even though the ΔB_{macro} component can be substantially minimized by successful B₀ shimming,⁴⁵ the ΔB_{micro} component cannot be eliminated as it originates from intrinsic tissue heterogeneity on a cellular level. Therefore, the effect of ΔB_{micro} line broadening should be identical for metabolites and MM. Since MM peaks contain an overlap of multiple J-coupled resonances from different amino acids, and identical contributing amino acids as part of different proteins experience slightly different chemical shifts, an additional increase in the linewidths of MM peaks is expected compared with metabolites. Indeed, T₂ relaxation plus ΔB₀ component alone cannot account for the observed apparent linewidth of MM peaks.²⁵

When assuming high-quality B₀ shimming, the apparent M_{0.94} signal linewidth can be approximated by a simple equation:

\[
\text{FWHM} = 1/(πT₂) + Δν B₀
\]

where the term Δν" corresponds to a line broadening per Tesla (microscopic heterogeneity and chemical shift differences). The contribution of J-coupling was neglected and not included in this simplified formula. The M_{0.94} signal linewidths (in Hz) assessed from human and animal experimental MRS data follow a linear relationship with B₀ from 1.5 to 16.4 T (Figure 1A). The linewidth was calculated assuming T₂ = 32 ms (section 2.2) and Δν" = 4.73 Hz/T. The M_{0.94} signal linewidth in ppm (Figure 1B) is determined primarily by T₂ relaxation at low B₀, while it reduces rapidly with increasing B₀, where it becomes nearly B₀-independent and approaches the value 2π Δν"/γ. As J-couplings are independent and T₂S of MM are nearly independent of the B₀ strength, the multiplet widths (in ppm) decrease with B₀, which consequently improves the apparent resolution of
MM spectra at high B0. In addition, increased B0 transforms complex higher-order spin systems of strongly coupled resonances into first-order multiplets that also may contribute to improve MM spectral resolution at high B0. Such an effect of B0 on strongly coupled MM multiplets can be observed between 3 and 4 T MM spectra in the region 1.0-1.8 ppm (Figure 1C). Only minor improvements in MM spectral resolution can be expected at 7 T or higher B0.

2.2 B0 dependence of MM relaxation

MM signals are typically eliminated or isolated from metabolite signals based on differences in T1 and/or T2 relaxation (Appendix S1, Tables S2 and S3), although differences in molecular diffusion have also been used.2,49

Figure 2 illustrates the B0 dependence of T1 and T2 for singlet metabolite resonances and MM (Matlab code is provided in Appendix S2). The metabolites include NAA (CH₃), tCr (CH₃) and tCho (CH₃) from a range of publications and B0. Most J-coupled metabolites show shorter T2 than singlets,52–56 while T1 for Cr (CH2), GSH and taurine [Tau] (CH2) are noticeably different, falling either below or above this range.2,50,57 The MM range includes T1 and T2 values for the M₀.₉₄ (M₁) to M₁.₇₀ (M₄) signals2,2₇ measured in rat brain. Measuring T1 and apparent T2 (J evolution not considered) of MM other than M₀.₉₄ to M₁.₇₀ is not straightforward due to a strong overlap with metabolites, and requires more sophisticated approaches using either double inversion recovery (IR) with optimized combinations of inversion times (TIs) and additional elimination of metabolite residuals5₈,5₉ or careful elimination of metabolite residuals during postprocessing2,3₀ or combining IR with a diffusion module1,4₉,6₀ (section 3).

Overall, T₁ time constants increase and T₂ time constants decrease with increasing B₀. The slower T₁ relaxation of metabolites is in agreement with the Bloembergen-Purcell-Pound (BPP) dipolar relaxation theory.5₁ The T₂ of MM increase more strongly with B₀, which is also in qualitative agreement with BPP theory for molecules with a longer rotation correlation time.² The apparent T₂ time constants of metabolites are shorter than those anticipated by BPP theory. The disagreement can be explained by a loss of phase coherence due to diffusion through microscopic susceptibility gradients.5₁ The T₂ of MM have a very mild B₀ dependence.² For any value of B₀, the T₂ of most metabolites is longer than
for MM, such that effective suppression of MM can be achieved at longer TEs (see section 3.2). The main problems of long TE scans are (1) the loss of potentially important MM resonances, (2) the loss of many scalar-coupled metabolite signals, (3) substantial decrease in SNR and (4) the introduction of T2-weighting, which requires a T2 correction when attempting quantification.

3 | MEASUREMENT OF MM IN VIVO

MM detection and suppression based on differences in $T_1$ have been reported using single and multiple IR methods (Appendix S1, Table S4). For inversion of magnetization the use of an adiabatic pulse is highly recommended due to broader bandwidth and insensitivity to $B_1$ inhomogeneity.

Figure 3 summarizes the MM signal recovery (A-C) and MM suppression efficiency (D-F) as achieved in metabolite-nulled and MM-nulled MRS. In general, double IR methods (Figure 3B,E) give improved metabolite (Figure 3B) or MM (Figure 3E) suppression over a wider range of $T_1$ times than single IR methods (Figure 3A,D). However, the improved suppression comes at the cost of reduced MM (Figure 3B) or metabolite (Figure 3E) signal recovery and increased $T_1$-weighting. As the difference between MM and metabolite $T_1$ decreases at higher $B_0$, it is harder to suppress one without affecting the recovery of the other. For metabolite-nulled MRS the optimal TIs have only a mild $B_0$ dependence (Figure 3C), whereas for MM-nulled MRS the optimal TIs rapidly increase with $B_0$ (Figure 3F). In all cases, one should be aware of metabolites that are outside the considered $T_1$ range (e.g., tCr methylene, Tau, GSH) and their residual signals have to be removed by postprocessing.
Diffusion-weighting (DW) combined with IR is another method to measure MM in vivo\textsuperscript{1,49,60} since MM are expected to have a 10 to 20 times slower diffusion than metabolites.\textsuperscript{49,62} By combining IR with DW (b value of 10 to 11.8 ms/μm\textsuperscript{2}),\textsuperscript{1,60,63} it was shown in rat brain that a significant attenuation of metabolite residuals can be achieved while the MM signals were almost unaffected.\textsuperscript{1} This eliminates the need for any post-processing.\textsuperscript{1,49,60} However, only a few published studies have used this method until now, mainly in rodents. The main limitation of this technique is the low SNR (due to the combination of DW at high b-values and IR), which might lead to difficulties in scan-to-scan phasing before averaging. Furthermore, reaching sufficiently high DW cannot be achieved in some sequences (in particular with short TE spin echo sequences), thus making that approach not a general strategy.

### 3.1 | Removal of residual metabolites

Theoretically, due to faster T\textsubscript{1} relaxation of MM compared with metabolites, metabolites are nullled at a specific TI with an almost fully recovered MM. In practice, independently of the type of IR method, small residuals of metabolites are still observed in the metabolite-nulled spectrum due to variability in T\textsubscript{1} relaxation of metabolites, as previously mentioned. These residuals strongly depend on the sequence used and its parameters (TE, TR, TI), on the transmit B\textsubscript{1} inhomogeneity and on B\textsubscript{0}. Therefore, some studies identified and removed the residuals of the main metabolites such as tCr, NAA and Tau, while others identified additional residuals from tCho, glutamate and glutamine (Glu/Gln) and myo-inositol (Ins) (Appendix S1, Table S4). Residual metabolite signals should be experimentally verified based on: (1) the T\textsubscript{1} relaxation times of the metabolites; (2) acquisition of a series of IR spectra using a full range of TIs (ie, 100-1200 ms) where the evolution of the metabolite intensities, changes from negative to positive; and (3) acquisition of an IR spectrum with the selected TI but longer TE (~40 ms) to confirm the residual metabolite signals\textsuperscript{46,64,65} (Figure 4A,B). Ideally, MM spectra in vivo should be acquired from reasonably small VOIs using high-quality B\textsubscript{0} shimming to optimize the spectral resolution, SNR, water suppression, and minimize baseline distortions or subcutaneous lipid contamination. The contamination of MM spectra by the residual metabolite signals can be efficiently reduced by shortening of the TR in the single IR method. The use of a short TR leads to partial saturation of magnetization of metabolites with longer T\textsubscript{1}, which reduces the sensitivity of metabolite nulling to T\textsubscript{1} differences.\textsuperscript{46,64,66} Moreover, TR shortening improves the SNR efficiency for a fixed measuring time.

Different approaches/algorithms can be used to eliminate the contribution of metabolite residuals in postprocessing. For example, Hankel-Lanczos singular value decomposition (HLSVD) was one of the first algorithms used, but cannot consider the known prior knowledge on the residual metabolites. More recently, Advanced Method for Accurate, Robust and Efficient Spectral fitting (AMARES)\textsuperscript{67} was used with constraints on the peak frequency, phase, linewidth and amplitude to fit the residual metabolites more robustly\textsuperscript{21} (eg, in AMARES fitting prior knowledge is provided only for the residual metabolite peaks to be removed; Figure 4C). This set of prior knowledge needs to be built by the user. In Figure 4B a spectrum acquired with TI = 750 ms was chosen as the one with the least metabolite residuals at 9.4 T in the rat brain after acquiring a series of IR spectra (Figure 4A). For the identification of metabolite residuals, in addition to the series of IR spectra where the evolution of the metabolite intensities is changing from negative to positive (Figure 4A, dotted lines), an IR spectrum with TE = 40 ms was also acquired (TI = 750 ms, TR = 2500 ms). In order to build the set of prior knowledge, special care has to be taken to analyze the behavior of each peak individually at a given TI and TE (the multiplicity of the peak, phase, estimated amplitude based on previously reported relaxation times and linewidths\textsuperscript{53,68}). The following steps and iterations can be performed for fixing the prior knowledge: (1) a flexible prior knowledge and manual inspection to avoid overfitting is used first to remove individually every metabolite residual from the MM spectrum; (2) the obtained results are then used to construct rigorous prior knowledge of all the metabolite residual peaks combined (still leaving some freedom for the peaks to adjust to different spectra); (3) after removing the peaks the remaining MM can also be fitted to make sure that the final residual is free of any artifacts indicating over- or underestimation of metabolite residuals; (4) if step (3) is validated then the residue from step (2) (the MM spectrum free of residual metabolites; Figure 4C) is saved separately and included in the metabolite basis set. This process requires multiple iterations, but once an adequate set of prior knowledge is built it can be efficiently reused and applied to different spectra with minor adaptations. By using a rigorous prior knowledge and by fixing the phase of each peak, AMARES fits peaks on a nonzero baseline especially when fitting several peaks at the same time. An alternative is to define the MM spectrum by simultaneously fitting a series of IR time spectra where residual metabolite signals are accounted for automatically\textsuperscript{49,70} or by using a residual metabolite basis set; however, to date very few studies have been published using these approaches. As such, AMARES or similar/alternative approaches appear to be favorable for the postprocessing of MM components\textsuperscript{21,30,69,70}.

### 3.2 | TE dependence of MM

The MM pattern and MM contribution to the overall spectrum depend on the TE and the sequence used (Figure 5). At short TEs, MM signals contribute significantly throughout the whole ppm range. At longer TEs, the MM contribution relative to metabolites decreases due to shorter T\textsubscript{2}s. For most nonediting sequences, \textit{TE} \geq 150 ms at 3 T and 4 T\textsuperscript{54,55} and \textit{TE} \geq 100 ms at 7 T\textsuperscript{56} in human brain and 9.4 T in rat brain are generally...
FIGURE 4  A, a series of IR spectra from rat brain in vivo with TI ranging from 420 to 1000 ms revealing the evolution of metabolite intensities as a function of TI (all the spectra were acquired with TE/TR = 2.8/2500 ms at 9.4 T using the SPECIAL sequence in a voxel of 3 x 3 x 3mm³ centered in the hippocampus); B, spectra acquired with a selected TI (750 ms) and TE of 2.8 ms (taken from A) as well as with TE of 40 ms (5x magnified, TE = 40 ms spectrum from 2.2-3.8 ppm is shown on the top); C, original spectra acquired at TI of 750 ms and TE of 2.8 ms (shown in black), estimated fits of the residual metabolites using AMARES (shown in red), and the residue obtained after subtraction of the estimated metabolite signals from the original spectrum (shown in blue). All spectra were acquired in vivo in the rat brain at 9.4 T.

sufficient to permit neglecting the MM contribution during quantification (Figure 5). Therefore, the assumption that MM contribution at TEs of ~40-80 ms is negligible, justifying the noninclusion of MM in the basis set during quantification might not be correct for high SNR spectra. Systematic studies on MM contributions at intermediate and long TEs in humans and animals are missing. Because the MM spectral pattern changes with TE due to the $J$-couplings between different MM resonances4,54 (section 1), MM spectra should be acquired for each specific TE and sequence.

4  |  MATHEMATICAL MODELING OF MM FOR METABOLITE AND MM QUANTIFICATION

4.1  |  Quantification/parameterization of MM

Quantitative comparison of MM content or MM composition between cohorts of subjects or different brain locations is facilitated by modeling the experimental MM in terms of interpretable MR signals. Signal integration of the raw measured MM spectrum or after postprocessing71 is also a possibility, though it is less flexible and accurate. Thus, most often the MM spectrum is parameterized into a number of Lorentzian, Gaussian or Voigt lines representing easily interpretable MR signal entities. However, parameterization of the MM into regular MR signal components is non-trivial since the number and nature of contributing chemical entities is a priori unknown.72 The best chemical information for parameterization still dates back to the pioneering work of Behar et al.,4 where ex vivo NMR showed signals at 14 frequencies, with the seven main peak groups labeled...
as M1 (M_{0.94}) through M7 (M_{3.00}) (see above). However, this original signal model was often not used directly for parameterization, mostly because the appearance of the MM is T_{1}-dependent, not all peaks are easy to identify and the nomenclature was arbitrary. Most researchers have thus devised heuristic models based on the visual appearance of their own MM spectra or closely matching previous data, although very often the original labeling of peak groups as M1 (M_{0.94}) through M7 (M_{3.00}) and later up to M10 (M_{4.20}) has been maintained in many reports. The actual models used between four and 32 Gaussian or Lorentzian lines\textsuperscript{27,29–35,73} (Appendix S1, Table S4). It is likely that future improvements in SNR and spectral resolution will warrant more complex mathematical models to model the MM profile accurately (Table 1).

Alternative parameterizations without a predefined choice of a number of interpretable component peaks have also been suggested. To that end, MM spectra have been either described point-by-point in the frequency domain (from saturation recovery data)\textsuperscript{28} or as a sum of overlapping densely and equally spaced Voigt lines,\textsuperscript{27,69,70} which can be grouped into interpretable features with common characteristics in hindsight. Both approaches have the advantage of being model-free, but are a mathematical representation rather than a physical or physiological model. Thus they are well suited to represent single MM spectra or a set of interrelated MM spectra recorded at specific acquisition parameters, but do not yield models that are generalizable. None of the above approaches can fully represent J-coupling modulations with T_{E} in the case of editing experiments or 2D J-resolved spectra.

As previously mentioned, the MM pattern is also influenced by the sequence used and its parameters (ie, T_{E}, T_{R}). Hence MM have also been parameterized in terms of relaxation times. An effective T_{2} (T_{2}^{\text{eff}}) that includes both relaxation as well as J-evolution effects has mostly been determined from metabolite-nulled scans with different TEs (Appendix S1, Table S3), and T_{1} has been derived from scans with multiple inversion or saturation recovery periods (Appendix S1, Table S2). One approach tried to include the entire set of TE and IR series into one spectral fit model to simultaneously quantify metabolites and macromolecules.\textsuperscript{69,70,76} Another recent approach used measured T_{1} and T_{2} times of all MM resonances to derive a MM model that can be adapted to any sequence and scan parameters from experimentally acquired MM spectra obtained by one specific sequence.\textsuperscript{77} Both approaches are still under development.

4.2 | Consideration of MM signal during quantification of metabolites

For a reliable quantification of metabolites from brain \textsuperscript{1}H MR spectra containing MM contributions, the MM spectrum has to be subtracted before spectral fitting\textsuperscript{78,79} or the MM spectrum or its components have to be included in the basis set used for linear combination model fitting\textsuperscript{30,35,36,47,65,73–80}. The second option is more common.

A widely used approach in estimating MM models is to suppress the metabolite signals using an IR sequence to determine a single spectrum containing only MM signals at fixed relative amplitudes (section 3). This MM model spectrum is subsequently included in the basis set used by the fitting algorithm, incorporating prior knowledge of the MM signals and therefore improving fitting stability. While the IR sequence reduces the SNR, the reduced overlap between metabolites and MM signals improves model accuracy over the use of a purely mathematically estimated MM spectrum.\textsuperscript{35,36,47,65,81,83} To create a single MM basis spectrum that includes all MM resonances, it may be sufficient to average the metabolite-nulled MM spectra acquired in vivo from several healthy subjects assuming the residual metabolite signals have been removed. A further approach involves averaging the parameterized MM signals after fitting the MM spectrum acquired in vivo (see below).
Alternatively, a parameterization into independent MM signals provides a higher level of analysis flexibility and yields noise-free MM models when compared with the direct use of experimentally acquired MM data in the fitting process. To that end, the experimental MM spectra can be modeled as either a sum of splines or a combination of broad symmetric resonances with Lorentzians, Gaussian or Voigt lines, each with characteristic frequency and lineshape parameters. Certain groups of subjects can be assumed to have identical MM profiles and therefore each individually modeled MM signal can then be combined at fixed proportions, which reduces the degrees of freedom in the fit and makes it more robust. Conversely, it may be known that a specific MM moiety is a disease biomarker. Then, a MM fitting model with the freedom to quantify this specific signal separately is useful. Alternatively, individually modeled MM signals can be included in the metabolite basis set and quantified together with metabolites. These components can also be combined into one or more signals and used with metabolite spectra for analysis. However, the increased number of fitted parameters without constraints may lead to overfitting. In such a case, the fitted amplitudes of MM signals may lose their biochemical meaning. Additional studies are required to evaluate the best prior knowledge or soft constraints to be used in the fitting process to avoid over-parameterization.

An alternative approach for estimating the MM signals exploits the short T2 relaxation of MM from the first time domain points of the MRS signal. The Subtract-QUEST algorithm was used to compare this approach with experimentally obtained spectra of MM in the quantification of in vivo 1H MRS data. Significant differences in the calculated concentrations were obtained when using the short T2 relaxation estimation of the MM.

In conclusion, accurate quantification of metabolites in short and intermediate TE 1H MR spectra require an equally accurate assessment of MM as demonstrated over a range of field strengths. While MM measured at a low B0 of 1.5-3 T appear smoother, the complexity of MM spectra increases at higher fields requiring additional scrutiny in their modeling. Differences in metabolic concentrations can be seen when comparing the mathematically generated MM models using different algorithms with the MM measured in vivo. The smooth approximation of spline or another type of mathematical fitting for MM does not completely reproduce the in vivo spectral pattern at higher B0. Therefore, experimentally measured MM are recommended for all B0.

A detailed description of different MRS quantification algorithms to handle MM quantification is provided in Appendix S3 and in a recent book chapter.

5 | MM-COEEDITING IN SPECTRAL EDITING (GABA+)

MRS of GABA in vivo, the major inhibitory neurotransmitter, is technically challenging because of the presence of overlapping resonances from metabolites, such as creatine, glutamate/glutamine, GSH, homocarnosine and NAA. For this reason, spectral editing is employed for GABA detection, including J-difference editing and the doubly selective multiple quantum filtering method. These methods exploit the J-coupling of GABA β and γ methylene protons resonating at 1.9 and 3.0 ppm. However, because of the finite bandwidth of the frequency-selective editing pulse set at 1.9 ppm, the MM resonance at 1.7 ppm, which is coupled to the MM resonance at 3.0 ppm, is also partially inverted (Figure 6), contributing to the net signal measured in the difference spectrum at 3.0 ppm. GABA+MM (or GABA+) values are widely reported in the literature.

In doubly selective, multiple quantum filtering methods, MM signals at 3.0 ppm are also partially coedited by the double-band selective pulse, but the MM signal contribution is smaller than for MEGA-PRESS because of the increased frequency selectivity of the double-banded selective pulse for refocusing both coupled partners. The measurement of GABA+ may be acceptable under certain conditions (eg, healthy controls or no MM changes expected), but when studying the change of GABA levels in certain disease conditions and different age groups, it is essential to account for the MM contributions. Quantification and comparison of GABA levels among different groups can be affected by possible differences in MM contamination. For example, studies conducted at 1.5, 4.1 and 7 T reported significantly higher MM levels in GM than in WM, whereas no difference was observed in another study conducted at 3 and 7 T. Another study involving GABA editing found M3.00 to be higher in WM than in GM. Any dependence of MM level on tissue composition will lead to differences in the calculated GABA level. Thus, variations in voxel positioning and inter-subject variability in GM/WM content will lead to increased variance in the measured GABA. In addition, because the 0.93 and 1.35 ppm coedited MM resonances are not related to the 3.0-1.7 ppm coupling, it is not possible at present to calculate the M3.00 contribution to the edited GABA+ signal in vivo by reference to other (non-overlapping) MM resonances.

The larger the bandwidth of the frequency-selective editing pulse at 1.9 ppm, the higher the excitation of MM and its coupled component, M3.00. However, for fixed bandwidth of the inversion pulse the editing selectivity (of GABA over MM) improves with increasing field strength. Conversely, a higher bandwidth will reduce the susceptibility to misadjustment of the editing pulse frequency caused by motion and field drift, which alters MM coediting. The relative contributions of GABA and MM to the edited GABA+ resonance also depend on the timing pattern of the editing pulse.

Improving the selectivity of the editing pulse by performing single quantum editing with a numerically optimized pulse has been used to reduce MM coediting. Additional strategies could be employed using multiple quantum filtering to reduce MM coediting by adjusting the frequency separation between the two frequency selection bands and by repeating the double-band selective pulse. MM contamination in...
MEGA-PRESS can be minimized by (1) performing an additional metabolite nulling scan\textsuperscript{92} using IR, (2) applying frequency-selective pulses symmetrically with respect to the 1.7 ppm MM resonance (ie, at 1.9 and 1.5 ppm, respectively; Figure 6),\textsuperscript{94} or (3) by using a longer TE than the proposed 68 ms (1/2 J).\textsuperscript{109}

When MM coediting cannot be avoided, the variance of possible MM coediting should be mitigated by standardization of editing pulses\textsuperscript{107} and real-time updating of the editing pulse frequency,\textsuperscript{110,111} or avoiding measurements after high gradient duty cycle sequences including functional and diffusion MRI with echo-planar sequences. A recent multi-site study has shown excellent stability and reproducibility of GABA\textsuperscript{+} measurements compared with MM-suppressed GABA measurements mostly due to site-to-site misadjustments in editing frequency.\textsuperscript{112} However, every effort should be made to acquire MM-suppressed GABA for a more unambiguous quantification.

### 6 | BRAIN REGIONAL DEPENDENCE OF MM

Due to the spectroscopic overlap and low SNR of MM in vivo, a precise characterization of their regional differences is yet to be established. Single voxel spectroscopy (SVS) has previously shown spatial/tissue differences in MM.\textsuperscript{28,37,113} Recent magnetic resonance spectroscopic imaging (MRSI) studies\textsuperscript{30,74,114} have provided an improved spatial coverage and indicate that these differences may be larger than expected.\textsuperscript{30,74} Thus, a careful characterization may be important for MRS quantification of metabolites in both healthy and diseased brain.\textsuperscript{21,73,113,115}

In MRSI, spatial mapping of MM using IR-based metabolite nulling is complicated by low SNR and spatial B\textsubscript{1}-inhomogeneities. Free induction decay (FID)-MRSI is therefore particularly suited for mapping MM due to the complete absence of any TE (ie, no J-coupling and T\textsubscript{2}-related signal loss), resulting in the best possible sensitivity for these ultra-short T\textsubscript{2} MM components.\textsuperscript{2,8} Direct spectral fitting of individual MM as part of the basis set rather than analysis of the metabolite-nulled spectrum was therefore proposed.\textsuperscript{30} This requires a strict control of the fitting parameters. In particular, when the different MM components are mapped individually, one must be extremely careful about over-parameterization.\textsuperscript{30,31,73} Another postprocessing approach of extracting the MM contribution from FID-MRSI was proposed by Lam et al.\textsuperscript{114}

Mapping of individual MM by using them as part of the basis set has provided insights into the spectroscopic differences between GM and WM.\textsuperscript{30} The MM components in the ranges from 0.5-2.3 and 3.6-4.0 ppm, as measured by FID-MRSI at 7 T, tend to be higher in GM compared with WM (the MM/NAA ratios were 15% to 40% higher in GM than in WM),\textsuperscript{30} which is in agreement with the results of previous SVS studies.\textsuperscript{28,37} In contrast, the MM peaks at 3.0 and 3.2 ppm do not follow this trend, being higher in WM than in GM\textsuperscript{28,30} (Figure 7). The observation that MM contribution in this frequency range in WM is higher than in GM was recently replicated using MEGA-edited MRSI at 3 and...
and was also consistent with results from other "MM-sensitive" techniques (eg, T1rho). This has important implications for undesired coedited components of MM in spectral editing at ~3 ppm (eg, GABA+MM).

The regional differences may be linked to amino acids inside cytosolic proteins that contribute to the MM signals (Appendix S1, Table S1). Some of the regional and tissue differences that are revealed by MM mapping could be explained by different T1 relaxation of individual MM resonances. Overall, the reliability of mapping MM components varies significantly between different spectral ranges:

- 0.94 ppm: the M0.94 peak is large and does not overlap with metabolites, making the mapping of this component straightforward. If lipids are present in the spectrum due to disease or outer-volume contamination, the M0.94 peak may be affected.
- 1.2-1.7 ppm (M1.22-M1.70): lipids present in the spectrum due to disease or outer-volume contamination may impede reliable mapping despite a lack of overlap with abundant metabolites.
- 2-4 ppm (M2.05-M3.97): these MM overlap strongly with metabolites. Hence, correlations with metabolite concentrations should be checked carefully.

Although preclinical studies become attractive due to a large number of disease models, little attention has been given to preclinical MM. In animal models, the typical assumption that no substantial differences exist between regions and species has been evaluated in rodents in hippocampus, cortex and striatum. This is mainly due to the fact that rodent brains contain mostly GM and only minor variability of MM in rats and mice has been observed. No significant differences in metabolite concentrations were found when different MM spectra were used for the metabolite quantification of a given brain region. However, care has to be taken when removing residual metabolites, since this procedure can lead to a slight variability in the shape of the MM.

### 7 | AGE DEPENDENCE OF MM

In the rodent brain, the MM content has been shown to increase during postnatal development with no changes to the macromolecular pattern. The MM content was quantified from spectra in three brain regions, cortex, striatum and hippocampus, using LCModel and corrected for age-dependent changes in brain water content. At the time of writing, there are no published reports on the MM content in aging rodent brain.

In human brain, the MM content and pattern have been shown to differ with age. Higher MM content was observed in middle-aged (aged 25-55 years) compared with young (aged <25 years) subjects in centrum semiovale and in older (aged 67-88 years) compared with young (aged 19-31 years) adults in the occipital and posterior cingulate cortex (Figure 8). The greatest MM pattern differences associated with age...
occurred around 1.7 ppm. In the occipital cortex and the posterior cingulate cortex, the largest differences in intensities were observed for the MM resonances around 1.7 and 2.0 ppm. These age-associated differences in MM pattern and content could not be explained by differences in the tissue content (lower GM content in older adults). The differences in MM pattern require the use of age-specific MM spectra for quantification. However, because the patterns were the same in two brain regions, there is no indication that region-specific MM spectra are needed when studying these brain regions.

8 | DISEASE DEPENDENCE OF MM AND ML

The clinical literature is extremely limited on the application of MM contributions using MRS with most reports on the MM of healthy people. Even though in preclinical studies there are pulse sequences available for MM measurements in vivo, to date few studies on MM in disease have been published.

Research publications reporting on the measurement of MM in human brain pathologies have included brain tumors, MS and stroke. With the onset of disease, signals from ML appear that overlap with peaks of MM, thus mainly changes in ML + MM were reported and more precisely in the region 0.9 to 1.9 ppm due to the easier accessibility. These ML consist mainly of neutral triglyceride and cholesterol esters in the form of cytoplasmic lipid droplets, rather than membrane lipids, although mobile components of membrane phospholipid (choline methyl, 3.2 ppm) might contribute. ML are not subcutaneous lipid signals arising from imperfect localization (considered as artifacts in MR spectra), thus these terms should not be used interchangeably. Proton signals of mobile lipids have been assigned to methyl –CH₃ (0.9 ppm) and methylene –(CH₂)n– (1.3 ppm), allylic (2.05 ppm), α-acyl (2.3 ppm), bi-allylic (2.8 ppm) methylene and vinylic (5.4 ppm) methine, which overlap the MM signals.

Mobile lipids can be distinguished qualitatively from mobile proteins/peptides by the higher proton density of methylene (1.3 ppm) over terminal methyl (0.9 ppm) groups, expressed as the CH₂/CH₃ ratio. The relative intensities of the 1.3 and 0.9 ppm lipid peaks may reflect differences in chain lengths, correlation times (and visibility) of the methylene protons along the chain length, as well as the presence of cholesterol esters representing different classes of lipid or proteolipid involved.

There is a few reports of MM + ML in ¹H MRS of MS. One study of acute MS reported changes in ML + MM at 1.3 and 0.9 ppm. In another study, acute and chronic MS lesions were compared with healthy brain tissue, finding an increase in MM + ML in the acute (but not chronic) lesions at 0.9 and 1.3 ppm and no changes at 2.1 and 3.0 ppm. The changes seen in the acute MS lesions were suggested to arise from both ML and MM, the latter comprising proteolipid protein from myelin fragments.

There is substantial literature reporting the potential use of ¹H MRS to classify brain tumors. In low grade glioma, ML is low, whereas in high grade gliomas ML can dominate the spectrum, depending on the level of necrosis. Different types of brain tumors have been discriminated based on the spectrum profile, including MM + ML, and classifiers built for computer-aided diagnostics allowing the prediction of the tumor type and grade. In a study on the relationship between distance to the malignant glioma core and spectral pattern, ML + MM were an important factor for demarcation of the solid brain tumor. Short and long TE spectroscopic patterns of normal-
<table>
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<th>Recommendations</th>
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<tbody>
<tr>
<td>1. If the objective of a study is to determine metabolite concentrations and/or metabolite ratios, then the MM contribution has to be removed or included in the basis set used for quantification, especially for TEs below 80 ms.</td>
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<tr>
<td>2. We recommend to use the unified nomenclature of the MM components (Table 1), which can be easily expanded to new peaks.</td>
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<tr>
<td>3. The MM spectrum is different from a spectral baseline. These terms should not be used interchangeably.</td>
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<td>4. Since the shape, amplitudes and relaxation times of different MM contributions vary with $B_0$, we recommend to measure the MM spectra for metabolite quantification specifically for each $B_0$ field strength and each acquisition sequence used.</td>
</tr>
<tr>
<td>5. We recommend to acquire MM spectra in vivo using adiabatic RF pulse(s) and with the highest possible spatial and spectral resolution (ie, high quality $B_0$ shimming), high SNR, efficient water suppression and no baseline distortions, and to avoid subcutaneous lipid contamination.</td>
</tr>
<tr>
<td>6. We recommend to identify properly and eliminate residual peaks of metabolites from any MM spectrum measured in vivo (section 3.1). Some residual peaks of metabolites are always present independent of the sequence used to measure them.</td>
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<tr>
<td>7. Both single and double inversion methods of MM measurement are recommended since they provide good MM signal recovery, while the double inversion methods provide improved metabolite suppression (eg, for mapping in the presence of $B_1^+$ – inhomogeneities) at the expense of stronger $T_1$-weighting (eg, reduced MM signal recovery).</td>
</tr>
<tr>
<td>8. If the goal of the study is to estimate individual MM peaks, care has to be taken to avoid over-parameterization of the fitting, for instance via including some soft constraints on the relative amplitudes, frequencies and linewidth of the different MM components. One possible indication for overfitting is the presence of strong correlation between a MM component and an overlapping metabolite peak.</td>
</tr>
<tr>
<td>9. In human brain, the MM content and pattern change in older adult subjects (aged ≥60 years), therefore age-specific MM spectra are required for metabolite estimation (ie, a spectrum per 5 years after the age of 60).</td>
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<tr>
<td>10. It should be kept in mind that the MM content of some individual MM peaks varies across brain regions in humans. This should be particularly considered for low abundant metabolites, such as GABA + in spectral editing, where the contribution of MM to GABA+ seems to be higher in WM than in GM.</td>
</tr>
<tr>
<td>11. The MM content and spectral pattern does not seem to differ in hippocampus, cortex and striatum in healthy rodents (rats and mice). Thus, fitting metabolite concentrations assuming the constant shape of the MM spectrum can be a practical approach. Therefore, we recommend to use one single MM spectrum and to provide a clear description of this MM spectrum when publishing the data.</td>
</tr>
<tr>
<td>12. The MM and ML content and pattern change in disease in both humans and rodents. We recommend characterizing the MM and ML contribution in each specific disease by measuring the MM or MM + ML spectrum in vivo.</td>
</tr>
<tr>
<td>13. The choice of the approach to handle the MM highly depends on the circumstances: (1) a single MM basis spectrum when metabolite concentrations are of primary interest; (2) individual MM components if MM measures are desired as disease biomarkers.</td>
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<tr>
<td>14. To avoid duplicated effort, we recommend sharing of the various MM models through a list of parameters or data points.</td>
</tr>
<tr>
<td>15. We recommend that each publication contains a clear description of how MM were handled during the metabolite quantification step and also a brief description of the parameters used for acquisition, voxel position and size, quality of the shimming reported as water linewidth, the names of metabolite residuals eliminated and the type of postprocessing used.</td>
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<tr>
<th>Open issues in the field of MM</th>
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<tr>
<td>1. Identification of the biological background of individual MM peaks, especially those yet unknown MM peaks observed at ultra-high $B_0$.</td>
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<tr>
<td>Improvement of the assignment of the individual MM peaks to particular amino acids and further investigate the origin of the MM signal with respect to contributions from structured versus unstructured cytosolic proteins, membrane-bound proteins and large protein complexes in order to better understand any spatial/tissue differences.</td>
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<tr>
<td>Investigation of the contribution of other types of macromolecules, such as sugars or DNA/RNA.</td>
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<tr>
<td>Performing additional ex vivo validation studies.</td>
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<tr>
<td>2. Measurement of $T_1$ and $T_2$ relaxation times of different MM peaks ($M_{1,81}$ to $M_{4,20}$) at different $B_0$ in humans and rodents.</td>
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<tr>
<th>Open issues in the field of MM</th>
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<tr>
<td>3. Analyze the possible contribution of signals of metabolites with short T$_2$ relaxation times, such as GSH, in the measured metabolite-nulled MM spectrum.</td>
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<tr>
<td>4. Performing additional studies on how MM vary with TE using different types of acquisition sequences (ie, LASER, STEAM, PRESS, SPECIAL) and determine a threshold of TE above which the MM contribution is insignificant and does not need to be considered in the quantification step.</td>
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<tr>
<td>5. Improvement of IR or DIR methods for MM measurement in vivo. Development of alternative methods for MM mapping, which will not be based on IR-nulling or spectral quantification.</td>
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<tr>
<td>6. Identification of possible soft constrains and systematic errors in fitting individual MM peaks.</td>
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<tr>
<td>7. Confirmation of the observed regional differences in MM spectra in humans. Measurement of MM in additional brain regions in rodents (ie, cerebellum, thalamus) to confirm the lack of brain regional changes in MM in rodent brain.</td>
</tr>
<tr>
<td>8. Performing additional studies on quantification of individual MM peaks in normal versus diseased brain tissue. Rapid changes (in hours/days) in the 0.9-1.8 ppm range are observed in response to (systemic) hypoxia, and slow changes in relative amplitudes are observed on the timescale of several weeks. Prediction of patient outcome seems possible, and further systematic investigations are needed. Continuation of the studies on ML, MM + ML and MM only changes in pathologies and performing a precise identification of the origins of these MM peaks and the mechanisms behind. Determination of the most suitable approach on how to handle the MM contributions in pathologies.</td>
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<tr>
<td>9. Evaluate whether the age-associated MM pattern and content differences observed in the occipital cortex and posterior cingulate cortex are more widely spread throughout the brain. Evaluate whether changes with age occur in GM, WM or in both tissue types. Identify the causes of these differences. Assess the effect of age-associated differences in the MM spectral pattern on quantification of nonedited and edited spectra. Acquire data from neo-natal through to young adult subjects to fully characterize the age-dependence of the MM contribution to human/rodent brain spectra.</td>
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appearing WM, meningioma, metastases, low grade astrocytoma, anaplastic astrocytoma and glioblastoma were (visually) compared, while the complete MM + ML signal of different types of human brain tumors has also been investigated. Moreover, the apparent $T_2$ of the ML component at 1.3 ppm was shown to be different between glioblastoma and brain metastases in patients. A preclinical study performed in a mouse glioblastoma model showed MM + ML and MM changes being partially consistent with in vivo and ex vivo HRMAS results from humans. Besides a large variation of MM + ML at 1.3 ppm in the tumor region induced by glioma-initiating cells, changes also appeared at 2.8 ppm (similar chemical shift to polyunsaturated fatty acids) and 3.6-3.7 ppm. The origin of these signals remains to be characterized more thoroughly.

In stroke, only a few studies have been performed, showing changes mainly in signals from ML, which in subacute stroke patients may represent ML in macrophages or other cells. A preclinical study performed in the rat hippocampus after mild and moderate traumatic brain injury revealed a substantial change at 1.3 ppm. To demonstrate the potential of $^1$H MRS analysis including the ML and MM contributions in the clinical setting, a clinical case showing the longitudinal effects of a transient, but severe systemic hypoxia on the ML, MM and metabolites in the human brain, is described in Appendix S1 using the SpectrIm MM model presented in Appendix S3.

Nonradiological ex vivo clinical applications of the metabolic and biopsy data have been performed using HRMAS spectroscopy, and differences in metabolites, MM and ML between various brain tumors have been measured.

The number of studies evaluating MM or ML + MM changes in disease is still limited, but they provide substantial evidence that MM + ML changes are relevant and should be taken into account in the quantification step. Suggestions on how to handle them would be: (1) to use a MM fitting model with the freedom to measure this signal separately is required (ie, add a separate simulated MM or lipid component as already done in patients with adrenoleukodystrophy); or (3) to use a parameterized MM spectrum with well-defined soft constraints to avoid overparameterization (ie, fix ratios of all MM peaks in the parameterized MM spectrum, except for a small number of specific ones). In this context, future studies should focus on evaluating MM changes in additional pathologies together with a precise identification of the origins of these MM peaks and the underlying mechanisms.

## 9 | DISSEMINATION

In order to streamline and standardize the analysis of MM contribution to $^1$H MRS spectra without duplicating effort, we recommend the sharing of MM models with the MRS community. Dissemination of sequence- and field strength-specific MM models can be accomplished through sharing either the parameterization of MM resonances or complete experimentally measured MM basis functions for linear-combination analysis, along with settings and control files, as well as a complete documentation of how these data were acquired (see Table 2, recommendations 14 and 15). Preferably, MM data should be collected in a centralized public repository, and made available free of charge or license. We encourage the use of the MRSHub (https://www.mrshub.org), a resource designed by the recently established Committee for MRS Data and Code Sharing, a standing committee under the auspices of the ISMRM MRS Study Group. The MRSHub features resources for dissemination of analysis software as well as data, and also includes a discussion forum to address open issues in the field of MM in an open and collaborative fashion.

## 10 | CONCLUDING REMARKS AND RECOMMENDATIONS

This paper was written as an experts’ consensus recommendation and aims to summarize the present knowledge in the field of MM contribution in brain $^1$H MRS measurements. At the time of writing, the authors, experts in the MM field, agreed on several recommendations and provided a list with future studies needed to improve the general knowledge about MM. The recommendations and problems to be addressed in the future are summarized in Table 2.

## ACKNOWLEDGEMENTS

These experts’ consensus recommendations were arrived at from senior members of the authorship, who are all longtime experts in MRS methodology in general and this special topic in particular. The trainee members of the authorship are also well familiar with this topic and have fulfilled the requirements for authorship by contributing data, literature search and/or assisting in drafting, discussing, and revising the manuscript; namely, Tamas Borbath, Saipavitra Murali-Manohar, Veronika Rackayová, Dunja Simicic and Andrew Martin Wright contributed to Appendix S1 and Appendix S3 and to the figures presented in this paper. A further group of MRS experts, all of them with years of expertise in the field of MRS methodology and/or macromolecules handling in $^1$H MRS brain spectra, was collected to support the recommendations as the “Experts Collaborators Group on Contribution of macromolecules to brain $^1$H MR spectra”. The members of the group are listed in Appendix S1, Table S5.
authors are grateful for the input of Dr J Valette in section 3, Prof. R. Bartha and Dr. S. Provencher in Appendix S3, and Dr Georg Oeltzschner in the Dissemination (section 9) and Appendix S3. The preparation of this paper was supported by the Swiss National Science Foundation Grant numbers: 320030–175.984 (RK) and 310030_173222/1 (CC); Horizon 2020 / CDS-QUAMRI Grant number: 634541 (AH, TB and SMM); Horizon 2020 / SYNAPLAST Grant number: 679927 (AH and AMW); US National Institutes of Health Grant numbers: P41- P41EB015909 (MP); R01 MH109159 (KLB); P41-EB027061, P30-NS076408, (MM and IT); Austrian Science Fund (FWF) Grant number: P 30701-B27 (WB); Ministry of Education, Youth and Sports of the Czech Republic Grant number: CZ.02.1.01/0.0/0.0/16_013/0001775 (ZS and JS).

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