Hyperpolarized $^{13}\text{C}$ MR metabolic imaging can detect neuroinflammation in vivo in a multiple sclerosis murine model

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Proinflammatory mononuclear phagocytes (MPs) play a crucial role in the progression of multiple sclerosis (MS) and other neurodegenerative diseases. Despite advances in neuroimaging, there are currently limited available methods enabling noninvasive detection of MPs in vivo. Interestingly, upon activation and subsequent differentiation toward a proinflammatory phenotype MPs undergo metabolic reprogramming that results in increased glycolysis and production of lactate. Hyperpolarized ($^{13}\text{C}$) magnetic resonance spectroscopic imaging (MRSI) is a clinically translatable imaging method that allows noninvasive monitoring of metabolic pathways in real time. This method has proven highly useful to monitor the Warburg effect in cancer, through MR detection of increased $^{13}\text{C}$pyruvate-to-lactate conversion. However, to date, this method has never been applied to the study of neuroinflammation. Here, we questioned the potential of $^{13}\text{C}$ MRSI of HP $^{13}\text{C}$pyruvate to monitor the presence of neuroinflammatory lesions in vivo in the cuprizone mouse model of MS. First, we demonstrated that $^{13}\text{C}$ MRSI could detect a significant increase in HP $^{13}\text{C}$pyruvate-to-lactate conversion, which was associated with a high density of proinflammatory MPs. We further demonstrated that the increase in HP $^{13}\text{C}$lactate was likely mediated by pyruvate dehydrogenase kinase 1 up-regulation in activated MPs, resulting in regional pyruvate dehydrogenase inhibition. Altogether, our results demonstrate a potential for $^{13}\text{C}$ MRSI of HP $^{13}\text{C}$pyruvate as a neuroimaging method for assessment of inflammatory lesions. This approach could prove useful not only in MS but also in other neurological diseases presenting inflammatory components.

Multiple sclerosis (MS) is a multifaceted disorder of the CNS and is one of the most common causes of neurological disability in young adults (1, 2). Cortical and white-matter demyelination occurs early in MS pathogenesis and has been associated with disease progression and subsequent cognitive impairment (3). In the vast majority of cases, demyelinating lesions present a high inflammatory component, with elevated density of activated microglia/macrophages (mononuclear phagocytes, MPs) (4–6). Importantly, evidence suggests that proinflammatory MPs are one of the most abundant sources of reactive oxygen species (7), which mediate demyelination and axonal injury (8). Due to their central role in MS pathogenesis, noninvasive imaging of proinflammatory MPs would be of high importance for monitoring progression and response to antiinflammatory therapeutic approaches.

Several recent studies have demonstrated that, upon activation and differentiation toward a proinflammatory phenotype, MPs undergo metabolic reprogramming toward enhanced glucose uptake and increased glycolysis (9–14). As for the Warburg effect observed in cancer cells, this augmented glycolysis takes place under aerobic conditions and results in the majority of pyruvate being converted to lactate (15). However, the molecular mechanisms by which aerobic glycolysis is increased in MPs are still not fully understood. Novel studies have uncovered a potential underlying mechanism, showing pyruvate dehydrogenase kinase 1 (PDK1) up-regulation in proinflammatory MPs (13, 16). Because PDK1 inhibits pyruvate dehydrogenase (PDH), the enzyme that controls pyruvate entrance into the Krebs cycle, PDK1 up-regulation in activated MPs results in increased pyruvate-to-lactate conversion (12, 17, 18). In line with these observations, lactate has long been suggested as an imaging marker of neuroinflammation in the clinic. A few studies on MS patients have used $^1\text{H}$ magnetic resonance spectroscopy (MRS)/MRSI approaches and detected increased levels of $^1\text{H}$ lactate in gadolinium-enhancing lesions (19, 20) as well as in the cerebrospinal fluid of patients with such active lesions (21–24).

Over the last decade, dissolution dynamic nuclear polarization coupled with $^{13}\text{C}$ MRSI has developed as a metabolic imaging approach of high potential (25, 26). Through increase in the MR-detectable signal of $^{13}\text{C}$ compounds by up to 50,000 [so-called hyperpolarized (HP) probes] this methodology has enabled the noninvasive monitoring of several metabolic reactions in vivo (27–32). In particular, in the context of cancer, HP $^{13}\text{C}$ MRSI has been shown to improve the noninvasive detection of hyperpolarized $^{13}\text{C}$ MR spectroscopy | multiple sclerosis | neuroinflammation | metabolism | macrophages

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cancerous lesions through the monitoring of the increased HP [1-13C]pyruvate-to-lactate conversion, not only in preclinical cancer models but also in the clinical setting (33).

To date, 13C MRSI of HP [1-13C]pyruvate has only been applied to investigate inflammation in three studies: a rat model of radiation-induced lung injury (34), a mouse model of inflammatory arthritis (35), and a mouse model of acute liver injury (36). In all three cases, 13C MRSI of HP [1-13C]pyruvate was capable of detecting an increase in the HP [1-13C]lactate-to-pyruvate ratio in the inflammatory region, which was associated with an increased number of macrophages as detected by H&E staining. These three studies are in line with the literature of increased glycolysis in activated MPs. However, to date, no studies have reported the use of 13C MRSI of HP [1-13C]pyruvate to study neuroinflammation, and underlying biochemical mechanisms linked to increased HP [1-13C]lactate production in inflammatory models are still lacking.

**Fig. 1.** Histological and MRI characterization of CPZ mice. (A) Myelin (MBP), microglia/macrophages (Iba-1, MPs), oligodendrocyte progenitor cells (OPCs, PDGFRα), and astrocytes (GFAP) staining of the corpus callosum (dashed lines) prior to (W0) and following CPZ diet (W4, W6), and after recovery (W6 + W6 recovery). (Scale bar: 100 μm.) (B) Immunofluorescence staining illustrates the massive cell infiltration of activated MPs (Iba-1; nuclear staining Hoechst) in the corpus callosum (white arrows) at W4 of CPZ compared with CTRL. (Magnification: 10×; scaling per pixel, 0.645 μm × 0.645 μm.) (C) Quantitative analyses of the corpus callosum confirm demyelination (MBP, P < 0.0001) and maximal microgliosis (Iba-1, P < 0.0001) after W4 of CPZ. Remyelination and decreased MP levels are observed following W6 of CPZ diet (P = 0.0285 and P < 0.0001, respectively). OPCs (PDGFRα) accumulate at W4 of CPZ (P = 0.0299) and return to CTRL levels at W6 + W6 recovery (P = 0.0479). Astrocyte (GFAP) staining quantification shows highest astrogliosis after W6 of CPZ (P < 0.0001), which persists after the recovery period (P < 0.0001) (n = 3–5 mice per group). All values are reported as mean ± SD. (D) T2-weighted MR images show hyperintensity in the corpus callosum at W4 and W6 of CPZ diet (white arrows). Corresponding nT2w values increased at W4 of CPZ (P ≤ 0.0003), in line with demyelination and neuroinflammation at these time points. At W6 + W6 recovery, hypointense contrast can be observed in the corpus callosum, resulting in a decrease of nT2w toward CTRL values (P = 0.0011), indicating remyelination and decreased neuroinflammation (n = 5–7 mice per group). In the CTRL group, the nT2w values decreased over time, reflecting myelination of the corpus callosum in young adult mice. All values are reported as mean ± SD (one-way ANOVA and two-Way ANOVA, Tukey HSD post hoc test *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; unpaired t test **P < 0.01; repeated measures ANOVA *P < 0.05). n.s., not significant. A.U., arbitrary unit.
In this context, we questioned whether $^{13}$C MRSI of HP [1-13C]pyruvate could monitor the presence of activated MPs in neuroinflammatory lesions in the mouse brain. We conducted a longitudinal study of the toxin-induced cuprizone (CPZ) mouse model, a well-established MS model characterized by high spatial and temporal reproducibility of neuroinflammatory lesions (37; 38). Our results demonstrated a significant increase in HP [1-13C]pyruvate-to-lactate conversion in the corpus callosum of CPZ mice as detected by $^{13}$C MRSI at 14.1 T. Using immunofluorescence, we then showed that increased HP [1-13C]lactate production was associated with a high density of activated proinflammatory MPs that overexpressed PDK1. We further showed that the enzymatic activity of PDH was significantly down-regulated in neuroinflammatory lesions, likely due to PDK1 up-regulation, thus providing a putative mechanism for the increased HP [1-13C]lactate production observed by $^{13}$C MRSI. Finally, using transgenic mice exhibiting an impaired microglial function following CPZ diet, we further confirmed that the increased HP [1-13C]lactate conversion was primarily driven by the presence of activated MPs in cerebral lesions.

**Results**

**Histological and MRI Characterization of the CPZ Mouse Model.** In the initial part of this study our goal was twofold. First, we wanted to confirm that, in our hands, the CPZ mouse model was behaving as previously described in terms of spatial and temporal distribution of lesions (37, 39–42). Next, we evaluated the potential of T2-weighted MRI at high field strength (14.1 T) to detect cerebral lesions in CPZ mice.

As shown in Fig. 1 A–C, histological analyses confirmed our CPZ model reproduced findings reported in the literature, presenting demyelination (myelin basic protein, MBP), recruitment of MPs (ionized calcium-binding adapter molecule 1, Iba-1) into the corpus callosum, accumulation of oligodendrocytes progenitor cells (OPCs) (PDGF receptor alpha chain, PDGFR-α), and astroglisis (glial fibrillary acid protein, GFAP). After 4 wk (W4) of CPZ diet, myelin content was significantly decreased by 86 ± 9% of control (CTRL) level ($P < 0.0001$) and MP number significantly increased by a dramatic 5.230 ± 762% ($P < 0.0001$). This massive infiltration of MPs after W4 of CPZ was mainly restricted to the corpus callosum and surrounded the dorsal hippocampal formation (Fig. 1B). In contrast, at 6 wk (W6) of CPZ diet, recruitment of activated proinflammatory MPs (Iba-1) was strongly diminished to 30 ± 6% of W4 ($P = 0.0285$ and $P < 0.0001$, respectively). At the end of the recovery period both myelin content and MP numbers reached CTRL levels. In addition, significant accumulation of OPCs (PDGFR-α) into the corpus callosum was observed at W4 of CPZ (668 ± 500%, $P = 0.0299$) and was later followed by a decrease to CTRL levels once remyelination had occurred at W6 + W6 recovery ($P = 0.0479$). Astrogliosis, as demonstrated by an increase of GFAP, was present after W4 of CPZ (362 ± 200%, $P = 0.0004$), peaked after W6 of CPZ (645 ± 200%, $P < 0.0001$), and remained elevated after the recovery period, eventually forming a glial scar (410 ± 225%, $P < 0.0001$). When looking at all cell types it is important to note that the maximum level of MP staining reached 83 ± 2% area at W4 (Iba-1), whereas the maximum level of astrocytes reached 54 ± 3% area at W6 (GFAP+), and OPC staining was maximum at W4 with 2.2 ± 1% area (PDGFR-α+)

On T2-weighted MR images, hyperintensity could be observed in the corpus callosum of CPZ mice following W4 and W6 of CPZ administration (Fig. 1D, arrows). Upon quantification, our results showed that normalized T2-weighted (nT2w) signals from the corpus callosum were significantly different between CPZ and CTRL groups ($P < 0.0001$ for group and time effects and for group and time interaction). The nT2w signals from the corpus callosum of CPZ-treated mice were significantly higher than those from the CTRL mice at any time point following CPZ diet ($P < 0.0001$ for W4, W6, and W6 + W6 recovery). Furthermore, nT2w signals from CPZ mice were increased by 42 ± 13% after W4, 33 ± 16% after W6 of CPZ diet, and 12 ± 9% after recovery compared with W0 ($P < 0.0001$ for W4 and W6 and $P = 0.0174$ for W6 + W6 recovery). However, no significant difference in nT2w signal values could be observed between W4 and W6 CPZ ($P = 0.0562$), despite the differences in underlying pathophysiological events observed at these two time points. In the CTRL group, nT2w signals significantly decreased over time ($P = 0.026$, likely reflecting myelination of the corpus callosum in young adult mice (40, 43).

**Increased Production of HP [1-13C]Lactate Following MP Activation in CPZ-Induced Lesions.** Next, we evaluated the potential of HP [1-13C]pyruvate to monitor the presence of proinflammatory MPs. To do so, as shown in Fig. S1, MR experiments were performed at W0, W4, and W6 of CPZ diet and W6 of CPZ diet followed by W6 of standard rodent chow. As shown in Fig. 2 A and B, the level of HP [1-13C]pyruvate detected in the corpus callosum voxel remained stable over time in CTRL and CPZ mice. In contrast, a significant increase of HP [1-13C]lactate was observed in the corpus callosum region following W4 of CPZ administration (Fig. S2A). Upon full quantification, our results showed that the ratio of HP [1-13C]lactate to pyruvate was significantly different between CPZ and CTRL groups over time ($P = 0.0014$ for group effect, $P = 0.045$ for time effect, and $P = 0.0004$ for group and time interaction). Specifically, when comparing CTRL and CPZ groups, the HP [1-13C]lactate-to-pyruvate ratio was more than threefold higher in the corpus callosum of CPZ mice after W4 of CPZ diet compared with age-matched CTRL ($P = 0.0006$), a time point that corresponds to the highest level of activated proinflammatory MPs (Fig. 2C). At that time point, T1-weighted MRI after injection of a gadolinium-based contrast agent showed similar signal enhancement in the corpus callosum of CTRL and W4 CPZ mice (Fig. S2). Next, longitudinal evaluation revealed a significant increase of the HP [1-13C]lactate-to-pyruvate ratio after W4 of CPZ compared with W0 (96 ± 74% of W0, $P = 0.0002$), followed by a significant decrease after W6 of CPZ (42 ± 59% of W4, $P = 0.0194$), when MPs numbers are strongly decreased and remyelination is occurring. By the end of the recovery period, the HP [1-13C]lactate-to-pyruvate ratio remained significantly decreased compared with W4 CPZ (131 ± 24% of W4, $P = 0.0346$) and was no longer significantly different from W0 values ($P = 0.2050$) (Fig. 2D). The two animals that were not imaged at W6 + W6 were excluded from the statistical analysis between W4 and W6 + W6. As shown on the heat maps of HP [1-13C]lactate-to-pyruvate ratio, the increase in HP [1-13C]lactate production was localized to the corpus callosum voxel only, in line with the high level of activated MPs in that cerebral region (Fig. 3A). To further assess the specificity of $^{13}$C MRSI of HP [1-13C]pyruvate to detect proinflammatory MPs, we examined the levels of HP [1-13C]lactate and HP [1-13C]pyruvate in a thalamic voxel (dark blue) as well as in an heterogeneous neck voxel outside the brain (light blue), regions that do not display a large recruitment, proliferation, and activation of MPs (Fig. 3B). In line with the HP [1-13C]lactate maps, the grid of the HP [1-13C]lactate maps showed an increased HP [1-13C]lactate in the region that contains the demyelinated and inflamed corpus callosum (Fig. 3B, red) but not in the adjacent brain regions (Fig. 3B, dark and light blue). In both the thalamic and the neck region the level of HP [1-13C]pyruvate remained stable over time for both CPZ and CTRL groups (Fig. 3 C and D) and the HP [1-13C]lactate-to-pyruvate ratio did not significantly differ between CPZ and age-matched CTRL (Fig. 3 E and F). T1-weighted postcontrast also showed no differences in thalamic kinetic parameters between CTRL and W4 CPZ mice (Fig. S2).

**The Increase in HP [1-13C]Lactate Signal Is Associated with Up-Regulation of PDK1 in Activated MPs and Decreased PDH Activity in the Corpus Callosum.** We performed immunofluorescence for PDK1, a protein that inhibits PDH and thus controls pyruvate.
entrance into the Krebs cycle. Interestingly, we demonstrated a significant up-regulation of PDK1 (PDK1) in activated MPs (Iba-1) following W4 of CPZ administration in the corpus callosum, but not in other brain regions (cortex and thalamus), as shown in Fig. 4A and Fig. S3. Whereas only 12 ± 7% of Iba-1 were also PDK1+ at W0, this number significantly increased at W4 (87 ± 7% of MPs are PDK1+/Iba-1+, P < 0.0001) and W6 after CPZ diet (82 ± 8% of MPs are PDK1+/Iba-1+, P = 0.001), in line with a higher density of activated MPs at these time points. Cellular colocalization of PDK1 and Iba-1 was further confirmed by confocal microscopy (Fig. 4B). Importantly, GFAP/PDK1 and PDGFβR/α/PDK1 costaining revealed that neither astrocytes nor OPCs overexpressed PDK1 at any time point (Fig. 4B and Fig. S3), providing additional evidence that these other cell types do not play a major role in the HP [1-13C]lactate signal increase at W4 of CPZ.

Next, we wanted to investigate whether PDK1 up-regulation by Iba-1 MPs was associated with a modulation in the activity of its downstream target PDH. As shown in Fig. 4C, PDH activity was significantly decreased in the corpus callosum of W4 mice compared with W0 (57 ± 15% decrease, P = 0.0026), but not in the thalamus (Fig. S3), suggesting a regional effect of Iba-1+ MPs PDK1 up-regulation on PDH activity. Further in line with this putative link, after W6 of CPZ and after the recovery period the number of PDK1+/Iba-1+ MPs in the corpus callosum significantly decreased and PDH activity no longer differed from CTRL levels. We also investigated lactate dehydrogenase A (LDH-A), the iso-enzyme that converts pyruvate to lactate and is overexpressed in most cancer types. Unlike in cancer, we did not observe an up-regulation of LDH-A staining in the corpus callosum (Fig. S3) or a change in LDH-A activity at any time point (Fig. 4D), thus ruling out overexpression of this enzyme as a main player in the observed increased HP [1-13C]lactate production at W4 CPZ.

Finally, we measured serum lactate levels in CPZ and CTRL mice at all time points. As shown in Fig. 4E, serum lactate levels were significantly increased at W4 and W6 of CPZ (161 ± 62% W4 P = 0.0014; 166 ± 48% W6 P = 0.0007) and returned to CTRL levels by the end of the recovery period (P = 0.0024), confirming the systemic effect of CPZ diet (38, 44, 45).

**HP [1-13C]Lactate Levels Are Not Increased in the Corpus Callosum of CXCR1CR1GFP/GFP Transgenic Mice Harboring Microglial Activation Deficiency, Suggesting a Central Role of MPs to the Detected HP [1-13C]Lactate Signal.** To confirm that the production of HP [1-13C]lactate was driven by the presence of MPs into the corpus callosum following CPZ diet, we studied CXCR1CR1GFP/GFP mice, which exhibit highly reduced or absent microgliosis, astrogliosis, and demyelination at W4 of CPZ diet, as previously described (39). In these mice, the CXCR1 receptor, which is highly expressed in microglia, is replaced by GFP, allowing one to easily monitor microglia levels by immunofluorescence (46).

As shown in Fig. S4, CPZ diet did not induce hyperintensities characteristic of demyelination and inflammation on T2-weighted MR images in the corpus callosum of CXCR1CR1GFP/GFP mice. Quantitative analyses of the nTW values from the corpus callosum showed that nTW were significantly decreased at W4 in CPZ and CTRL CXCR1CR1GFP/GFP, as well as in CTRL nontransgenic mice, reflecting normal myelination throughout development (40, 43). In contrast to nontransgenic CPZ mice, however, no differences in nTW
MR contrast could be detected between CTRL and CPZ groups at W0 or W4. Immunofluorescence analyses further confirmed the absence of microgliosis (CX3CR1GFP/GFP labeled microglia), cell infiltration, demyelination, and astrogliosis (Fig. S4) in the corpus callosum of CX3CR1GFP/GFP mice, despite W4 of CPZ diet, and in line with previous reports (37, 39).

Importantly, PDH (Fig. 5B) and LDH-A (Fig. 5C) activities measured from the corpus callosum of CX3CR1GFP/GFP mice were not significantly different between CTRL and CPZ animals at W4. As expected however, and similar to the nontransgenic model, the serum lactate levels (Fig. 5D) were increased by 173 ± 79% (P = 0.0079) in CX3CR1GFP/GFP mice after W4 of CPZ diet compared with CTRL mice, confirming the systemic effect of the CPZ diet.

We then performed HP[1-13]C MRSI to further confirm that increased HP[1-13]C lactate was associated with the presence of proinflammatory MPs. HP[1-13]C spectra acquired in the corpus callosum of CX3CR1GFP/GFP mice show no evident differences between CTRL and CPZ groups (Fig. 5E). Spectra quantification showed that, in contrast to the nontransgenic mice, HP[1-13]C lactate-to-pyruvate ratios for the corpus callosum were similar between CTRL and CPZ groups at W4 of CPZ diet, in line with the absence of MP activation and infiltration in this transgenic model (Fig. 5F). Similarly, HP[1-13]C lactate-to-pyruvate ratios from the thalamic area (Fig. 5G) and the neck (Fig. 5H) were not significantly different between CTRL and CPZ groups at W4. HP[1-13]C pyruvate delivery was also unchanged in all regions (Fig. S4).

**PDH1 Is Up-Regulated in Activated MPs in Experimental Autoimmune Encephalomyelitis Lesions.** Finally, to further assess the validity of our findings, we investigated whether the up-regulation of PDH1 by MPs was also present in demyelinated lesions in another animal model for MS. For this, we performed immunofluorescence analyses on tissues from experimental autoimmune encephalomyelitis (EAE) mice. Most neuroinflammatory lesions were found in the spinal cord and brainstem in close vicinity to blood vessels. We found that 45 ± 8% of MPs present in the EAE group expressed PDH1 in the spinal cord (Fig. 6A–C), 28 ± 8% in the brainstem, and 25 ± 7% in the cerebellar white matter (Fig. 6B and C), whereas the level of detection for PDH1 was below detection for age-matched CTRL (Fig. 6A).

**Discussion**

In this study we applied a metabolic imaging method, 13C MRSI of HP[1-13]C pyruvate, to the longitudinal study of the CPZ model of MS. HP[1-13]C pyruvate-to-lactate conversion increased in the corpus callosum of CPZ mice at W4 of CPZ diet, a time point presenting the highest density of proinflammatory MPs. In contrast, in knockout CX3CR1GFP/GFP animals with low...
microglial activation HP $[1^{-13}C]$lactate levels were not increased, suggesting a central role for MPs in the detected HP signal. We further showed that, at W4 in the nontransgenic model, the majority of activated MPs present in the corpus callosum overexpressed PDK1 and that PDH activity was significantly decreased in this region, providing a plausible mechanism for the observed increased HP $[1^{-13}C]$lactate levels.

We opted to investigate the well-established CPZ mouse model for MS, whose strengths lie in the localization of the lesions, their well-known pathogenesis, and their reproducibility in time and space, allowing for longitudinal validation of imaging methods (37, 47, 48). In this model, the vast majority of activated MPs are endogenous microglia, not peripheral macrophages (49–52). At W4 of CPZ diet, cerebral lesions show a large recruitment and proliferation of MPs, which induce demyelination and can reach up to 50 times the density of microglia present under normal conditions (40). By W6 of CPZ diet, most of the MPs have disappeared and remyelination is ongoing despite continuous administration of the toxin (57, 58). After the 6-wk recovery period, remyelination has occurred, MP number has returned to baseline, and only astrogliosis remains. These marked differences between the cellular events happening at W4, W6, and W6+W6 recovery justifies the longitudinal follow-up of CPZ mice at these three time points and enabled us to evaluate the potential of $^{13}C$pyruvate to detect the presence of proinflammatory MPs.

Current clinical evaluation of MS brain lesions essentially includes conventional T2-weighted and gadolinium-enhanced T1-weighted MRI (59, 60). Although these methods provide high spatial sensitivity, prediction of outcome and response to treatment remains challenging. Gadolinium-enhanced T1 lesions

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**Fig. 4.** Increased HP $[1^{-13}C]$lactate production is associated with up-regulation of PDK1 in activated MPs and decreased PDH activity in the corpus callosum. (A) Immunofluorescence staining for PDK1 (green) and MPs (Iba-1, red) reveal overexpression of PDK1 in MPs after W4 of CPZ diet (yellow). Quantitative analyses confirmed the significant up-regulation of PDK1 after W4 of CPZ ($P < 0.0001$), (n = 3 mice per time point). Scale bar: 100 μm.) (B) Confocal microscopy confirmed cellular colocalization (yellow, arrows) of PDK1 (green) in MPs (Iba-1, red) at W4 of CPZ diet. In contrast, astrocyes (GFAP, red, arrow) and OPCs (PDGFR-α, red, arrow) staining did not colocalize with PDK1 staining (green) at W4 of CPZ diet, as shown by confocal microscopy. (Scale bar: 10 μm.) (C) Quantitative analyses revealed a significant decrease of PDH enzyme activity at W4 of CPZ diet ($P = 0.0028$) and (D) no significant change in LDH-A enzyme activity (reported as $V_{\text{max}}$). (E) Serum lactate levels were significantly increased at W4 and W6 of CPZ diet (161 ± 62% W4 $P = 0.0014$; 166 ± 48% W6 $P = 0.0007$) and returned to CTRL levels by the end of the recovery period ($P = 0.0024$), (n = 4–6 mice per time point). All values are reported as mean ± SD (one-way ANOVA, Tukey HSD post hoc test **$P < 0.01$, ***$P < 0.001$).
are indicative of altered blood–brain barrier (BBB) and are considered the main radiologic marker of active MS lesions (59, 61). However, whereas density of active lesions are linked to treatment efficacy in some cases, formation of new T1 lesions may occur subclinically and thus more frequently than clinical relapses, limiting treatment monitoring (62). Furthermore, noninvasive methods for assessment of neuroinflammatory status in chronic, nonenhancing lesions in gray and white matter are still lacking. Such limitations represent a major drawback for evaluation of lesion formation and monitoring of regenerative and antiinflammatory therapies. In this study we showed that, whereas T1-weighted postcontrast MRI confirmed the lack of BBB breakdown in the CPZ model, nT2W values enabled the detection of CPZ-induced lesions and long-lasting changes associated with a demyelinating event. However, this measure could not readily distinguish between lesion stages linked to different underlying cellular events, notably acute inflammation at W4 CPZ. In contrast, using 13C MRSi of HP [1-13C]pyruvate, we were able to detect an increase of HP [1-13C]lactate production specifically at W4 CPZ in lesions presenting a high level of proinflamatory MPs, thus highlighting the potential added value of this metabolic imaging strategy compared with conventional MR methods.

Following i.v. injection, we detected delivery of HP [1-13C]pyruvate in the corpus callosum of CTRL and CPZ mice. Interestingly, levels of HP [1-13C]pyruvate were comparable between these two groups for all time points. This result is in line with the fact that, unlike in the EAE model, the induction of lesions following CPZ diet occurs without BBB disruption (49–51, 58, 63). Furthermore, and importantly, we were able to detect production of HP [1-13C]lactate from HP [1-13C]pyruvate in the corpus callosum of both CTRL and CPZ animals and a significant increase in HP [1-13C]lactate production at W4 in the CPZ group only. In agreement with other HP 13C MRSi

![Diagram](Image)

Fig. 5. HP [1-13C]lactate levels are not increased in the corpus callosum of CXCR1Gfp/GfP transgenic mice harboring microglial activation deficiency, suggesting a central role of MPs to the detected HP [1-13C]lactate signal. (A) T2-weighted MRI of CXCR1Gfp/GfP mice show similar hypointense contrast of the corpus callosum regardless of diet (CTRL or W4 CPZ). nT2W were significantly decreased at W4 in CPZ and CTRL CXCR1Gfp/GfP, reflecting normal myelination (P ≤ 0.0079). Immunofluorescence images of CXCR1Gfp/GfP microglia show no evident microglialis of the corpus callosum of CXCR1Gfp/GfP mice despite the CPZ diet. (Scale bar: 100 μm.) (B) PDH and (C) LDH-A activities from the corpus callosum were not significantly different between CPZ and CTRL groups. (D) Serum lactate levels were significantly increased following W4 CPZ, in line with a systemic toxic effect of CPZ diet (173 ± 79%), P = 0.0079. (E) T2-weighted image overlaid with the HP 13C grid is shown for a W4 CPZ CXCR1Gfp/GfP mouse. HP 13C spectra for CTRL and W4 CPZ CXCR1Gfp/GfP mice show no evident differences between CTRL and CPZ groups in any studied voxel (corpus callosum red; thalamic region dark blue; neck voxel light blue). HP [1-13C]lactate-to-pyruvate ratios for (F) the corpus callosum, (G) the thalamic area, and (H) the neck were not significantly different between CTRL and CPZ groups at any time points (n = 5–7 mice per group). All values are reported as mean ± SD (two-way ANOVA, Tukey HSD post hoc test **P < 0.05; unpaired t test ***P < 0.001). A.U., arbitrary unit; Lac, lactate; Pyr, pyruvate.

![Diagram](Image)

Fig. 6. Detection of PDK1-expressing MPs after EAE induction. (A) Immunofluorescence staining for MPs (Iba-1, red) shows increased number of MPs and expression of PDK1 (green) in the spinal cord 30 d after EAE induction compared with age- and sex-matched CTRL. (B and C) Quantitative analyses reveal that 45 ± 8% of MPs present in the lesions coexpress Iba-1 and PDK1 (yellow) in the spinal cord, 28 ± 8% in the brainstem, and 26 ± 7% in the cerebellum, whereas the level of PDK1 in the CTRL group was below detection. All values are reported as mean ± SD (n = 3 mice).
From a technical perspective, we evaluated the dynamic conversion of HP [1,3-13C]pyruvate into lactate by acquiring spectra every 3 s for a period of a minute. This achievement comes with the trade-off of a lower spatial resolution, which may result in lower sensitivity to subtle lactate changes. For instance, after W6 of CPZ administration we observed a strong reduction of MPs in the corpus callosum. Although most of the remaining MPs still express PDK1 and there is a trend toward decreased PDH activity compared with CTRL, we did not detect a significantly increased production of HP [1,3-13C]lactate at that time point, which may be explained by the limited sensitivity of HP [1,3-13C]MRSI to lower MP number. Furthermore, the lack of increase in the HP [1,3-13C]lactate-to-pyruvate ratios in two of seven mice at W4 (Fig. 2D), despite the likely infiltration of MPs in these animals, should be acknowledged as a probable technical failure of the DNP methodology. Future improvements in hardware and MR acquisition schemes will likely help in reaching a higher sensitivity specificity and improve the detection of smaller lesions.

In conclusion, this paper shows that metabolic imaging using HP [1,3-13C]MRSI can be used to monitor neuroinflammatory lesions noninvasively in a preclinical model for MS. Increase in HP [1,3-13C]lactate production can be detected in cerebral lesions and is associated with high levels of PDK1-activated MPs and subsequent regional PDH inhibition. Altogether, our results demonstrate a potential for [1,3-13C]MRSI of HP [1,3-13C]pyruvate as a neuroimaging method for assessment of inflammatory lesions. Because HP [1,3-13C]MRSI is clinically translatable (35) and expanding rapidly, this study provides high significance for future clinical trials not only on MS but also all neurological diseases presenting an inflammatory component (66). When used in conjunction with conventional MRI techniques, such a method would enhance diagnosis and help refine therapeutic regimens, which will ultimately improve clinical outcome and patient care.

Materials and Methods

Animals and Experimental Outline. All animal research was approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco. For CPZ studies, 8-wk-old B6(Cg-Tyr tm1Litt/J) (stock no. 000058) albino female mice and 8-wk-old B6.129P4-Cx3cr1tm1Litt/J (stock no. 005382) mice (referred to above as Cx3cr1(GFP/GFP)) were purchased from Jackson Laboratories. Mice [n = 43 B6(Cg-Tyr tm1Litt/J); n = 6 Cx3cr1(GFP/GFP)] received a 0.2% wt/vt CPZ (Sigma-Aldrich) diet for up to 6 wk. Age-matched CTRL mice received a standard rodent chow [n = 22 B6(Cg-Tyr tm1Litt/J); n = 6 Cx3cr1(GFP/GFP)]. For B6(Cg-Tyr tm1Litt/J) mice, a subset of animals were imaged longitudinally prior to death (W0), and after 4 and 6 wk of CPZ (W4 and W6) as well as after 6 wk of CPZ followed by 6 wk of standard rodent chow (W6 + W6 recovery) [n = 7 CPZ and 7 CTRL]. For Cx3cr1(GFP/GFP) mice, all animals were imaged at W0 and W4 (n = 6 CPZ and 6 CTRL) for immunofluorescence staining, enzyme activity assays, and serum lactate levels, a subset of mice was killed at each time point (n = 3–6 CTRL, 3–6 W4, 3–6 W6, and 3–6 W6 + W6 recovery). For EAE induction, 8-wk-old C57BL/6J female mice were injected s.c. with 100 μg of myelin oligodendrocyte glycoprotein (MOG35-55), in complete Freund’s adjuvant (Difco Laboratories) (67). After immunization and 2 d later mice received 400 ng of pertussis toxin i.p. At 30 d postinjection mice (n = 3 EAE and 3 age-matched CTRL) were killed and CNS tissue was collected. For all experiments, animals were observed daily, and clinical signs were assessed as follows: 0, no signs; 1, decreased tail tone; 2, mild monoparesis or paraparesis; 3, severe paraparesis; 4, paraplegia; 5, quadraparesis; and 6, moribund or death. Mice with an EAE score of 2.5 ± 0.3 at 30 d postinjection were used for the experiments.

In Vivo MR Acquisition. All in vivo MR experiments were conducted on a 14.1 T vertical MR system (Agilent Technologies) equipped with 100 Gcm gradients and a dual tune 1H-13C volume coil (Q0 = 40 mm). For each imaging session, mice were anesthetized using isoflurane (1-3% in O2) and a 27-gauge catheter was secured in the tail vein to allow for i.v. injection of the HP probe. Animals were placed in a dedicated cylindrical cradle allowing for reproducible positioning of the mouse head; the cradle was then tightly inserted inside the volume coil, which was secured to the MR bore to ensure similar positioning between experiments. Respiration and temperature were continuously monitored to ensure animal well-being and data reproducibility.

T2-weighted MRI was acquired using the following parameters: TE/TR = 201/200 ms, slice thickness = 0.5 mm, number of averages = 2, matrix = 256 × 256, and field of view (FOV) = 30 × 30 mm2. For HP [1,3-13C]MRSI acquisitions, 24 μL...
of [1-13C]pyruvate preparation was hyperpolarized using a Hypersense DNP polarizer (Oxford Instruments) for 1 h (29). Note that all pyruvate preparations used in this study were prepared as large batches and underwent quality control to ensure reproducible polarization level between experiments (10–12%). After dis- solution, HP [1-13C]pyruvate was rapidly dissolved in isotonic buffer (pH ~7) to a final concentration of 80 mM. A final volume of 300 μL of the solution was then injected i.v. over 12 s through the tail vein catheter. From the beginning of the iv injection, 2D dynamic chemical shift imaging (CSI) [13C] data were acquired using the following parameters: T/ΔT = 1.260 ms; spectral width ~2,500 Hz; 128 points; flip angle (FA) = 10°; FOV = 24 × 24 mm²; matrix 8 × 8 × in-plane resolution 3 × 3 mm²; 5-mm slice thickness; scan time 3 × 3 time point; and 16 time points (68). T2-weighted images (T/ΔT = 1.14/39 ms, FA = 40°, 10 slices, thickness = 1.5 mm, NA = 1, matrix = 128 × 128, and FOV = 22.4 × 22.4 mm²) were ac- quired prior (five images) and after (25 images) i.v. injection of Magnevist (gadopentetate dimeglumine, gadolinium-based contrast agent, 1 mmol/kg).

MR Data Analysis. The corpus callosum was defined as the region of interest and manually delineated on the T2-weighted images according to the Franklin and Paxinos anatomical mouse brain atlas (69) with AMIRA soft- ware (Mercury Computer Systems). The average signals from the corpus callosum were normalized to the mean cerebrospinal fluid signal of the third ventricle as signal value standard. In a similar manner, the corpus callosum and the thalamus were delineated on T1-weighted images and simple kinetic pa- rameters of contrast enhancement (area under the curve, slope, and relative signal enhancement ([MR signal postcontrast – MR signal precontrast]/MR signal precontrast)*100) were calculated, as surrogate values of global per- fusion, permeability, and blood flow, as previously described (70, 71).

HP [13C] MRSI datasets were analyzed using the in-house SIVIC software (https://sourceforge.net/apps/trac/sivic/) and custom-built programs written in MATLAB (MATLAB R2011b; The MathWorks Inc.). HP [1-13C]lactate-to- pyruvate ratio were generated using a sinc-based interpolation of the 13C 2D CSI data to the resolution of the anatomical images using custom-built programs written in MATLAB and SIVIC.

Immunofluorescence Acquisition and Analysis. All analyses were performed according to previously described procedures (72). Mice were first perfused with an ice-cold 0.9% NaCl solution followed by an ice-cold 4% paraformaldehyde (PFA) solution. Next, brains were dissected and further fixed in 4% PFA for 2 h then dehydrated through a sucrose gradient (2 h at 5%, 2 h at 10%, and overnight at 20%). Af- terward, brain tissue was snap-frozen in liquid nitrogen and kept at ~80 °C until further processing. Ten-micrometer-thick cryosections were collected. Immunofluo- rescence staining was performed on brain slides using the following antibody combinations: a primary chicken anti-myelin basic protein (MBP) antibody (AB9348; 1:200 dilution); Millipore; a secondary donkey anti-chicken DyLight 549 anti- body (A21205; 1:200 dilution; Jackson ImmunoResearch) or with a secondary donkey anti-rabbit Alexa Fluor 555 (A31572, 1:1,000 dilution; Invitroge) and a secondary goat anti-rabbit Alexa Fluor 488 (A11008, 1:1,000 dilution; Invitrogen) or with a secondary donkey anti-rabbit Alexa Fluor 555 (A31572, 1:1,000 dilution; Invitrogen); and a primary rat anti-mouse CD40a PDGFR-α (clone APAS, 1:500 dilution; BD Pharmingen) with a secondary goat anti- rat 570 (1:50, 112-297-002; Jackson ImmunoResearch). Slides were counter- stained using Hoechst 33342 (H3570, 1:2,000 dilution; Invitrogen). For confocal microscopy and custom software written in Python. Quantitative analyses of immunofluorescence images were performed using NIH ImageJ (v1.46r). The levels of microglia/ macrophages (Iba-1), astrocytes (GFAP), OPCs (PDGFR-α), PKD1, and myelin (MBP) were determined based on the image-covering staining and expressed as percentage of the total area (39, 40). For confocal microscopy, images were acquired using a Zeiss LSM780 controlled by Zen software (Zeiss 2014). Only linear adjustments were made during image acquisition. Spectrophotometric Assay. A subset of mice were killed at each time point of interest ([W0 (CTRL), W4, W6, and W6 + W6 recovery]) and brains were rapidly dissected. The corpus callous was then isolated and snap-frozen and samples were stored at ~80 °C until further processing. The enzymatic activity of PDH was assessed using spectrophotometric assay kit (ab109902; Abcam), according to manufacturer’s guidelines and normalized to the concentration of protein de- termined by the Bradford method (n = 4–6 mice per group). The enzymatic activity of LDH-A (Vmax) was assessed as previously shown (73) (n = 4–6 mice per group).

Serum Lactate Levels. A subset of mice was anesthetized at each time point ([W0 (CTRL), W4, W6, and W6 + W6 recovery]) and blood was collected through the tail vein. Serum lactate levels were measured using an assay kit (%–lactate assay kit, colorimetric, ab65331; Abcam) (n = 4–6 mice per group).

Statistical Analysis. For all experiments sample sizes were determined based on previous experience of tumor-bearing mice imaging using [1-13C] MRSI of HP [1-13C]pyruvate at 14.1 T (32, 68). Based on this experience, we assumed that seven to eight animals per experimental group studied longitudinally would suffice to get significant HP data using a two-way repeated measure ANOVA test with P = 0.05. For immunohistochemistry and activity assays we de- termined that three to four animals per group would suffice to get signifi- cant data using a one-way ANOVA test with P = 0.05. With such group sizes, we would have 80–90% power to detect an effect size of 1.25.

Results are expressed as mean ± SD. Two-Way ANOVA was used to determine statistical significance of T2-w MR and HP [13C] MRSI between CPZ and CTRL groups and P values were corrected for multiple testing using the Tukey honest signifi- cance test. For HSD post hoc test was used (P < 0.05, **P < 0.01, ***P < 0.001). To compare nonlongitudinal data between W6 + W6 recovery and all other time points (W0, W4, and W6) unpaired t test was used (P < 0.05, **P < 0.01, ***P < 0.001). Additionally, a repeated measures ANOVA, with P values corrected for multiple testing using the Tukey HSD post hoc test, was used to evaluate a sta- tistical significance in the CTRL group (P < 0.05, **P < 0.01, ***P < 0.001). Statistical analyses of immunofluorescence, enzyme activity assays, and serum lactate levels between W0, W4, W6, and W6 + W6 recovery were performed using a one-way ANOVA, and the given P values were corrected for multiple testing using the Tukey HSD post hoc test (P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001). To evaluate statistical significance of immunofluorescence, enzyme activity assays and serum lactate levels between CTRL and W4 CPZ groups of CX3CR1<tg> mice we used an unpaired t test (P < 0.05, **P < 0.01, ***P < 0.001).

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Thind K, et al. (2014) Mapping metabolic changes associated with early radiation
17. Jha MK, Jeon S, Suk K (2012) Pyruvate dehydrogenase kinases in the nervous system:
their principal functions in neuronal-glial metabolism and neuro-metabolic
pyruvate dehydrogenase kinases in metabolic flexibility. Nutr Metab (Lond) 11:10.
magnetic resonance imaging and 1H-magnetic resonance spectroscopy study. J
Neural Sci 182:143–150.
fluid in inflamed plaques. Comparison with biochemical changes in demyelinating plaques.
degenerative dementias obtained by high resolution proton magnetic resonance
28. Chaumeil MM, et al. (2012) Hyperpolarized 13C MR spectroscopic imaging can be used
to monitor evolets treatment in vivo in an orthotopic rodent model of glioblastoma.
34. Gudi V, et al. (2009) Regional differences between grey and white matter in
35. Matsushima GK, Morell P (2001) The neurotoxicant, cuprizone, as a model to study