Effects of fasting on serial measurements of hyperpolarized [1-13C]pyruvate metabolism in tumors

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Imaging of the metabolism of hyperpolarized [1-13C]pyruvate has shown considerable promise in preclinical studies on oncology, particularly for the assessment of early treatment response. The repeatability of measurements of 13C label exchange between pyruvate and lactate was determined in a murine lymphoma model in fasted and non-fasted animals. The fasted state showed lower intra-individual variability, although the [1-13C]lactate/[1-13C]pyruvate signal ratio was significantly greater in fasted than in non-fasted mice, which may be explained by the higher tumor lactate concentrations in fasted animals. These results indicate that the fasted state may be preferable for the measurement of 13C label exchange between pyruvate and lactate, as it reduces the variability and therefore should make it easier to detect the effects of therapy. © 2016 The Authors. NMR in Biomedicine published by John Wiley & Sons Ltd.

Keywords: repeatability; hyperpolarization; fasting; pyruvate; lymphoma; cancer

INTRODUCTION

The introduction of hyperpolarized 13C-labeled cell substrates has created a renewed interest in the field of MRS by allowing the real-time assessment of tissue metabolism in vivo (1). [1-13C]Pyruvate ([1-13C]Pyr) is the most well-studied substrate, having shown promise in preclinical studies in oncology, particularly as an imaging marker for the detection of early response to therapy and as a possible marker for tumor grading and early lesion detection (2–5). Pyr has also translated to the clinic, with a study in prostate cancer (6). As the end product of glycolysis, Pyr can be reduced reversibly by NADH to generate lactate (Lac), in a reaction catalyzed by lactate dehydrogenase (LDH), or transaminated by glutamate to form alanine, in a reaction catalyzed by alanine aminotransferase (ALT). Irreversible decarboxylation of Pyr to carbon dioxide, in a reaction catalyzed by mitochondrial pyruvate dehydrogenase (PDH), may also occur and, in some tissues, is sufficiently fast to give observable labeling of carbon dioxide and bicarbonate with a hyperpolarized 13C label (7). Data from hyperpolarized 13C MRSI measurements are often presented as either signal ratios (8) or apparent exchange rates when the metabolite signals are analyzed as a function of time. Both measurements are affected by Pyr delivery to the tumor, tumor cell uptake and subsequent enzyme-catalyzed exchange (2,9–12).

Despite extensive preclinical use of hyperpolarized [1-13C]Pyr, with promising future clinical applications in oncology (2,13,14), notably in the serial assessment of treatment response, there is a lack of information regarding the repeatability of these measurements. The determination of repeatability is important for an understanding of whether the observed changes in kinetics of hyperpolarized [1-13C]Pyr conversion to Lac reflect real
effects of therapy or, rather, changes in physiology and/or method variability.

18F-Fluorodeoxyglucose positron emission tomography (18F-FDG-PET), similarly to hyperpolarized [1-13C]Pyr, also assesses an aspect of glycolysis. Several studies have evaluated the repeatability of 18F-FDG-PET, as well as the main factors underlying the observed variability. A major factor affecting 18F-FDG uptake is high blood glucose levels (15), as a result of competition between glucose and 18F-FDG for transport into the cell and subsequent phosphorylation. Therefore, it is recommended that, for 18F-FDG-PET examinations, there is some control over blood glucose levels, either by overnight fasting (16) or by the measurement of blood glucose levels at the time of injection (17).

We have investigated here whether the variability and repeatability of measurements with hyperpolarized [1-13C]Pyr are also affected by fasting.

**MATERIALS AND METHODS**

**Tumor implantation and animal preparation**

Tumors were established by subcutaneous inoculation of a suspension of 5 × 10^6 EL4 murine lymphoma cells (in a volume of 100 μL) in the left flank of 8–10-week-old C57/Blk6 female mice. Tumors were allowed to grow for 10 days (volume, ~2 cm³; maximum diameter, 1.5 cm), after which the mice were divided into two cohorts: fasted and non-fasted. Fasted mice were deprived of food for 18 h prior to the first 13C spectroscopic examination. Experiments were conducted in accordance with project and personal licenses issued under the United Kingdom Animals (Scientific Procedures) Act, 1986. Protocols were approved by the Cancer Research UK, Cambridge Institute Animal Welfare and Ethical Review Body.

**MRI/MRS protocols**

Tumor-bearing mice were anesthetized initially by inhalation of a mixture of O₂ (0.3 L/min) and air (0.9 L/min) containing 3% isoflurane (Isoflo, Abbotts Laboratories Ltd, Maidenhead, UK) and were maintained during the MRS experiment with 1–2% isoflurane in O₂ and air, which was delivered via a facemask. Animals were taped into a cradle to minimize breathing-related motion, and placed in a heated MR probe, which maintained the core body temperature at ~37 °C (monitored by a rectal probe). Respiratory rate and body temperature were monitored during the experiment using a BioTRIG physiological monitor (Small Animal Instruments, Stony Brook, NY, USA). A cannula was inserted into a tail vein and its patency was maintained through the use of heparin diluted in sterile physiological saline (100 U/mL). A 20-mm-diameter curved surface coil (Rapid Biomedical GmbH, Rimpar, Germany) was placed over the tumor. The entire assembly was placed in a 13C/1H volume coil (Rapid Biomedical GmbH) in a 7-T horizontal bore magnet (Agilent, Palo Alto, CA, USA). The tumor was localized using transverse 1H images acquired using a spin-echo pulse sequence (TR = 1.5 s; TE = 10 ms; field of view, 40 mm × 40 mm; data matrix, 128 × 128; slice thickness, 2 mm; 15 slices).

| Table 1. Mean coefficient of variation of k_P and the hyperpolarized [1-13C]lactate/[1-13C]pyruvate signal ratio in fasted and non-fasted tumor-bearing mice following repeated measurements |
|-----------------|-------------------|-----------------|-----------------|-----------------|
| HP [1-13C]pyruvate | (95% CI) | Mean | SD | COV (%) |
|-----------------|-------------------|-----------------|-----------------|-----------------|
| Fasted k_P      | 0.89 (0.60–0.97)  | 0.141           | 0.016           | 11.773*         |
| Lac/Pyr         | 0.83 (0.44–0.95)  | 5.671           | 0.942           | 14.998          |
| Non-fasted k_P  | 0.62 (0.03–0.89)  | 0.1             | 0.021           | 24.767          |
| Lac/Pyr         | 0.47 (−0.14–0.82) | 3.563           | 0.934           | 29.499          |
| Fasted 1st measurement | 0.137 ± 0.05 | 5.49 ± 2.23** |
| 2nd measurement | 0.138 ± 0.06 | 5.41 ± 3.04 |
| Non-fasted 1st measurement | 0.108 ± 0.04 | 3.67 ± 1.7 |
| 2nd measurement | 0.107 ± 0.03 | 3.89 ± 1.25 |

CCC, concordance correlation coefficient; CI, confidence interval; COV, coefficient of variation; HP, hyperpolarized; k_P, apparent exchange rate constant of conversion of pyruvate to lactate; Lac, lactate; Pyr, pyruvate; SD, standard deviation.

k_P and [1-13C]Lac/[1-13C]Pyr signal ratios were calculated from the time courses of 13C labeling in lactate and pyruvate in tumors in fasted and non-fasted mice, acquired 4 h apart. The mean, SD, intra-individual COV and CCC were calculated from 16 measurements for both the fasted and non-fasted animals.

*p = 0.01, significantly different from non-fasted animals

**p = 0.02, significantly different from non-fasted animals**
Pyr was hyperpolarized as described previously (1). Briefly, [1-13C]pyruvic acid samples (44 mg, 14 mol/L; 91% 13C), containing 15 mmol/L of trityl radical, tris(8-carboxy-2,2,6,6-tetra-(hydroxyethyl)-benzo-(1–5)-bis-(1,3)-dithiole-4-y)-methyl sodium salt (OX063; GE Healthcare, Amersham, Buckinghamshire, UK) and 1.5 mmol/L gadolinium chelate (Dotarem), were polarized using a microwave source at 93.982 GHz and 100 mW for 1 h in a 3.35-T Hypersense (Oxford Instruments Molecular Biotools Ltd., Abingdon, Oxfordshire, UK) polarizer. The frozen sample was then dissolved in a solution containing 40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 94 mM NaOH, 30 mM NaCl and 50 mg/L ethylenediaminetetraacetic acid (EDTA) at ~180 °C and ~1 MPa. Polarization levels ranged from 16% to 25%, measured using a bench-top polarimeter (13C-MQC polarimeter, Oxford Instruments Molecular Biotools Ltd., Abingdon, Oxfordshire, UK).

NMR spectroscopy of tumors

From the total number of animals (n = 30), paired data were only obtained from 16 animals, as the remainder showed a poor signal-to-noise ratio in the 13C spectra from one of the examinations, reflecting the difficulty in obtaining good sequential tail vein injections over such a relatively short period of time. Paired 13C spectroscopic data, acquired 4 h apart, were obtained from eight fasted and eight non-fasted animals (Table 1). The mice were examined on the same day as the tumor model showed rapid growth (18,19), which could alter the metabolic status of the tumor if the animals were examined on subsequent days. After the first examination, mice were recovered from anesthesia and food was provided only to the non-fasted group (Fig. 1).

Following intravenous injection of hyperpolarized [1-13C]Pyr (10 mL/Kg, 82 mmol), single transient spectra from a 6-mm-thick slice through the tumor were acquired using a slice-selective excitation pulse with a nominal flip angle of 10°. One hundred and twenty 13C spectra were acquired every 2 s beginning 10 s after injection. The processing of 13C spectroscopic data was performed in Matlab (Mathworks, Massachusetts, USA). The integrated peak intensities of hyperpolarized [1-13C]Pyr and [1-13C] Lac were fitted to the modified Bloch equation for two-site exchange to calculate the rate constants $k_P$ and $k_L$ and the apparent spin–lattice relaxation rates, as described previously (2).

$$\frac{dL_z}{dt} = -R_{1,L}(L_z - L_w) + k_P P_z - k_L L_z$$  

$$\frac{dP_z}{dt} = -R_{1,P}(P_z - P_w) + k_L L_z - k_P P_z$$

where $L_z$ and $P_z$ are the z magnetizations of the 13C nucleus in the Lac and Pyr carboxyl carbons, respectively, $R_{1,L}$ and $R_{1,P}$ are the spin–lattice relaxation rates (1/T1), and $L_w$ and $P_w$ are the equilibrium magnetizations of Lac and Pyr, respectively, which are effectively equivalent to their concentrations. The relaxation rates for Lac and Pyr were assumed to be the same. We have shown previously that this assumption has little effect on the fitted rate constants (see supplementary fig. 2 in ref. 2). $L_w/P_w$ was obtained from the ratio of the fitted rate constants. The 13C-labeled Lac/Pyr ratios were calculated from $L_w/P_z$ at 30 s.

Dynamic contrast-enhanced MRI (DCE-MRI)

Separate cohorts of mice, fasted (n = 7) and non-fasted (n = 7), were used for DCE-MRI measurements of tumor perfusion, in which the mice were injected with Gd3+-DTPA (diethylenediaminetetraacetic acid; 200 mmol/kg; Magnevist, Bayer Schering Pharma, Leverkusen, Germany) (Table 2). The imaging protocol simulated the protocol used for the 13C hyperpolarized measurements. Briefly, for each cohort (seven fasted, seven non-fasted), four mice were injected with contrast agent (1st scan) and three mice were injected with unlabeled Pyr (82 mm, 10 mL/kg), followed by injection of contrast agent 4 h later (2nd scan) (Fig. 1). Before each injection, animals were submitted to at least 30 min of anesthesia, which is the same time for which the animals were anesthetized for the HP [1-13C]Pyr studies.

DCE-MRI data were acquired using a T1-weighted spin-echo pulse sequence, as described previously (12). Briefly, first an inversion recovery fast low-angle shot (FLASH) pulse sequence was used to measure the native spin–lattice relaxation rates ($R_1 = 1/T_1$). These inversion recovery data were fitted, pixel-by-pixel, to a mono-exponential function to obtain a pre-contrast $R_1$ map. A dynamic T1-weighted spin-echo pulse sequence was then used to collect baseline images (6 or 10) prior to injection of contrast agent. Diluted Gd3+-DTPA in sterile saline (0.9% histological examination of the tumors (20).
Figure 2. Repeatability of $k_p$ and $[1^{-13}C]$lactate/$[1^{-13}C]$pyruvate ($[1^{-13}C]$Lac/$[1^{-13}C]$Pyr) signal ratio measurements in fasted mice (a–d) and non-fasted mice (e–h) (see Fig. 1). Measurements in a single animal from the 1st scan (measurement 1) are plotted against measurements from the same animal from the 2nd scan (measurement 2). The full line indicates the best-fit line between these two measurements (a & c, e & g). The broken lines indicate the 95% confidence limits of the best-fit line. The dotted lines represent perfect agreement between the two measurements. For each mouse, the differences in $k_p$ between the 1st and 2nd scans are plotted against the average of $k_p$ from the 1st and 2nd scans (b & f). This Bland–Altman (BA) plot (23) was used to test the assumption of constant variances across the differences in $k_p$. The full line at 0.00 is that of perfect agreement between the measurements of $k_p$ from the 1st and 2nd scans, and the full line above is the mean difference of $k_p$ between the 1st and 2nd scans. The broken lines indicate the 95% confidence limits of agreement. The dotted lines represent the 95% confidence intervals for these limits of agreement. The BA plots for the $[1^{-13}C]$Lac/$[1^{-13}C]$Pyr ratios are shown in (d & h).
sodium chloride) was then injected as a bolus through a tail vein catheter over 2–3 s and dynamic T1-weighted spin-echo images were acquired for 10 min post-injection. These images were then converted to $R_1$ relaxation rate maps, as described previously (12). Gd³⁺–DTPA concentration curves were determined for all tumor-containing slices with a manually delineated region of interest (ROI) in the tumor and also in adjacent thigh muscle. The area under the uptake curve up to 45 s (AUC 45) was calculated for each ROI.

### Blood and tissue collection and analysis

In a separate set of experiments (Table 3), tumor-bearing mice ($n = 24$) were divided into two groups: fasted ($n = 12$) and non-fasted ($n = 12$). In each group, the mice were further divided into three subgroups: Group 0, anesthetized for 30 min; Group 1, 30 min of anesthesia plus a bolus injection of 10 mL/kg of 82 mM unlabeled Pyr; Group 2, same as Group 1, plus a 4-h recovery period, a second period of anesthesia for 30 min and another injection of 10 mL/kg of 82 mM unlabeled Pyr (Fig. 1). The timing of anesthesia and Pyr injection was the same as for the ³¹C NMR data acquisition protocol (Fig. 1). Blood was collected rapidly by cardiac puncture in all mice from each group (starting at 25 s after Pyr injection) and the tumors were quickly excised and freeze-clamped 30 s after Pyr injection.

Blood samples were centrifuged (18 800 g) at 4°C for 5 min in heparin-coated Eppendorf tubes (~5 μL of 1000 U/mL). Pyr was analyzed using a colorimetric assay kit (AbCam, Cambridge, UK) and Lac by an enzymatic assay kit (Siemens Healthcare, Sudbury, UK), using a Siemens Dimension RxL analyzer. Perchloric acid extracts were prepared from the tumor tissues of Groups 1 and 2 for both fasted ($n = 6$) and non-fasted ($n = 6$) mice using ice-cold 7% perchloric acid (1:8 w/v), which were then neutralized with KOH, lyophilized and dissolved in 99.9% deuterium oxide. High-resolution H NMR spectra of plasma and tumor extracts were obtained at 14.1 T (25°C, pH 7.2) in a Bruker 600-MHz NMR spectrometer (Bruker, Ettlingen, Germany) using a 5-mm probe. The acquisition conditions were as follows: 90° pulses; spectral width, 7.3 kHz; acquisition time, 4.5 s; 32 k data points; 64 transients; recycling time, 12.5 s. Chemical shifts were referenced to 3-(trimethylsilyl)-2,2′,3,3′-tetradeuteropropionic acid (TSP) at 0.0 ppm, which was added to the sample at a concentration of 5 mM. Data were analyzed using ACDSpecManager (ACD/Labs, Bracknell, UK). The free induction decays were zero-filled twice and multiplied by an exponential function prior to Fourier transformation. Peak integrals were normalized to the integral of the TSP resonance.

#### Table 2. Measurements of tumor perfusion using dynamic contrast agent-enhanced H MRI

|                  | Fasted 1st scan | Fasted 2nd scan | Non-fasted 1st scan | Non-fasted 2nd scan |
|------------------|----------------|----------------|---------------------|--------------------|
| AUC (tumor/muscle)| 6.8 ± 2.8      | 9.7 ± 4.7      | 12.7 ± 5.6          | 5.9 ± 3.0          |

Tumor/muscle (thigh) ratio for the area under the contrast agent concentration curve (AUC) at 45 s after injection of contrast agent for fasted and non-fasted EL4 tumor-bearing mice. Acquisitions were performed at the same time points as for the hyperpolarized [¹³C]pyruvate study, with mice from Group 2 having received a dose of unlabeled non-polarized pyruvate (10 mL/kg, 82 mM) and 30 min of anesthesia 4 h before the contrast agent-enhanced H images were acquired. Mean ± standard error of the mean.

### Statistical analysis

Data were analyzed using SPSS (v21, IBM SPSS, Chicago, IL, USA) and GraphPad Prism v6 (GraphPad Software, San Diego, USA). The repeatability of measurements of $k_P$ and the [¹³C]Lac/[¹³C]Pyr signal ratio, which was measured at 30 s after injection, were assessed from the two imaging examinations performed 4 h apart. Scatter plots (Measurement 1 versus Measurement 2) and Bland–Altman plots (difference versus mean) were generated. The concordance correlation coefficient (CCC), within-subject (intra-individual) standard deviation (SD) and coefficient of variation (COV) were calculated as described in refs. (20,21). The COVs of $k_P$ and the [¹³C]Lac/[¹³C]Pyr signal ratio were calculated, for each parameter, as the SD divided by the parameter mean, and expressed as a percentage. This was calculated for each individual and then averaged. COV can be used as a statistical measure of reliability (22) and has been used previously to assess reliability in FDG-PET studies (21). Data were reported as mean ± SD, unless stated otherwise. Statistical significance was tested with Prism using a two-tailed Student’s t-test or analysis of variance (ANOVA) (post-hoc test: Tukey) when appropriate. Paired t-tests were used when comparing paired data from the same mouse. The results were considered to be significant when $p < 0.05$.

### RESULTS

#### Repeatability of ¹³C MRS measurements with hyperpolarized [¹³C]Pyr

Intravenous injection of hyperpolarized [¹³C]Pyr (10 mL/kg, 82 mM) resulted in tumor signals from [¹³C]Lac and [¹³C]Pyr that were similar in intensity to those observed previously in this tumor model (2). The apparent rate constant describing the conversion of Pyr to Lac ($k_P$) and the [¹³C]Lac/[¹³C]Pyr signal ratios at 30 s were calculated. Previous studies in this tumor model have shown that there is substantial labeling of both the Pyr and Lac pools at 30 s after Pyr injection (1). The repeatability of measurements made 4 h apart is shown in Fig. 2 and Table 1. Non-fasted animals showed poorer correlations and higher mean differences between successive measurements than fasted animals. Repeatability was also assessed by calculating the COV for the mean $k_P$ and mean [¹³C]Lac/[¹³C]Pyr signal ratio using data from both time points, i.e. a total of 16 measurements for each group of animals (Table 1). Animals in the fasted state showed lower intra-individual variability than those in the non-fasted state, with significantly lower COV for the mean $k_P$ ($p = 0.01$). The higher metabolic rate of mice versus

|                  | Fasted 1st scan | Fast (fasted) | Non-fasted 1st scan | Non-fasted 2nd scan |
|------------------|----------------|--------------|---------------------|--------------------|
| AUC (tumor/muscle)| 6.8 ± 2.8      | 9.7 ± 4.7    | 12.7 ± 5.6          | 5.9 ± 3.0          |

Tumor/muscle (thigh) ratio for the area under the contrast agent concentration curve (AUC) at 45 s after injection of contrast agent for fasted and non-fasted EL4 tumor-bearing mice. Acquisitions were performed at the same time points as for the hyperpolarized [¹³C]pyruvate study, with mice from Group 2 having received a dose of unlabeled non-polarized pyruvate (10 mL/kg, 82 mM) and 30 min of anesthesia 4 h before the contrast agent-enhanced H images were acquired. Mean ± standard error of the mean.
Table 3. Blood and tumor metabolite concentrations and excised tumor and mouse weights in fasted and non-fasted mice

| Sample                  | Metabolite          | Fasted (baseline) | Fasted 1 | Fasted 2 | Non-fasted (baseline) | Non-fasted 1 | Non-fasted 2 |
|-------------------------|---------------------|------------------|----------|----------|-----------------------|--------------|--------------|
| Tumor concentration     | Lactate (μmol/g wet tissue) | 19.1 ± 2.7 (n = 3)*a | 15.5 ± 2.0 (n = 3) | 10.8 ± 4.1 (n = 3) | 15.8 ± 5.9 (n = 3) | 10.8 ± 4.1 (n = 3) | 15.8 ± 5.9 (n = 3) |
| Tumor concentration     | Alanine (μmol/g wet tissue) | 3.9 ± 1.4 (n = 3) | 2.9 ± 1.1 (n = 3) | 2.6 ± 0.9 (n = 3) | 5.3 ± 1.5 (n = 3) | 2.6 ± 0.9 (n = 3) | 5.3 ± 1.5 (n = 3) |
| Blood concentration     | Lactate (mmol/L)     | 10.1 ± 2.6 (n = 3) | 13.0 ± 1.8 (n = 4) | 8.7 ± 0.8 (n = 5) | 11.9 ± 1.3 (n = 4) | 8.7 ± 0.8 (n = 5) | 11.9 ± 1.3 (n = 4) |
| Blood concentration     | Pyruvate (μmol/L)    | 193 ± 35 (n = 3)*b | 183 ± 30 (n = 4) | 110 ± 14 (n = 5) | 135 ± 29 (n = 4) | 110 ± 14 (n = 5) | 135 ± 29 (n = 4) |
| Mouse weight (g)        |                     | 23.7 ± 1.7 (n = 3) | 20.3 ± 0.8 (n = 4) | 25.3 ± 0.6 (n = 4) | 24.4 ± 1.7 (n = 4) | 25.3 ± 0.6 (n = 4) | 24.4 ± 1.7 (n = 4) |
| Tumor weight (g)        |                     | 0.8 ± 0.5 (n = 3) | 0.9 ± 0.6 (n = 3) | 0.7 ± 0.1 (n = 3) | 1.3 ± 0.3 (n = 3) | 0.7 ± 0.1 (n = 3) | 1.3 ± 0.3 (n = 3) |

Blood metabolite concentrations were determined using spectrophotometric assays, and tumor alanine and lactate concentrations were measured by 1H NMR. Group 0 (baseline), anesthetized for 30 min; Group 1, 30 min of anesthesia plus a bolus injection of 10 mL/kg of 82 mM unlabeled pyruvate; Group 2, same as Group 1, plus a 4-h recovery period, a second period of anesthesia for 30 min and another injection of 10 mL/kg of 82 mM unlabeled pyruvate. Mean ± standard deviation; *p < 0.05.

a,b Significantly different from non-fasted 1.
Significantly different from fasted baseline.
Significantly different from fasted 2.
(Table 3). This is consistent with previous in vitro and in vivo studies (2,25,27), which have shown that the exchange rate increases with an increase in Lac concentration. There was no evidence for a difference in tumor perfusion between fasted and non-fasted animals or between the first and second scans in these animals (Table 2), although the error in these measurements was large. Therefore, the higher tumor Lac levels in fasted mice implies that these may have been caused by an increase in tumor glycolysis. This has been observed previously in tumors of fasting animals, where the tumor preferentially consumes glucose in comparison with other tissues and produces increased amounts of Lac (28). The fasted animals also showed higher blood Pyr levels after the first injection of [1-13C]Pyr. This may be explained by increased gluconeogenesis, which results in increased levels of blood Pyr and alanine (29,30), and occurs independently of the presence of a tumor (31,32).

The variability of hyperpolarized [1-13C]Pyr metabolism may depend on the organ and/or type and grade of tumor being assessed. Hyperpolarized [1-13C]Pyr was found to give reproducible results in the assessment of normal kidneys of non-fasted rats; however, the fasted state was not examined in this study (33).

In conclusion, if hyperpolarized [1-13C]Pyr is to be used in the clinic, especially for monitoring response to therapy or disease progression using serial measurements, a knowledge of repeatability is essential. The results presented here suggest that, similar to what is already performed in some clinical centers for 18F-FDG-PET examinations (16,17,34), fasting prior to the MR examination might be preferred because of the more uniform and constant metabolic status and lower intra-subject variability.

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Supporting information

Additional supporting information can be found in the online version of this article at the publisher’s website.

The raw data acquired during this study and on which the results presented in this paper are based can be found at http://content.cruk.cam.ac.uk/kblab/nbm3568.zip.