

PDF hosted at the Radboud Repository of the Radboud University Nijmegen

The following full text is a publisher's version.

For additional information about this publication click this link.

<http://hdl.handle.net/2066/167953>

Please be advised that this information was generated on 2019-02-18 and may be subject to change.

Monitoring creatine and phosphocreatine by ^{13}C MR spectroscopic imaging during and after ^{13}C creatine loading: a feasibility study

Barbara H. Janssen¹ · Saskia Lassche² · Maria T. Hopman³ · Ron A. Wevers⁴ · Baziel G. M. van Engelen² · Arend Heerschap¹

Received: 15 February 2016 / Accepted: 1 July 2016 / Published online: 11 July 2016
© The Author(s) 2016. This article is published with open access at Springerlink.com

Abstract Creatine (Cr) supplementation to enhance muscle performance shows variable responses among individuals and different muscles. Direct monitoring of the supplied Cr in muscles would address these differences. In this feasibility study, we introduce in vivo 3D ^{13}C MR spectroscopic imaging (MRSI) of the leg with oral ingestion of ^{13}C –creatine to observe simultaneously Cr and phosphocreatine (PCr) for assessing Cr uptake, turnover, and the ratio PCr over total Cr (TCr) in individual muscles. ^{13}C MRSI was performed of five muscles in the posterior thigh in seven subjects (two males and two females of ~20 years, one 82-year-old male, and two neuromuscular patients) with a $^1\text{H}/^{13}\text{C}$ coil in a 3T MR system before, during and after intake of 15 % ^{13}C –enriched Cr. Subjects ingested 20 g Cr/day for 4 days in four 5 g doses at equal time intervals. The PCr/TCr did not vary significantly during supplementation and was similar for all subjects and investigated muscles (average 0.71 ± 0.07), except for the adductor magnus (0.64 ± 0.03). The average Cr turnover rate, assessed in male muscles, was 2.1 ± 0.7 %/day. The linear uptake rates of Cr were variable between muscles, although not

significantly different. This assessment was possible in all investigated muscles of young male volunteers, but less so in muscles of the other subjects due to lower signal-to-noise ratio. Improvements for future studies are discussed. In vivo ^{13}C MRSI after ^{13}C –Cr ingestion is demonstrated for longitudinal studies of Cr uptake, turnover, and PCr/TCr ratios of individual muscles in one exam.

Keywords Creatine · MR spectroscopy · ^{13}C · Human muscle

Introduction

Creatine (Cr) occurs in fish and meat and is endogenously produced in the liver and kidneys (Guthmiller et al. 1994). It is taken up from blood vessels into tissues with high energy demands such as muscle and brain (Wyss and Kadurah-Daouk 2000). This uptake is facilitated by a creatine transporter protein (CrT), which transports the compound together with sodium into the cell against a Cr concentration gradient (Guerrero-Ontiveros and Wallimann 1998). Inside the cell, Cr is reversibly phosphorylated to phosphocreatine (PCr) in the creatine kinase (CK) reaction. This Cr–PCr system plays a key role in energy metabolism as a temporal energy buffer and by facilitating substrate diffusion (Meyer et al. 1984). A spontaneous reaction irreversibly degrades both Cr and PCr into creatinine, which is excreted via the kidneys (Wallimann et al. 2011; Hoberman et al. 1948). Because of low PCr levels in some dystrophic muscles (Kemp et al. 1993) and potential cellular protective effects (Pulido et al. 1998), Cr supplementation has been proposed as treatment for several neuromuscular disorders (Kley et al. 2013). Cr is also recognized as an effective nutritional supplement in other diseases and in sports

Handling Editor: T. Wallimann and R. Harris.

✉ Barbara H. Janssen
Barbara.Janssen@radboudumc.nl

¹ Department of Radiology, Radboud University Medical Center, Nijmegen, The Netherlands

² Department of Neurology, Radboud University Medical Center, Nijmegen, The Netherlands

³ Department of Physiology, Radboud University Medical Center, Nijmegen, The Netherlands

⁴ Department of Laboratory Medicine, Radboud University Medical Center, Nijmegen, The Netherlands

(Wyss and Schulze 2002; Gualano et al. 2012; Kley et al. 2013).

However, selection of an effective Cr loading protocol is hampered by a wide variation in responsiveness between individuals (Harris et al. 1992; Greenhaff et al. 1994). This may be due to various factors determining Cr uptake, such as intracellular Cr concentration, hormone status, and fiber type composition (Mesa et al. 2002; Snow and Murphy 2003). Moreover, the contribution of each factor may vary between different muscles and may change with age or due to disease (Coggan et al. 1992; Kreis et al. 1999; Dubowitz and Sewry 2007; McCall and Persky 2007). Therefore, an in vivo method to directly monitor the supplied Cr in individual muscles would be desirable to help to understand and optimize Cr loading.

Magnetic resonance spectroscopy (MRS) has the unique capability to measure Cr and PCr levels in human tissues non-invasively. With proton (^1H) MRS, the total amount of Cr (TCr) in muscles can be observed, and with phosphorus (^{31}P) MRS, it is possible to monitor PCr (De Graaf 2007). Both ^1H and ^{31}P MRS have been used in studies of TCr and PCr levels in skeletal muscle after Cr supplementation and revealed differential Cr increases ranging from about 5 % in the gastrocnemius to about 23 % in the quadriceps femoris (Vandenbergh et al. 1997; Kreis et al. 1999; Smith et al. 1998, 1999). To examine these two compounds individually in one exam are complicated as different hardware is required for each nucleus and quantitative sequential measurements are hampered by the required calibrations (Brault et al. 2007). By carbon (^{13}C) MRS, it is possible to observe separate signals for Cr and PCr simultaneously in skeletal muscle spectra, but because of the low natural abundance of ^{13}C in the body, this is not a very sensitive method. However, with oral ingestion of ^{13}C -4 labeled Cr, the signal-to-noise ratio can be increased. This was used to establish the PCr/TCr ratio and Cr turnover in the calf of a single individual only employing a surface coil adjacent to the calf for localization, mainly acquiring signal from the gastrocnemius (Kan et al. 2006). Multiple spatially resolved MR spectra can be acquired with MR spectroscopic imaging (MRSI) and, therefore, allow the detection of metabolites in individual muscles (Vermathen et al. 2012). Human muscles have not been assessed yet by ^{13}C MRSI to monitor compounds labeled with ^{13}C spins at thermal equilibrium.

The aim of the work described in this paper was to investigate the performance of three-dimensional ^{13}C MRSI at 3T of the posterior thigh during and after the ingestion of ^{13}C -4 labeled Cr. In particular, we wanted to know for different muscles what the PCr/TCr ratio is and how feasible it is to measure Cr turnover and uptake, all in one examination. To test this feasibility of the method for clinical research, we examined a variety of seven volunteers

representing potentially different leg muscle conditions, including two patients suffering from facioscapulohumeral muscular dystrophy (FSHD).

Materials and methods

Subjects

For this feasibility study, we included four healthy young volunteers (YH), one 82-year-old male (OH), and two genetically confirmed FSHD patients. We made sure that participants had no MR contraindication (pacemaker, metal implants or claustrophobia). None of the subjects performed sports at a professional level, were vegetarian, or were taking Cr as supplement.

Participants' demographics are listed in Table 1. Ethical approval was obtained through the institutional review board, and written informed consent was obtained from all participants.

Study design

Participants ingested 20 g Cr per day for 4 days in four 5 g doses (15 % ^{13}C enriched at the guanidino carbon, ^{13}C -4-Cr) at equally spaced intervals through the day. They were instructed to dilute the Cr in a glass of lukewarm water and to drink it along with 250 ml of standard carbohydrate solution to augment skeletal muscle Cr accumulation (Green et al. 1996).

After the first MR measurement on day 0, the participant was provided with 16 portions of the ^{13}C -enriched Cr, and was instructed to start the Cr supplementation the next day.

The full study design consisted of 12 MR measurements at day 0, 1, 2, 3, 4, 5, 7, 12 ± 1 , 14 ± 1 , 19 ± 2 , 41 ± 3 , 78 ± 3 , and 105 ± 3 . Day 0 was the baseline measurement, days 1–4 were during Cr supplementation, and the remaining measurements were after Cr supplementation. The measurement protocol was reduced to six MR exams

Table 1 Participants demographics

| | Age (years) | BMI (kg/m ²) | Weight (kg) | Gender |
|------------------|-------------|--------------------------|-------------|--------|
| Young healthy #1 | 23 | 20.94 | 57 | Female |
| Young healthy #2 | 21 | 20.62 | 58 | Male |
| Young healthy #3 | 21 | 22.06 | 63 | Female |
| Young healthy #4 | 21 | 21.90 | 85 | Male |
| Old healthy | 82 | 30.67 | 95 | Male |
| FSHD #1 | 72 | 33.46 | 90 | Female |
| FSHD #2 | 41 | 24.68 | 73 | Male |

Fat fractions were similar between young healthy man and women (1.2 ± 0.3 % vs. 2.8 ± 0.8 , $p = 0.08$ two-tailed t test)

for the older and FSHD subjects; one before Cr supplementation (day 0), two during (days 2 and 5), and three after (days 19, 41, and 78) Cr supplementation.

Blood and urine samples were taken on days 0, 3, and 7 to monitor kidney function during Cr supplementation. Venous blood was drawn directly into a $\text{K}_2\text{-EDTA}$ vacuum tube. Tubes were centrifuged at 3000 revolutions per minute for 10 min, and subsequently, the plasma was separated from the packed red blood cells and was stored at $-80\text{ }^\circ\text{C}$ until analysis. Cr and ^{13}C -4-Cr concentrations were determined with mass spectroscopy.

MR protocol

MR exams were performed on a clinical 3Tesla MRI system (Tim Trio, Siemens, Erlangen, Germany) with a ^1H birdcage volume coil (diameter 25 cm) and a circularly polarized ^{13}C half-volume coil ($13 \times 15\text{ cm}$) inserted into the ^1H coil. A fish oil capsule served as a matching landmark between the MR exams and was positioned at one-third the distance between the anterior superior iliac spine and the patella. Subjects were placed feet first supine inside the magnet bore, and the coil was placed around the right upper leg. After scout imaging, the protocol consisted of T2 multi-spin-echo imaging (T2-MRI; Kan et al. 2009) (field of view $175 \times 175\text{ mm}$, voxel size $0.68 \times 0.68\text{ mm}$ repetition time (TR) 3000 ms, 16 equally spaced echo times (TE) 7.7–123.2 ms, 8 slices, slice thickness/gap 6/9 mm).

Followed by 3D, ^{13}C MRSI with an adiabatic BIR45 excitation pulse centered at 157 ppm, which is between the resonance frequencies of Cr and PCr. The FOV was $200 \times 200 \times 400\text{ mm}$, and the matrix size was $10 \times 10 \times 8$, resulting in nominal voxels of $20 \times 20 \times 50\text{ mm}$. Furthermore, Hamming-weighted k-space acquisition was applied with TR 1000 ms, bandwidth 10 kHz, vector size 1024, and 30 averages with WALTZ16 proton decoupling during acquisition.

In one subject, a T_1 measurement was performed at day 10, by acquiring ^{13}C free induction decays (with adiabatic excitation and 150 averages) at different repetition times (TR = 0.75, 1, 2, 3, and 8 s).

Because of the ^{13}C coil sensitivity profile only spectra from the posterior thigh muscles, more specifically, the biceps femoris (BF), semimembranosus (SM), semitendinosus (ST), adductor magnus (AM), and the gracilis (G) were collected (Fig. 1). The entire MR examination took about 1 h, including subject placement. All MRSI data sets were saved as raw files for off line processing.

Data analyses

Muscle specific fat-fractions were determined by fitting the signal of the multi-spin-echo images to a biexponential

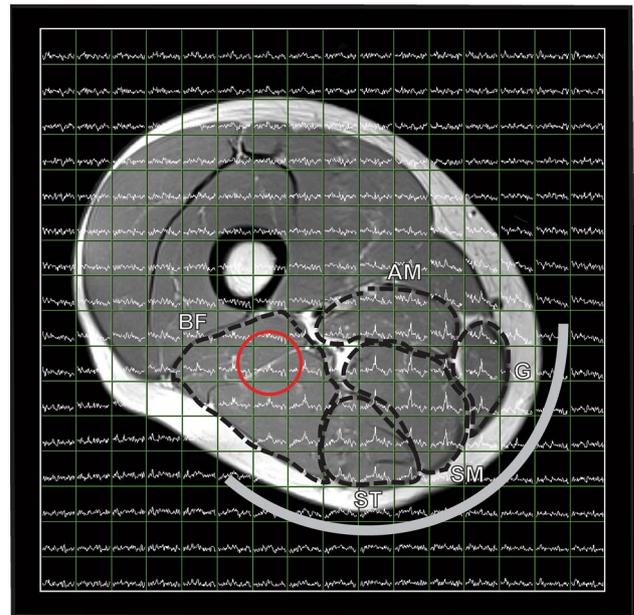


Fig. 1 ^{13}C MRSI grid with voxels overlaid on T2-weighted MR image of the right thigh of a healthy young male after 4 days of creatine supplementation. Because of the k space sampling, the true voxel shape is more a sphere, which is approached in the image by a red circle. The ^{13}C half-volume coil, positioned adjacent to the hamstring muscles, is indicated. The spectra are zoomed on the creatine signals. The muscles that are in the field of view of the coil include the biceps femoris (BF), semi-tendinosus (ST), semi-membranosus (SM), gracilis (G), and adductor magnus (AM), as delineated in the image (color figure online)

function with fixed relaxation times for fat and water as described previously (Kan et al. 2009), classifying severe fatty infiltration as a fat fraction above 80 % and minor as below 20 %.

For the ^{13}C MRSI data, the matrix dimension was zero-filled to $16 \times 16 \times 8$. A spatial 100 % Hamming filter was applied to the ^{13}C MRSI k-space data before voxel selection. From every slice in the field of view of the ^{13}C coil, one voxel was selected per muscle using the program 3DiCSI (Zhao et al. 2005). To position the voxel in the center of the muscle, grid-shifting was applied. Only voxels were selected that were completely lying within one muscle, resulting in three voxels per muscle on average. The corresponding free induction decays (FID) were exported to jMRUI 4.0 for spectral fitting (Naressi et al. 2001).

Spectral fitting

Voxels from the same muscle were summed, subsequently, unwanted lipid resonances were removed from the ^{13}C spectra with an HLSVD filter (Pijnappel et al. 1992), and the spectra were apodized by 10 Hz. From unlocalized high SNR MR spectra, a line width ratio of 0.81 and a

frequency difference of 30 Hz between Cr (157.5 ppm) and PCr (156.6 ppm) were obtained as prior knowledge that was used in the fit of the signals to Gaussian line shapes using AMARES software (Vanhamme et al. 1997). With the Cr and PCr pools in equilibrium (Brault and Terjung 2003), the summed signal intensities of Cr and PCr served as TCr. Spectral quality was assessed by comparing the TCr signal intensity to the standard deviation (SD) of the noise (defined as SD of the last 10 % of the FID). Only spectra in which the TCr signal intensity was larger than the SD of the noise were included for further analysis. In addition, we excluded spectra in which the fitted line width of the ^{13}C creatine signals was more than 50 Hz.

Creatine uptake

TCr amplitudes of individual muscles at days 0 till 5 were fitted to a linear function (Eq. 1) where $\text{TCr}(t)$ is the ^{13}C signal intensity at measurement day t . This was only calculated for the muscles that had at least 3 measurement days with spectra that passed the quality criteria (see above). The fitted slope gives the Cr uptake rate (k_{up}) and the intercept the initial TCr value (TCr_0), both in arbitrary units (a.u.). The final uptake rate is presented as $k_{\text{up}}/\text{TCr}_0$ to give a value expressed in percentage per day. The error of the fit was treated as the standard deviation.

$$\text{TCr}(t) = k_{\text{up}}t + \text{TCr}_0 \quad (1)$$

Creatine turnover

TCr signal intensities from day 5–105 were used to calculate the turnover rate. Cr breakdown is assumed to follow first order kinetics (Walker 1979), so TCr signal intensities were fitted to a mono-exponential function (Eq. 2), under the assumptions described in (Kan et al. 2006). The maximal ^{13}C TCr signal intensity is TCr_{max} , and k_{br} is the breakdown rate (% per day). The error of the fit was treated as the standard deviation. All curve fitting was done using Prism 5.0 (GraphPad Software, San Diego, CA, USA).

$$\frac{\text{TCr}(t)}{\text{TCr}_0} = \frac{(\text{TCr}_{\text{max}} - \text{TCr}_0)e^{-k_{\text{br}}t}}{\text{TCr}_0} + 1 \quad (2)$$

PCr/TCr ratio

The PCr/TCr ratio was determined per muscle over time for every measurement point unless the Cr and PCr signals could not be resolved properly (i.e., one or both signals fitted with amplitude 0). To compare the PCr/TCr ratios between individual muscles, the PCr/TCr ratios were averaged over all these measurement points.

Table 2 Plasma creatine concentrations and percentage ^{13}C -4-Cr (mean \pm SD)

| | Day 0 | Day 3 | Day 7 |
|-----------------------------------|---------------|-----------------|----------------|
| Plasma creatine (μM) | 38 \pm 23 | 668 \pm 432** | 41 \pm 6 |
| % ^{13}C | 6.0 \pm 0.6 | 19 \pm 6.1** | 10 \pm 0.5** |

Those at day 3 and day 7 are compared with day 0 by means of a one-sided paired t test

** $p < 0.01$

T_1 measurement

The Cr and PCr signal intensities were fitted as a function of the repetition time according to Eq. 3, giving a value for the T_1 relaxation time in ms.

$$\frac{M_z(\text{TR})}{M_0} = 1 - e^{-\text{TR}/T_1} \quad (3)$$

Statistics

A one-sided paired t test was used to compare blood plasma concentrations to day 0, and to assess differences between male and female muscles. Errors are given as standard deviation (SD) unless presented otherwise. Muscle specific effects of Cr uptake and PCr/TCr ratios were assessed by a one-way ANOVA. All statistical analysis were done using Prism 5.0 (GraphPad Software, San Diego, CA, USA), and statistical significance was set at $p < 0.05$.

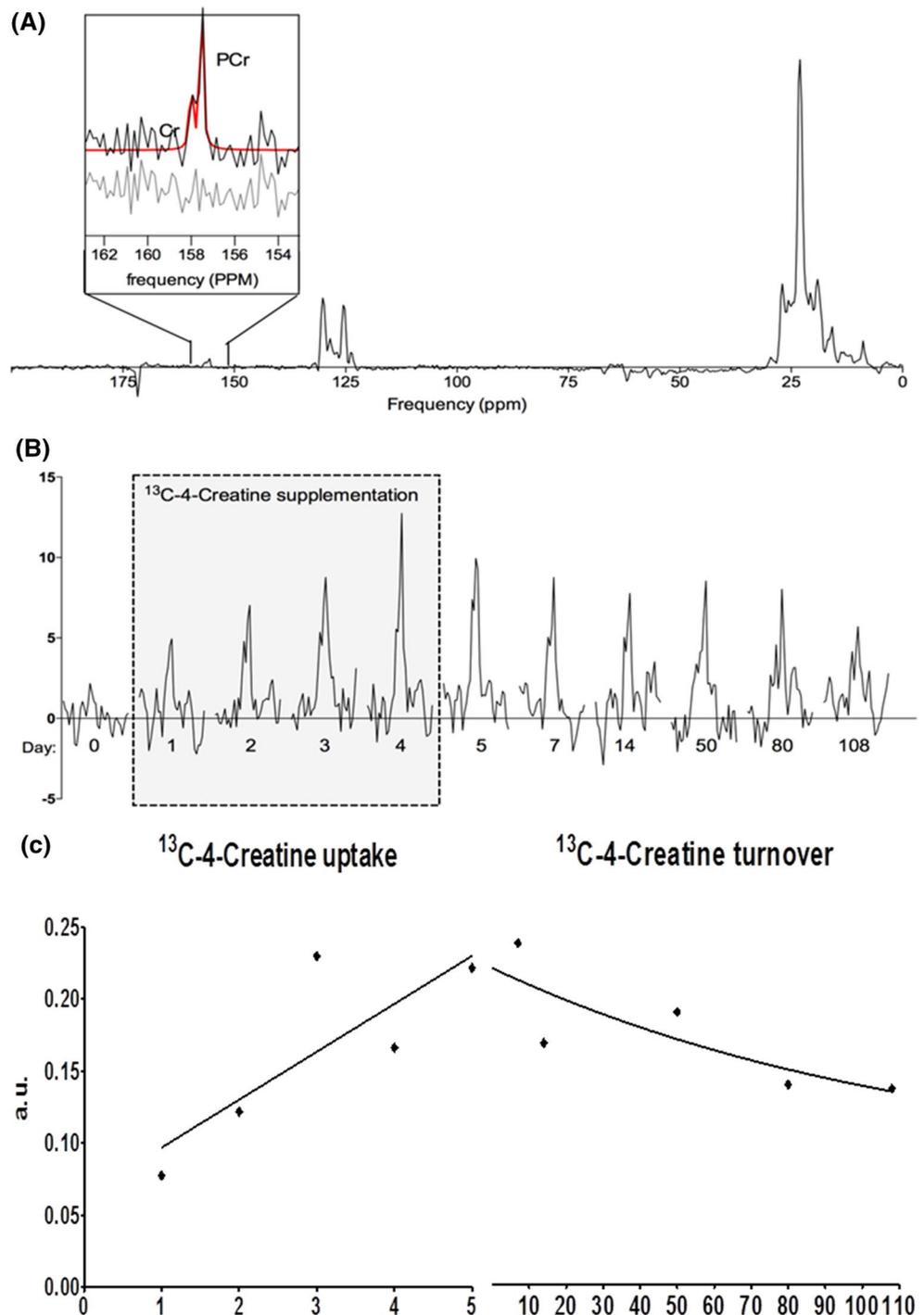
Results

General observations

None of the subjects reported side effects of the creatine supplementation. Blood analysis of the creatine content and ^{13}C enrichment indicated that all subjects adhered to the prescribed intake of the supplementation (Table 2). At day 3, these values were comparable for young males and females, i.e., average Cr content was 929 and 711 μM , respectively, and average ^{13}C enrichment 22 and 20 %, respectively. Two days after the supplementation period the plasma creatine levels had returned to normal values, but the ^{13}C enrichment was still increased.

During the ingestion of 20 g/day 15 % enriched ^{13}C -4-Cr for 4 days, the ^{13}C signals for PCr and Cr increased in muscles within the sensitive profile of the $^{13}\text{C}/^1\text{H}$ coil (Figs. 1, 2b). The separate signals of PCr and Cr were integrated by fitting, using the prior knowledge described above (Fig. 2a, inset), for further analyses.

Fig. 2 ^{13}C MR spectra before, during and after creatine supplementation in the ST muscle of a healthy young male subject. **a** Full ^{13}C MR spectrum of this muscles at day 7, 3 days after the creatine supplementation period. Cr and PCr resonate at 157.5 and 156.6 ppm, respectively. Even though creatine is 15 % ^{13}C enriched the signal intensity is still much smaller than that of natural abundance ^{13}C lipid resonances. The *inset* shows the fit of the PCr and Cr resonances with the residual below. **b** Starting with day 0 a clear increase in signal intensity is observed during the 4 days of ^{13}C -4-Cr supplementation followed by a slower decrease; the signal is still elevated up to day 108. **c** The uptake rate is fitted with a linear function over the first 5 days, and the breakdown rate was fitted by a mono-exponential decay. Note that for the uptake part, the integral of the creatine signals at day 0 is not included as the fit of these signals did not pass the quality criteria



Creatine uptake rate

The summed intensities of the ^{13}C signal of Cr and PCr determined at day 0 till day 5 were fitted to a linear function (Fig. 2c) to yield a subject and muscle specific Cr uptake rate. Following the spectral quality criteria, this analysis could be performed in 17 out of the 20 investigated muscles of the young healthy volunteers. In males, a significant

Cr uptake rate was observed in the SM, ST, and AM muscles with less in the other two muscles (Fig. 3). In females, we could observe Cr uptake in all investigated muscles, except that the G muscle of one female was too thin to place a voxel solely in that muscle. The data of the BF and ST muscles of this female did not pass the quality criteria to obtain a Cr uptake rate mainly because of low signal-to-noise ratio in some spectra. Taking all remaining muscles

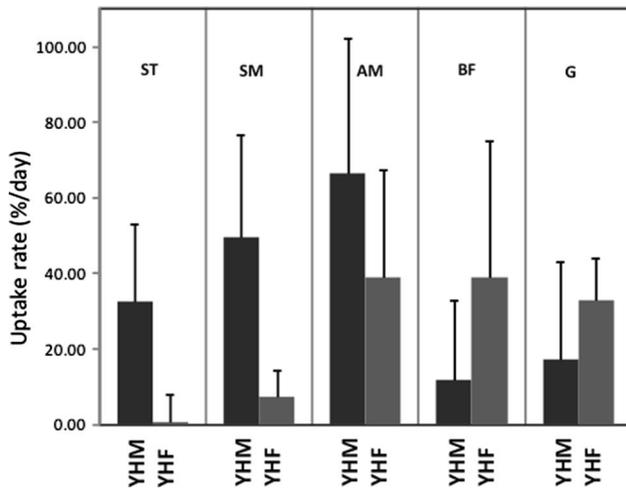


Fig. 3 Muscle specific creatine uptake rates (% per day) in four volunteers: two young healthy males (YHM; black bars) and two young healthy females (YHF; gray bars), from left to right: semi-tendinosus (ST), semi-membranosus (SM), adductor magnus (AM), biceps femoris (BF), and gracilis (G). Each uptake rate is the average of two muscles, except for the ST, BF, and G in YHF, for which the bars represent the uptake rate in one muscle. For the latter muscles, the error of the linear fit (see Fig. 2c) is given as SD. In the presented SD of the other muscles, these fitting errors have been considered

together the mean uptake rate in females was lower than in males although not significant (17 ± 26 vs. 36 ± 22 %/day with SD; $p = 0.25$).

To test the feasibility of the ^{13}C MRSI assessment of muscular creatine uptake in more fragile persons, i.e., a 82-year-old male and two FSHD patients, it was necessary to decrease the number of exams during the supplementation and thereafter. The creatine supplementation was well tolerated by all participants, and no side effects were reported.

The female FSHD patient (FSHD#1) showed fatty infiltration in the AM and SM muscles, and the male FSHD patient (FSHD#2) showed severe fatty infiltration in the SM muscle, and minor fatty infiltration in the ST and BF muscles. Following the spectral and fitting quality criteria, creatine uptake rates could be determined for FSHD#1 in the ST (48 ± 101 %/day), AM (27 ± 5 %/day), and the BF (9 ± 12 %/day), and for FSHD#2 in the BF (77 ± 184 %/day). For the 82-year-old male, we observed Cr uptake in four of the five investigated muscles of which three passed the quality criteria. The SM had an uptake rate of 8 ± 15 %/day, the BF 9 ± 28 %/day, and the G 24 ± 8 %/day.

Creatine turnover

The Cr turnover was determined by fitting the TCr signal intensities from day 5–105 after start of supplementation,

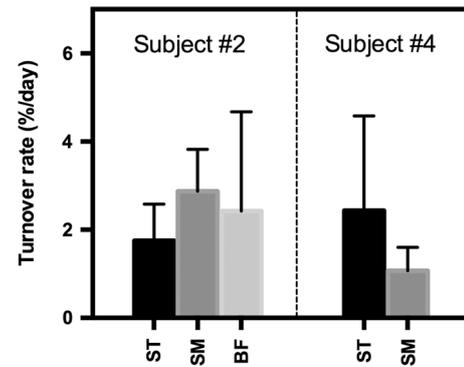


Fig. 4 Muscle specific Cr turnover rates (% per day) in young male volunteers. Only muscles in which the turnover signals were found to follow a mono-exponential decay were included. Turnover rate could not be determined if muscles showed relatively low Cr uptake. Bars indicate the mean uptake rate with standard error of the mean

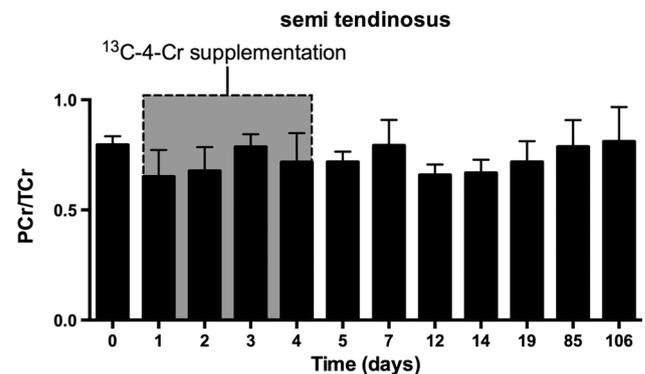


Fig. 5 PCr/TCr ratios of the semi-tendinosus muscle before, during and after Cr supplementation of the four young healthy volunteers. PCr/TCr ratios were determined from ^{13}C spectra acquired and analyzed as described in the materials and methods section. Bars indicate the mean values, and the error bars indicate the standard deviation

to a mono-exponential decay function (Fig. 2c). Because all Cr curves will fit to this function, even when no Cr uptake had taken place, we added a fit quality criterion that R^2 has to be larger than 0.5 to ensure that the fitted values indeed follow a mono-exponential decay. Only data of three muscles of male subject #2 and two muscles in the other male fulfilled this criterion. No turnover rates fulfilling the fit quality criteria could be determined for the female volunteers. For the muscles in which the Cr turnover could be determined, the rates were comparable with an average of 2.1 ± 0.7 % per day (Fig. 4).

T_1 relaxation times of ^{13}C -4 spins

To verify that the pulse repetition time for MRSI acquisition did not cause signal saturation, we measured the T_1 relaxation times of the ^{13}C spins in the hamstring muscle

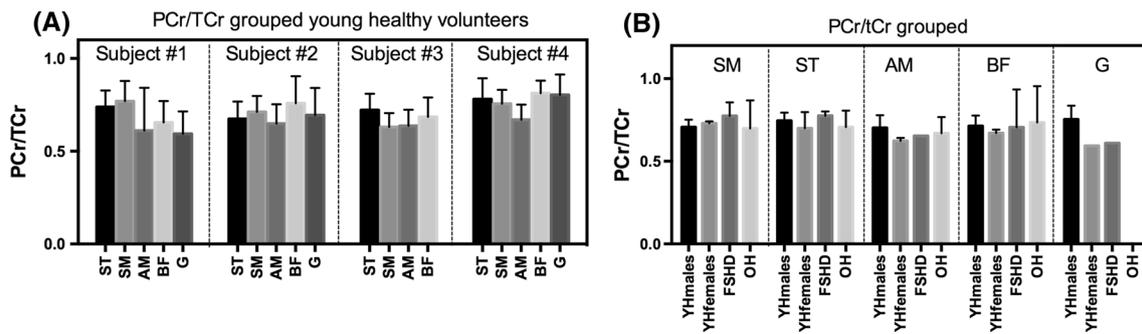


Fig. 6 PCr/TCr ratios. **a** Muscle specific ratios in young healthy volunteers ($n = 4$). One-way ANOVA did not reveal statistical significant muscle specific effect. *Bars* indicate the mean PCr/TCr per volunteer and the *error bars* indicate standard deviation. **b** Grouped data of the PCr/TCr ratio of the young healthy subjects, the FSHD

patients, and the old volunteer. The PCr/TCr ratios of the FSHD and old subjects are within the range found in the young healthy subjects. *YHmales/females* young healthy subjects, *FSHD* FSHD patients, *OH* old healthy subject. *Error bars* indicate standard deviation

Table 3 Average muscular PCr/TCr ratios of the four young volunteers (mean \pm SD)

| Muscle | Mean |
|--------|-----------------|
| ST | 0.75 ± 0.04 |
| SM | 0.73 ± 0.03 |
| AM | 0.65 ± 0.03 |
| BF | 0.75 ± 0.07 |
| G | 0.70 ± 0.10 |

With correction for the T_1 values of Cr and PCr

of one healthy young male subject by analyzing the ^{13}C -4 signals of Cr and PCr as a function of the repetition time. The T_1 for Cr was 341 ± 36 ms and for PCr 413 ± 49 ms. This implies that with a 45° excitation pulse and a TR of 1000 ms for the ^{13}C MRSI acquisition, there will be little spin saturation.

PCr/TCr ratio

To investigate if creatine supplementation would change the PCr/TCr ratio, we determined this ratio for every time point during and after the supplementation. For none of the examined muscles in the healthy young volunteers, we observed a significant change in the PCr/TCr ratio during this period. An example is shown in Fig. 5 for this ratio in the ST muscle, which is around 0.75.

To investigate if the PCr/TCr ratio is the same as determined in other studies, we assessed this ratio for each investigated muscle in the young volunteers averaged over all time points (Fig. 6a; Table 3). Testing each muscle against the other revealed that the PCr/TCr of the AM is significantly lower than that of the other muscles ($p < 0.05$), but a one-way ANOVA did not show a muscle specific effect for the PCr/TCr ratio ($p = 0.32$). For

all subjects and all muscles, together the average PCr/TCr ratio was 0.71 ± 0.07 (including a small correction for differences in T_1 between PCr and Cr).

The average PCr/TCr ratios of all investigated muscles in the old healthy male and FSHD patients were similar to the values of the young healthy subjects (see Fig. 6b), with T_1 -corrected values of 0.71 ± 0.03 and 0.71 ± 0.07 , respectively.

Discussion

In this feasibility study, we demonstrate that 3D ^{13}C MRS imaging with oral ingestion of ^{13}C -4-creatine can be used to monitor Cr uptake and turnover rates as well as PCr/TCr ratios in individual muscles of the human leg. Previously, we showed with unlocalized ^{13}C MRS, and an RF coil next to the gastrocnemius of a male subject that the non-invasive monitoring of Cr and PCr during and after supplementation of ^{13}C -4-labeled Cr is feasible (Kan et al. 2006). Given the substantial variation in creatine uptake in different muscles, it is relevant to demonstrate that with ^{13}C MRS in spectroscopic imaging mode, we can spatially resolve information of ^{13}C labeled metabolites in multiple individual muscles in one exam. We applied this technique in a diverse group of subjects, including four young and one old healthy volunteer, and two patients with FSHD. In this way, the supplied Cr, and its immediate cellular product PCr, is assessed instead of the total Cr or PCr pool, as has been done with ^1H or ^{31}P MRS after oral Cr ingestion, e.g., (Kreis et al. 1999; Braut et al. 2007).

It is a common finding in human muscular studies of creatine uptake, assessed with biopsies, that TCr increases more than PCr during the supplementation period, e.g., (Hultman et al. 1996; McKenna et al. 1999). A study combining ^1H and ^{31}P measurements to determine TCr and PCr

in the vastus lateralis (where most biopsies are taken from) did not find this (Brault et al. 2007). The authors attribute this discrepancy to higher PCr hydrolysis during the biopsy procedure because of the change in physiological condition of muscles loaded with Cr. Our single subject study using ^{13}C MRS also did not find a change in the PCr/TCr ratio of the gastrocnemius during Cr uptake (Kan et al. 2006). In the present study, we assessed this for a few other muscles in the thigh and found no preferential increase in TCr over the supplementation period within the error of the measurements. These findings indicate a general preservation of the overall thermodynamic equilibrium of the creatine kinase reaction in muscle cells at rest, also during Cr loading.

The range of average PCr/TCr ratios of the investigated muscles (from 0.65 to 0.75) is within the range reported in the literature for this ratio from 0.6 to 0.8 (Casey et al. 1996; Mesa et al. 2002) and comparable with our previous unlocalized study of the gastrocnemius (Kan et al. 2006). The PCr/TCr ratio is similar for all the investigated muscles in the young volunteers with a somewhat lower value for the adductor magnus, which may be due to a different fiber type distribution (Johnson et al. 1973; Mesa et al. 2002).

Interestingly, the average PCr/TCr ratios in the older subject and FSHD patients were not different from the young volunteers indicating that energy balance and CK activity in the muscle of these subjects result in a similar thermodynamic equilibrium of the reaction at rest. In principle, muscle specific PCr/TCr ratios can also be determined non-invasively with ^1H and ^{31}P MRS recordings of the same muscle, but this has not yet been reported as such.

The average muscular Cr turnover rate of 2.1 ± 0.7 % per day is in agreement with the results of our previous ^{13}C MRS study of a single subject and with the reported non-enzymatic breakdown rates of creatine to creatinine (Kan et al. 2006; Walker 1979; Wyss and Kaddurah-Daouk 2000).

In particular, for muscles with relatively high SNR spectra (SM and AM muscles of young volunteers), the data points indicate an apparent linear uptake of labeled Cr during the 4 days supplementation. This is in agreement with previous supplementation studies and has been associated with near-saturation of the Cr transporters (Brault et al. 2007), which would suggest that uptake rates reflect creatine transporter capacity of muscles.

Cr uptake rates were variable among the five thigh muscles and subjects and varied between 0 and about 80 % per day over all subjects and muscles. It is not possible that the lack of Cr uptake in some muscles is caused by subjects not taking ^{13}C -labeled Cr as the blood plasma samples analyzed before and during the Cr supplementation period showed similar increase in Cr and ^{13}C -4-Cr concentration for all subjects during the supplementation period proving that they did ingest the provided ^{13}C -Cr.

Differences in Cr response have been observed in rat hind limb muscles, with significant uptake in the gastrocnemius muscle but no response in the soleus muscle, an effect attributed to a higher contribution of type II fibers in the gastrocnemius (McMillen et al. 2001). In humans, the increases in (relative) Cr or PCr level after supplementation appear to differ among leg muscles with reported values from about 4 % for the gastrocnemius (Vandenberghe et al. 1997) to about 20 % for quadriceps muscles (Kreis et al. 1999; Smith et al. 1999). The origin of these differences is unknown; in studies addressing this issue, the proposed ^{13}C MRSI method to assess uptake rates could be helpful.

Based on the reported values for Cr turnover rates, we estimate that to keep up with spontaneous constant Cr breakdown, a ^{13}C signal increase in muscle spectra of 10–30 %/day is expected during the supplementation. This implies that higher uptake rates are reflecting an increase of Cr levels in the muscles. It also suggests that for muscles with an uptake rate below about 10 %/day in this study, the SNR and/or the reproducibility of the measurement and/or fitting procedure of the Cr signal was inadequate.

For this ^{13}C MRSI study, we increased the field strength from 1.5T to 3T and the ^{13}C enrichment from 10 to 15 %, compared with our previous ^{13}C MRS study (Kan et al. 2006). The higher sensitivity allowed us to study ^{13}C uptake and metabolism in individual muscles of young male subjects with sufficient SNR. However, this was less so in the females and older person and in FSHD patients, in which the quality filter only allowed to assess an uptake rate in four out of ten muscles. In the latter cases, this could be due to less Cr uptake or a lower muscular fraction. In a ^{31}P MRS study on FSHD patients, a preferential decrease of the SNR and the PCr/ATP ratio was observed in fat infiltrated muscles (Kan et al. 2010). These results imply that for future clinical research studies with ^{13}C MRSI, the SNR has to be improved, which can be achieved in several ways. First, the relative short T_1 values of the ^{13}C spin systems in Cr and PCr (≈ 400 ms) at 3T allow for a shorter T_r than the currently used 1000 ms, so that more scans can be acquired in the same amount of time. Second, the voxel size can be enlarged in the length direction of the muscles. Third, improved MR instrumentation can be used, such as array RF coils or higher field strength, which requires substantial investments. Fourth, the ^{13}C enrichment of the creatine can be increased, which, however, will increase the cost of the experiment. In addition, calibrations and registrations for better reproducibility, and signal fitting with more restrictive prior knowledge, such as line width limitations, may help.

For a full non-invasive investigation of muscular creatine uptake, it would be important to also know the total amount of creatine during and after the supplementation (Nabuurs et al. 2013). As we already employ a proton RF

coil in the current setup, this can be most easily achieved by simultaneous or alternate ^1H MRS measurements (Kreis et al. 1999).

In summary, we introduce an in vivo ^{13}C MRS imaging method to study intake and conversion of ^{13}C labeled metabolites in skeletal muscles. We demonstrate that this allows for direct monitoring of supplied ^{13}C -labeled Cr for subject and muscle specific determination of PCr/TCr ratios, and Cr turnover and uptake rates.

Acknowledgments We thank Arno van Rooij for performing mass spectroscopy experiments, Andi Boss for helpful conversations, Mark van Uden for hardware support and Sjaak van Asten for help with data processing.

Compliance with ethical standards

Funding This study was funded by Prinses Beatrix Spierfonds (PBF) (The Dutch Public Fund for Neuromuscular Disorders) with Grant WAR08-15, FSHD Global Research Foundation and Stichting FSHD with Grant WP15.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from all participants.

Open Access This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

References

- Brault JJ, Terjung RL (2003) Creatine uptake and creatine transporter expression among rat skeletal muscle fiber types. *Am J Physiol Cell Physiol* 284(6):C1481–C1489. doi:10.1152/ajpcell.00484.2002
- Brault JJ, Towse TF, Slade JM, Meyer RA (2007) Parallel increases in phosphocreatine and total creatine in human vastus lateralis muscle during creatine supplementation. *Int J Sport Nutr Exerc Metab* 17(6):624–634
- Casey A, Constantini-Teodosiou D, Howell S, Hultman E, Greenhaff PL (1996) Creatine ingestion favorably affects performance and muscle metabolism during maximal exercise in humans. *Am J Physiol* 271(1 Pt 1):E31–E37
- Coggan AR, Spina RJ, King DS, Rogers MA, Brown M, Nemeth PM, Holloszy JO (1992) Histochemical and enzymatic comparison of the gastrocnemius muscle of young and elderly men and women. *J Gerontol* 47(3):B71–B76
- De Graaf RA (2007) *In vivo* NMR spectroscopy: principles and techniques. Wiley, Chichester, West Sussex, England
- Dubowitz V, Sewry CA (2007) *Muscle biopsy: a practical approach*, 3rd edn. Saunders/Elsevier, Philadelphia
- Green AL, Hultman E, Macdonald IA, Sewell DA, Greenhaff PL (1996) Carbohydrate ingestion augments skeletal muscle creatine accumulation during creatine supplementation in humans. *Am J Physiol* 271(5 Pt 1):E821–E826
- Greenhaff PL, Bodin K, Soderlund K, Hultman E (1994) Effect of oral creatine supplementation on skeletal muscle phosphocreatine resynthesis. *Am J Physiol* 266(5 Pt 1):E725–E730
- Gualano B, Roschel H, Lancha-Jr AH, Brightbill CE, Rawson ES (2012) In sickness and in health: the widespread application of creatine supplementation. *Amino Acids* 43(2):519–529. doi:10.1007/s00726-011-1132-7
- Guerrero-Ontiveros ML, Wallimann T (1998) Creatine supplementation in health and disease. Effects of chronic creatine ingestion in vivo: down-regulation of the expression of creatine transporter isoforms in skeletal muscle. *Mol Cell Biochem* 184(1–2):427–437
- Guthmiller P, Van Pilsom JF, Boen JR, McGuire DM (1994) Cloning and sequencing of rat kidney L-arginine:glycine amidinotransferase. Studies on the mechanism of regulation by growth hormone and creatine. *J Biol Chem* 269(26):17556–17560
- Harris RC, Soderlund K, Hultman E (1992) Elevation of creatine in resting and exercised muscle of normal subjects by creatine supplementation. *Clin Sci* 83(3):367–374
- Hoberman HD, Sims EA, Peters JH (1948) Creatine and creatinine metabolism in the normal male adult studied with the aid of isotopic nitrogen. *J Biol Chem* 172(1):45–58
- Hultman E, Soderlund K, Timmons JA, Cederblad G, Greenhaff PL (1996) Muscle creatine loading in men. *J Appl Physiol* 81(1):232–237
- Johnson MA, Polgar J, Weightman D, Appleton D (1973) Data on the distribution of fibre types in thirty-six human muscles. An autopsy study. *J Neurol Sci* 18(1):111–129
- Kan HE, van der Graaf M, Klomp DW, Vlak MH, Padberg GW, Heerschap A (2006) Intake of ^{13}C -4 creatine enables simultaneous assessment of creatine and phosphocreatine pools in human skeletal muscle by ^{13}C MR spectroscopy. *Magn Reson Med: Off J Soc Magn Reson Med/Soc Magn Reson Med* 56(5):953–957. doi:10.1002/mrm.21068
- Kan HE, Scheenen TW, Wohlgemuth M, Klomp DW, van Loosbroek-Wagenmans I, Padberg GW, Heerschap A (2009) Quantitative MR imaging of individual muscle involvement in facioscapulohumeral muscular dystrophy. *Neuromuscul Disord: NMD* 19(5):357–362. doi:10.1016/j.nmd.2009.02.009
- Kan HE, Klomp DW, Wohlgemuth M, van Loosbroek-Wagemans I, van Engelen BG, Padberg GW, Heerschap A (2010) Only fat infiltrated muscles in resting lower leg of FSHD patients show disturbed energy metabolism. *NMR Biomed* 23(6):563–568. doi:10.1002/nbm.1494
- Kemp GJ, Taylor DJ, Dunn JF, Frostick SP, Radda GK (1993) Cellular energetics of dystrophic muscle. *J Neurol Sci* 116(2):201–206
- Kley RA, Tarnopolsky MA, Vorgerd M (2013) Creatine for treating muscle disorders. *The Cochrane database of systematic reviews* 6:CD004760. doi:10.1002/14651858.CD004760.pub4
- Kreis R, Kamber M, Koster M, Felblinger J, Slotboom J, Hopfeler H, Boesch C (1999) Creatine supplementation—part II: in vivo magnetic resonance spectroscopy. *Med Sci Sports Exerc* 31(12):1770–1777
- McCall W, Persky AM (2007) Pharmacokinetics of creatine. *Sub-Cell Biochem* 46:261–273
- McKenna MJ, Morton J, Selig SE, Snow RJ (1999) Creatine supplementation increases muscle total creatine but not maximal intermittent exercise performance. *J Appl Physiol* 87(6):2244–2252

- McMillen J, Donovan CM, Messer JJ, Willis WT (2001) Energetic driving forces are maintained in resting rat skeletal muscle after dietary creatine supplementation. *J Appl Physiol* 90(1):62–66
- Mesa JL, Ruiz JR, Gonzalez-Gross MM, Gutierrez Sainz A, Castillo Garzon MJ (2002) Oral creatine supplementation and skeletal muscle metabolism in physical exercise. *Sports Med* 32(14):903–944
- Meyer RA, Sweeney HL, Kushmerick MJ (1984) A simple analysis of the “phosphocreatine shuttle”. *Am J Physiol* 246(5 Pt 1):C365–C377
- Nabuurs CI, Choe CU, Veltien A, Kan HE, van Loon LJ, Rodenburg RJ, Matschke J, Wieringa B, Kemp GJ, Isbrandt D, Heerschap A (2013) Disturbed energy metabolism and muscular dystrophy caused by pure creatine deficiency are reversible by creatine intake. *J Physiol* 591(2):571–592. doi:[10.1113/jphysiol.2012.241760](https://doi.org/10.1113/jphysiol.2012.241760)
- Naressi A, Couturier C, Devos JM, Janssen M, Mangeat C, de Beer R, Graveron-Demilly D (2001) Java-based graphical user interface for the MRUI quantitation package. *Magma* 12(2–3):141–152
- Pijnappel WWF, Vandenboogaart A, Debeer R, Vanormondt D (1992) Svd-based quantification of magnetic-resonance signals. *J Magn Reson* 97(1):122–134. doi:[10.1016/0022-2364\(92\)90241-X](https://doi.org/10.1016/0022-2364(92)90241-X)
- Pulido SM, Passaquin AC, Leijendekker WJ, Challet C, Wallimann T, Rungg UT (1998) Creatine supplementation improves intracellular Ca²⁺ handling and survival in mdx skeletal muscle cells. *FEBS Lett* 439(3):357–362
- Smith SA, Montain SJ, Matott RP, Zientara GP, Jolesz FA, Fielding RA (1998) Creatine supplementation and age influence muscle metabolism during exercise. *J Appl Physiol* 85(4):1349–1356
- Smith SA, Montain SJ, Matott RP, Zientara GP, Jolesz FA, Fielding RA (1999) Effects of creatine supplementation on the energy cost of muscle contraction: a 31P-MRS study. *J Appl Physiol* 87(1):116–123
- Snow RJ, Murphy RM (2003) Factors influencing creatine loading into human skeletal muscle. *Exerc Sport Sci Rev* 31(3):154–158
- Vandenbergh K, Goris M, Van Hecke P, Van Leemputte M, Vangerven L, Hespel P (1997) Long-term creatine intake is beneficial to muscle performance during resistance training. *J Appl Physiol* 83(6):2055–2063
- Vanhamme L, van den Boogaart A, Van Huffel S (1997) Improved method for accurate and efficient quantification of MRS data with use of prior knowledge. *J Magn Reson* 129(1):35–43. doi:[10.1006/jmre.1997.1244](https://doi.org/10.1006/jmre.1997.1244)
- Vermathen P, Saillen P, Boss A, Zehnder M, Boesch C (2012) Skeletal muscle (1)H MRSI before and after prolonged exercise. I. Muscle specific depletion of intramyocellular lipids. *Magn Reson Med: Off J Soc Magn Reson Med/Soc Magn Reson Med* 68(5):1357–1367. doi:[10.1002/mrm.24168](https://doi.org/10.1002/mrm.24168)
- Walker JB (1979) Creatine: biosynthesis, regulation, and function. *Adv Enzymol Relat Areas Mol Biol* 50:177–242
- Wallimann T, Tokarska-Schlattner M, Schlattner U (2011) The creatine kinase system and pleiotropic effects of creatine. *Amino Acids* 40(5):1271–1296. doi:[10.1007/s00726-011-0877-3](https://doi.org/10.1007/s00726-011-0877-3)
- Wyss M, Kaddurah-Daouk R (2000) Creatine and creatinine metabolism. *Physiol Rev* 80(3):1107–1213
- Wyss M, Schulze A (2002) Health implications of creatine: can oral creatine supplementation protect against neurological and atherosclerotic disease? *Neuroscience* 112(2):243–260
- Zhao Q, Patriotis P, Arias-Mendoza F, Stoyanova R, Brown TR (2005) An interactive software for 3D chemical shift imaging data analysis and real time spectral localization and quantification. In: Conference paper presented in annual meeting of the International Society of Magnetic Resonance in Medicine