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Creatine and creatinine quantified using nuclear magnetic resonance: A method validation study and clinical associations between circulating creatine and fatigue in kidney transplant recipients

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ABSTRACT

Background: A potential contributor to fatigue in kidney transplant recipients (KTR) may be impaired creatine homeostasis. We developed and validated a high-throughput NMR assay allowing for simultaneous measurement of circulating creatine and creatinine, and determined plasma creatine and estimated intramuscular creatine concentrations in KTRs, delineated their determinants and explored their associations with self-reported fatigue.
Methods: An NMR assay was developed and validated for measurement of circulating creatinine and creatine concentrations. Plasma creatine and creatinine concentrations were measured in 618 KTR. Fatigue was assessed using the checklist individual strength. Associations of creatine parameters with fatigue was assessed using linear mixed effect models.
Results: The NMR-based assay had good sensitivity, precision and demonstrated linearity across a large range of values. Among KTR, the mean age was 56 ± 13 years, 62% were men and eGFR was 54 ± 18 ml/min/1.73 m^2. Plasma creatine concentration was 27 [19–39] μmol/L. Estimated intramuscular creatine concentration was 27 ± 7 mmol/kg. Higher plasma creatine concentration and higher estimated intramuscular creatine concentration were independently associated with a lower total fatigue score and less motivation problems.
Conclusion: An NMR method for measurement of circulating creatine and creatinine which offers the potential for accurate and efficient quantification was developed. The found associations suggest that improving creatine status may play a beneficial role in mitigating fatigue.

1. Introduction

Kidney transplantation is a vital and life-saving procedure for individuals with kidney failure, as it partially restores kidney function and significantly reduces morbidity and mortality rates. However, despite these benefits, kidney transplant recipients (KTRs) often experience higher levels of fatigue compared to the general population [1]. Fatigue encompasses multiple domains and is influenced by physiological, psychological, and lifestyle factors. One potential contributor to fatigue in KTRs may be impaired creatine homeostasis. Creatine, a nitrogenous

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organic acid, plays a crucial role in cellular energy balance, particularly in muscles, the heart, and the brain. The majority of the bodily creatine pool is present in skeletal muscle. Approximately 1.7% of the total creatine pool per day is non-enzymatically converted to its waste product, creatinine [2–6]. To remain in steady state, this loss has to be compensated, either by ingestion of creatine-containing food (or supplements) or by endogenous synthesis. The rate of endogenous synthesis depends mostly on the enzyme arginine:glycine amidinotransferase (AGAT), which is predominantly expressed in the proximal tubular cells of the kidneys [7]. Although the importance of creatine in muscle function is widely recognized [8–10], there is currently a scarcity of information regarding circulating and intramuscular creatine concentrations in the post-transplant context or their relationships with fatigue.

The metabolic product of creatine, creatinine is widely used to assess kidney function and as such is used to calculate estimated glomerular filtration rate (eGFR). Jaffe-based assays utilizing alkaline picrate are currently the most widely used methods for quantifying circulating creatinine. However, it is well known that lipemia, hemolysis, and bilirubin can affect results from the alkaline pictrate-based colorimetric assays (Jaffe) [11]. Having an assay that is able to measure both creatine and creatinine, while avoiding the typical limitations of the enzymatic assays, would be valuable for assessment of various diseases as well as studies involving creatine supplementation. There are several benefits of nuclear magnetic resonance (NMR)-based assays, as they are reagentless and do not require hands-on manipulation of the specimens before placing them on the instrument for testing, thereby reducing laboratory technician time. With these benefits in mind, a high-throughput NMR assay was developed to simultaneously measure creatine and creatinine in serum or plasma.

In this study, we pursued two main objectives. The first objective was to introduce and validate a high-throughput NMR assay capable of simultaneously measuring creatine and creatinine in serum or plasma. This assay has the potential to provide accurate and efficient quantification, thereby reducing both technician time and instrument costs. The second objective was to assess plasma creatine concentration and estimate intramuscular creatine concentration in KTRs, delineating their determinants and exploring their associations with self-reported fatigue. By addressing these research objectives, our aim was to enhance our understanding of creatine metabolism in the context of kidney transplantation.

2. Materials and methods

2.1. Objective 1

2.1.1. Specimen collection and data analysis

To enable the analytical validation studies, de-identified residual clinical specimens were pooled at Labcorp (Morrisville, NC). In addition, fresh blood was collected when appropriate de-identified specimens were not available. Specimens drawn in Greiner Bio-One serum tubes (Part number 456293P), plain red-top serum tubes or EDTA purple-top plasma tubes were processed as per manufacturer's instructions. All volunteers for blood donations signed informed consent forms and all procedures were carried out in accordance with the Declaration of Helsinki. The clinical protocol (SQNM-RND-103; study #1329278) was approved for use by WCG Institutional Review Board (IRB) (tracking #520100174). All analytical validation analyses were conducted using Excel Analyse-it (v5.11.3) in Excel 2016 (v16.0.4266.1001) unless otherwise indicated.

2.1.2. NMR spectral acquisition and result generation

Serum or EDTA anti-coagulated plasma specimens were diluted on board the Vantera® Clinical Analyzer with citrate/phosphate buffer (3:1 v/v) to lower the pH to 5.3, which separates the creatine and creatinine peaks that overlap at physiological pH [12–14]. The Carr-Purcell-Meiboom-Gill (CPMG) technique, which suppress signals from proteins/lipoproteins, and the water suppression enhanced through T1 effects technique were utilized to collect one-dimensional (1D) proton (^{1}H) NMR spectra as previously described [12–14]. Spectra were acquired at 47 °C on a Vantera® Clinical Analyzer equipped with 400 MHz (9.4 T) Agilent spectrometer. Parameters for NMR data acquisition and signal processing have been reported previously [12–14]. Fig. 1 shows a representative 1D ^{1}H NMR CPMG spectrum for serum used to quantify circulating creatine and creatinine. The sample-to-result throughput is 6 min.

2.1.3. Quantification of creatinine and creatine by peak deconvolution

While the creatine peak location did not vary, the creatinine peak location within the NMR spectrum varied as a function of pH. Therefore, a linear equation relating the creatinine offset (distance between the pH invariant α-anomeric glucose and the creatinine peak) versus citrate offset (distance between the α-anomeric glucose and the leftmost citrate peak) was empirically established. This was then utilized by the algorithm to locate the creatinine peak for analysis. On the other hand, the α-anomeric glucose peak was used as reference to locate the creatine peak. Creatine and creatinine were quantified using a proprietary deconvolution algorithm that resolves each analyte signal into its spectral components. Each peak was modelled mathematically using a combination of Lorentzian and Gaussian lineshapes. To adequately create a model for the baseline and the residual background signal, linear and quadratic functions, along with experimental component (spectrum of serum devoid of small metabolites) were incorporated in the algorithm. Non-negative least squares fitting algorithm was used for lineshape deconvolution [15]. Fig. 1 shows the observed analyte signal (black) and mathematical model signal (red) for creatinine (inset A) and creatine (inset B) in serum. The spectral components used for construction of the observed NMR signal are also shown (Lorentzian (blue) and Gaussian (green) functions, experimentally acquired component (gray), quadratic (black dashed line) and linear (orange dashed line) functions). After subtracting the baseline/background signal, the analyte peak amplitude was converted to concentration units using a factor obtained from a calibration curve. The calibration curve was generated by relating the peak amplitude for serum spiked with creatine or creatinine standards and the amount of added standards.

2.1.4. Method comparison studies

Method comparison studies were performed as per Clinical and Laboratory Standards (CLSI) guidelines [16]. For the creatine method comparison study, serum specimens were obtained from multiple donors. Some samples were spiked with creatine in order to increase the range of concentrations within and above the normal range. A total of 50 samples with varying creatine concentrations were generated and aliquots were frozen at <-70 °C until the time of analysis. Samples were tested using NMR as well as an enzymatic creatinase spectrophotometric assay for creatine. For the enzymatic assay, creatine concentration was determined by a coupled enzyme reaction, which results in a colorimetric (570 nm)/fluorometric (λex = 535/λem = 587 nm) product which is proportional to the amount of creatine present in the sample.

Samples from the Catheterization Genetics (CATHGEN) study that had creatinine results generated by both the kinetic colorimetric assay (Jaffe method) and NMR (n = 1172) were used for the creatinine method comparison study. Briefly, the kinetic colorimetric assay (Jaffe method) entails the formation of a yellow-orange complex between creatinine and picrate (in potassium phosphate buffer) and the rate of dye formation is proportional to the creatinine concentration in the sample. Samples were run on a Roche cobas c702 as per manufacturer's instructions.

2.1.5. Analytical validation of the NMR-based dual creatine and creatinine assay

Linearity was evaluated using serially mixed source pools with low and high concentrations of creatine and creatinine according to CLSI

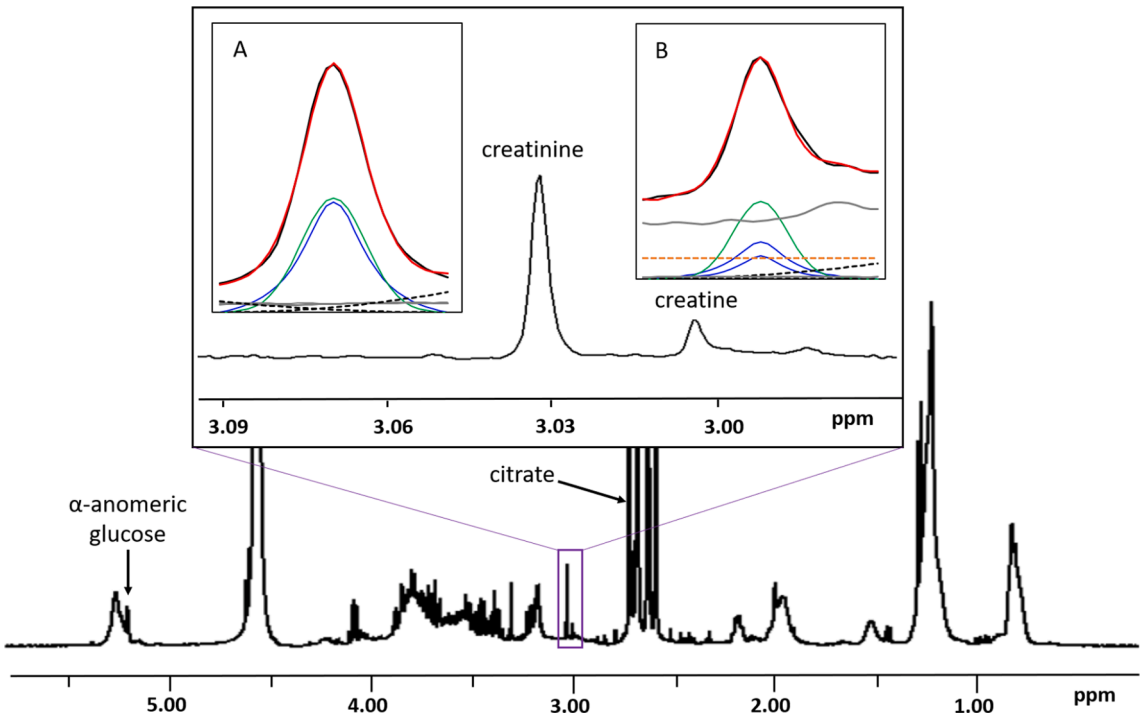

Fig. 1. 1D ^{1}H CPMG spectrum collected on a 400-MHz spectrometer used to quantify creatinine and creatine in serum/plasma. Expanded region shows creatinine and creatine peak locations at pH 5.3 that would otherwise overlap at physiological pH. Insets A and B show the superimposed observed signal (black) and the mathematical model (red) obtained from a composite of signal components (Lorentzian (blue) and Gaussian (green) functions, experimentally acquired component (gray), quadratic (black dashed line) and linear (orange dashed line) functions), for creatinine and creatine, respectively. The α-anomeric glucose and leftmost citrate peaks used as reference to locate the creatinine peak are also shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

guidelines [17]. A total of 9 mixed serum samples were generated and tested in quadruplicate on a single instrument. Assay linearity was assessed by linear and higher order polynomial regression of the test results of the prepared mixtures compared to the expected concentrations. To calculate the limits of blank (LOB), limits of detection (LOD) and the lower limits of quantification (LLOQ) of the assay, serum or EDTA plasma samples were prepared and tested in quadruplicate for three days according to CLSI guidelines, as previously described [12,18].

Serum or EDTA plasma pools with low and high levels of creatine and creatinine were prepared by mixing de-identified residual specimens and tested according to CLSI guidelines in order to determine imprecision [19]. Within-run (intra-assay) imprecision was determined by testing 20 replicates of each pool in a single day on one instrument. Within-laboratory (inter-assay) imprecision was determined by testing the low and high pools in duplicate, two times per day, for 19–20 days (n = 75–80). Mean, standard deviation (SD) and % coefficients of variation (%CV) were calculated.

For tube comparisons, blood was drawn from 29 volunteers into the following specimen collection tubes: Greiner Bio-One (also known as LipoTube) serum tubes, red-top plain serum tubes (without the gel barrier), and K_2EDTA purple-top plasma tubes. Specimens were processed immediately as per the manufacturer's directions for each specimen tube. Samples were tested in singlicate. As per CLSI guidelines [20], a bias of > 10% between collection tubes was considered technically significant. Fresh serum or EDTA plasma samples from 2 to 3 donors were used to assess the stability of creatine and creatinine at controlled room temperature (20–25 °C), refrigerated (2–8 °C) and frozen (−20 °C; <−70 °C) temperatures, as well as four freeze–thaw cycles. Creatine and creatinine were considered stable if the difference between the result at time 0 and the following time point was < 10%.

A total of 18 substances (6 endogenous and 12 common drugs/metabolites) were tested in vitro for potential analytical interference on the creatine and creatinine results according to CLSI guidelines [21]. Stock solutions and samples were prepared as previously described [18]. Substances eliciting a greater than 10% difference were claimed to interfere with test results.

2.2. Objective 2

2.2.1. Study design and population

For the current prospective cohort study, data were extracted from the ongoing TransplantLines Biobank and Cohort study of the University Medical Center Groningen (UMCG) (ClinicalTrials.gov Identifier: NCT03272841). All eligible transplant recipients who gave written informed consent were included in the TransplantLines cohort from June 2015 onward. The Medical Ethical Committee of the UMCG approved the TransplantLines study protocol (METc 2014/077), and all study procedures were performed in line with the principles of the Declaration of Helsinki. Further details on the design of the TransplantLines cohort have been described previously [22]. For the current study, we included all adult KTR (≥18 years) enrolled in the TransplantLines study between June 2015 and February 2021. KTRs with missing data regarding plasma creatine concentration, estimated intramuscular creatine concentration and fatigue were excluded, leaving 618 KTR eligible for further analysis who in total had 1007 outpatient clinic visits.

2.2.2. Data collection

Anthropometric measures, i.e., height and weight, were performed using a wall-secured stadiometer and a digital scale, respectively. Muscle mass was assessed using a multi-frequency bio-impedance analysis device (Quadscan 4000, Bodystat, Douglas, British Isles) and the equation by Janssen et al. 2000 [23]. The equation by Janssen et al. 2000 uses height, sex, age, resistance and reactance to estimate total skeletal muscle mass, rather than appendicular skeletal muscle mass, as assessed in many other equations.

Demographic variables and data on disease history, medication and transplant characteristics were extracted from electronic patient files. Fasting blood samples and 24-hour urine samples were collected and analyzed by using standard laboratory procedures, prior to the TransplantLines study visit. The serum creatinine-based Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) algorithm of 2021 was used to calculate the eGFR [24]. The total creatine pool was defined as the 24-h urinary creatinine excretion (mmol/day) divided by 0.017, assuming that 1.7% of the creatine pool is degraded to creatinine and excreted in the urine [8,25]. The estimated intramuscular creatine concentration was defined as the creatine pool divided by the total skeletal muscle mass, given that roughly 95% of all creatine pool is stored in skeletal muscle [26–30].

2.2.3. Assessment of fatigue

Fatigue was assessed using the checklist individual strength (CIS). The 20-item CIS is a self-reported multidimensional instrument to assess four qualitatively different domains of fatigue, including fatigue severity (range 8–56 points) physical activity (range 3–21 points), concentration problems (range 5–35 points) and reduced motivation (range 4–28 points). Higher CIS scores indicate higher fatigue burden, and less physical activity, more concentration problems, and/or reduced motivation, respectively.

2.2.4. Statistical analyses

Statistical analyses were performed using R version 4.3.0 (Vienna, Austria). Results were expressed as mean ± standard deviation (SD), median [interquartile range], or number (percentage) for normally distributed, skewed, and categorical data, respectively. $P < 0.05$ was considered to indicate statistical significance. To assess the associations of plasma creatine concentration and estimated intramuscular creatine concentration with potential determinants, linear mixed effect regression models implemented in the 'lme4′ R package were used. Analyses included a random intercept for each KTR to account for within-participant correlations and are adjusted for time between transplantation and visit, age, sex, height and weight. In these analyses, plasma creatine concentration and estimated intramuscular creatine concentration were taken as the dependent variables. Regression coefficients were given as standardized beta (Std. β) values, referring to the number of standard deviations a dependent variable changes per one standard deviation increase of the independent variable. Potential effect-modification by age or sex was explored by including product terms in the model.

To assess the associations of plasma creatine concentration and estimated intramuscular creatine concentration with self-reported fatigue, linear mixed effect regression models were also used. All models included a random intercept for each KTR to account for within-participant correlations and were stepwise adjusted for the a priori defined potential confounders time between transplantation and visit, age, sex (model 1), height, weight, eGFR, urinary protein excretion (model 2), anemia and diabetes (model 3). No covariance matrix was included in the analysis as our study design did not involve the examination of covariance effects. The focus of this analysis was primarily on estimating the fixed effects and examining the relationships between the predictor variables and the dependent variables. In these analyses, the various domains of fatigue were the dependent variables. To account for potential bias in the linear mixed effects regression analyses that could result from the exclusion of participants with missing values in potential confounding variables, multiple imputation was performed using fully conditional specification to obtain 10 imputed data sets. The algorithm was run for 10 iterations and convergence of the Markov chains was evaluated with trace plots of the mean and variance. Analyses were performed in each of the data sets and results were pooled using Rubin's rules. To assess the robustness of the findings, the following robustness analyses were performed: Analyses after excluding outliers (defined as values outside of two times the interquartile range below the first or above the third quartile), KTR with an age > 70 years and KTR with an eGFR < 25 ml/min/1.73 m^2. Furthermore, we performed analyses without using repeated measures, i.e. using only the first visit at > 1 year after transplantation per KTR.

3. Results

3.1. Objective 1

3.1.1. Development of a dual NMR-based assay to measure serum creatine and creatinine

The concentrations of creatine and creatinine were determined by modeling their respective NMR signal peaks, which arise from the methyl group protons and are illustrated in the CPMG spectrum (Fig. 1). The peaks were modeled to their component lineshapes and the creatine and creatinine concentrations were determined by converting the amplitudes of the signal peaks into concentration units. Given that the NMR spectra collected on Vantera® Clinical Analyzers are standardized each day and across instruments, the amplitudes of the spectral peaks are linearly related to the analyte concentrations [31]. Therefore, the signal peaks for creatine and creatinine were readily transformed into concentrations using conversion factors that were determined from standard curves with known creatine and creatinine concentrations. The units are mg/dL for use in clinical laboratories in the US and μmol/L for clinical laboratories in The Netherlands.

3.1.2. Comparison of NMR and enzymatic creatine and creatinine results

Method comparison studies were performed to compare serum creatine and creatinine results generated by the NMR assay versus the creatinase enzymatic assay for creatine (n = 50) and the kinetic colorimetric assay (Jaffe method) for creatinine (n = 1,172). Deming regression analysis of the creatine results produced an R value of 0.998, a slope of 0.990 and an intercept of 0.166 (Fig. 2**A**). The Bland-Altman plot revealed that there was a small bias for higher NMR results compared to the enzymatic assay results (Fig. 2**C**). Deming regression analysis of the creatinine results produced an R value of 0.990, a slope of 0.997 and an intercept of −0.149 (Fig. 2**B**). The Bland-Altman plot revealed that there was no significant bias between the results from the two assays; however, there were a few outliers in this large dataset (Fig. 2**D**).

3.1.3. Assay performance and stability of creatine and creatinine

To evaluate linearity, regression analyses were performed on the experimental results versus the expected concentrations in nine samples with varying levels of creatine and creatinine (Fig. 3). The line equation for the best fit for creatine was $Y = 0.996X + 0.165$ and the $R^2 = 1.000$. The equation for the best line for creatinine was determined to be $Y = 1.001X + 0.002$ and the R^2 value was 1.000. Linearity was demonstrated over a wide range of creatine 0.15 to 5.23 mg/dL (11.4 to 398.9 μmol/L) and creatinine 0.18 to 5.79 mg/dL (15.9 to 512.0 μmol/L) concentrations. The limit of blank (LOB), the analytical sensitivity or limit of detection (LOD), and the functional sensitivity or lower limit of quantitation (LLOQ) were determined to be: 0.08, 0.13 and 0.14 mg/dL (6.4, 10.2 and 10.5 μmol/L) for creatine and 0.06, 0.12 and 0.15 mg/dL (5.0, 10.9 and 13.3 μmol/L) for creatinine.

Intra-assay and inter-assay precision were evaluated using serum and EDTA plasma pools with low and high concentrations of creatine and creatinine. The results are summarized in Table 1. The serum %CV for creatine ranged from 1.4 to 5.7% for intra-assay and 3.5 to 6.0% for inter-assay precision, while the %CV for creatinine ranged from 1.3 to 6.4% for intra-assay and 2.6 to 6.3% for inter-assay precision. To be consistent with subsequent clinical data, precision was also calculated in EDTA plasma. Results were similar between serum (mg/dL) and plasma (μmol/L) (Table1). Reference intervals for NMR-measured creatine and creatinine were determined in an apparently healthy population. For creatine, the mean ± SD and reference interval in serum (n = 538) was

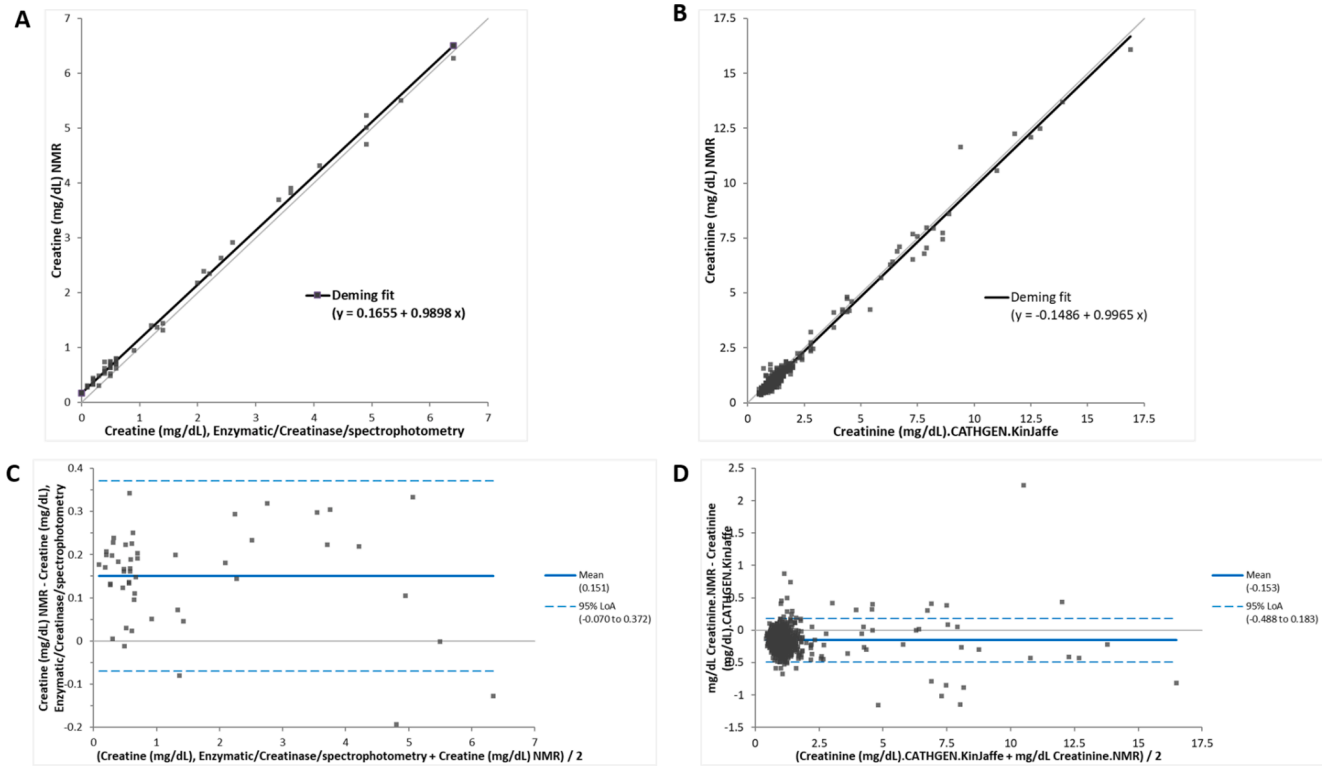

Fig. 2. Deming regression comparison between LC/MS/MS and NMR measured (A) creatine and (B) creatinine. Bland-Altman plots of the residuals for (C) creatine and (D) creatinine. The limits of agreement (LOAs) are depicted as dotted blue lines and 0% bias is a solid grey line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

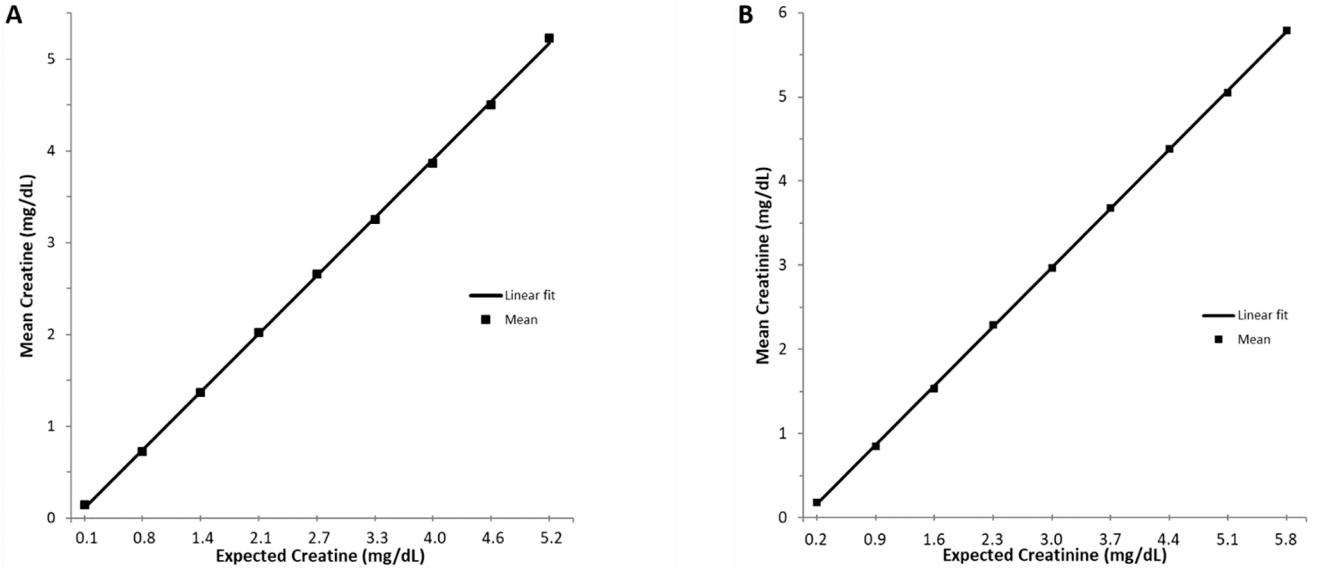

Fig. 3. Linearity of expected versus NMR-measured creatine (A) and creatinine (B).

0.63 ± 0.38 mg/dL (48.0 ± 29.0 μmol/L) and 0.18 to 1.51 mg/dL (13.7 to 116.0 μmol/L) and in plasma (n = 563) was 0.54 ± 0.34 mg/dL (41.1 ± 25.6 μmol/L) and 0.12 to 1.31 mg/dL (9.5 to 99.7 μmol/L). For creatinine, the mean ± SD and reference interval in serum (n = 538) was 0.90 ± 0.21 mg/dL (79.6 ± 18.6 μmol/L) and 0.56 to 1.36 mg/dL (49.5 to 120.3 μmol/L) and in plasma (n = 563) was 0.90 ± 0.22 mg/dL (79.7 ± 19.8 μmol/L) and 0.54 to 1.43 mg/dL (47.5 to 126.3 μmol/L).

Creatine and creatinine were measured in various specimen types in order to assess the suitability of standard specimen collection tubes for use with the assay. Results obtained in serum collected in LipoTubes were compared with those obtained for plain serum collected in red-top tubes (no gel barrier) and EDTA plasma collected in purple-top tubes. While no significant bias was observed for serum collected in LipoTubes vs. red top tubes (0.6% bias), creatine concentrations were lower in EDTA plasma by 11% compared to serum. There was no difference in creatinine concentrations between specimens collected in LipoTubes vs. red top serum tubes (−0.7% bias) or between specimens collected in LipoTubes and EDTA plasma tubes (−0.3%). Both creatine and

Table 1
Intra-assay and inter-assay imprecision measured in LipoTube serum and EDTA plasma pools with low and high concentrations of creatine and creatinine.

Specimen	LipoTube Serum				EDTA Plasma			
Analyte	Creatine (mg/dL)		Creatinine (mg/dL)		Creatine (μmol/L)		Creatinine (μmol/L)	
Pool	Low	High	Low	High	Low	High	Low	High
Intra-assay								
Mean	0.56	3.66	0.78	5.90	52.7	350.5	58.8	527.5
SD	0.03	0.05	0.05	0.08	3.6	8.8	6.0	13.1
%CV	5.7	1.4	6.4	1.3	6.8	2.5	10.2	2.5
Inter-assay								
Mean	0.57	3.88	0.76	5.87	53.0	337.9	60.9	527.7
SD	0.03	0.13	0.05	0.15	3.8	13.2	7.5	22.3
%CV	6.0	3.5	6.3	2.6	7.1	3.9	12.4	4.2

Abbreviations: CV, coefficient of variation; SD, standard deviation.

creatinine were stable for up to 15 days in serum (up to 8 days in plasma) when stored at controlled room temperature, up to 14 days for serum and plasma when refrigerated and 5 years when stored in a <−70 °C freezer. Stability may extend beyond the time points tested in the current study.

A total of 18 endogenous (conjugated and unconjugated bilirubin, hemoglobin, urea, uric acid, triglycerides) and exogenous (over-the-counter and prescription drugs: atorvastatin, fenofibrate, acetylsalicylic acid, acetaminophen, naproxen sodium, ibroprofen sodium salt, hydrochlorothiazide, metoprolol tartrate, nifedipine, enalaprilat diydrate, hydralazine hydrochloride, salicylic acid) substances were tested for potential interference with accurate creatine and creatinine assay results. Results revealed that none of these substances interfered with the assay results within the concentrations at which they occur naturally or within their therapeutic concentration range.

3.2. Objective 2

3.2.1. Baseline characteristics

A total of 618 KTR were included, whom in total had 1007 visits. Mean age was 56 ± 13 years, 62% were men and the mean eGFR was 54 ± 18 ml/min/1.73 m^2. The plasma creatine concentration was 27 [19–39] μmol/L and was higher in women than men (34 [24–50] versus 24 [18–32] μmol/L; P < 0.001). The estimated intramuscular creatine concentration was 27 ± 7 mmol/kg and was higher in women than men (29 [25–33] vs. 26 [22–30] mmol/kg; P < 0.001). The ratio between intramuscular creatine concentration and plasma creatine concentration was 1042 [739–1424] L/kg, which was lower in women than in men (904 [632–1263 vs 1123 [816–1557] L/kg; P < 0.001). An overview of all baseline characteristics is shown in Table 2.

3.2.2. Determinants of plasma creatine concentration and intramuscular creatine concentration

An overview of linear mixed-effects regression models of plasma creatine concentration and intramuscular creatine concentration with potential determinants is shown in Table 3. Plasma creatine concentration was positively associated with eGFR and with estimated intramuscular creatine concentration. Furthermore, plasma creatine concentration was negatively associated with male sex, height, and anemia. Estimated intramuscular creatine concentration was positively associated with weight and plasma creatine concentration. Furthermore, estimated intramuscular creatine concentration was negatively associated with age, male sex, time between transplantation and visit, and anemia.

3.2.3. Analyses of plasma creatine concentration and estimated intramuscular creatine concentration with self-reported fatigue

Fatigue was assessed using the four domains of the checklist individual strength (CIS) with higher scores implicating more impairment (Table 4). The total CIS score was 62 ± 26. The mean scores for the domains subjective fatigue and reduced activity were 27 ± 13 and 10 ± 5, respectively. The mean scores for the domains concentration problems and motivation problems were 13 ± 7 and 12 ± 6, respectively. Lower plasma creatine (Std. beta: −0.07, 95% CI: −0.14, −0.01; P = 0.023) and lower estimated intramuscular creatine concentration (Std. beta: −0.08, 95% CI: −0.14, −0.02; P = 0.009) were both associated with a lower total CIS score, indicating more fatigue. Plasma creatine concentration was not associated with the domains fatigue severity and reduced activity, whereas higher estimated intramuscular creatine concentration was associated with less fatigue severity and more physical activity, independent of potential confounders. Higher plasma creatine concentration was associated with less concentration problems, independent of potential confounders, whereas the estimated intramuscular creatine concentration was not. In addition, both higher plasma creatine concentration and higher estimated intramuscular creatine concentration were associated with less motivation problems.

A variety of sensitivity analyses were performed to assess the robustness of these findings (Table 5). After excluding outliers defined as two times the interquartile range above or below Q1 or Q3, plasma creatine concentrations remained negatively associated with total fatigue score. In analyses after excluding KTR aged >70 years and in analyses without using all available measurements (using only the first

Table 2
Characteristics of kidney transplant recipients (KTR) across 1008 patient visits in 618 KTR.

Variable	
Age, years	56 ± 13
Sex, n (%) male	629 (62)
Height, cm	174 ± 10
Weight, kg	82.1 ± 15.2
Time since transplantation, years	1.1 [0.7–5.0]
Anemia, n (%)	229 (25)
Diabetes, n (%)	266 (28)
eGFR, ml/min/1.73 m^2	54 ± 18
Urinary protein excretion, g/day	0.2 [0.1–0.3]
Plasma creatine, μmol/L	27 [19–39]
Plasma creatinine, μmol/L	114 [93–138]
Muscle mass (via BIA)[1], kg	28 ± 8
Creatine pool[2], mmol	735 ± 222
Estimated intramuscular creatine concentration, mmol/kg	27 ± 7
Creatine ratio muscle to plasma, L/kg	1042 [739–1424]
Checklist individual strength	
Fatigue severity	26 ± 13
Reduced activity	10 ± 5
Concentration problems	13 ± 7
Motivation problems	12 ± 6
Total score	62 ± 25

Abbreviations: BIA, bioelectrical impedance analysis; eGFR, estimated glomerular filtration rate.
[1] Estimated using the equation by Janssen et al. [23].
[2] Estimated using the 24 h urinary creatinine excretion.

Table 3
Linear mixed-effects regression models of plasma creatine concentration and estimated intramuscular creatine concentration with potential determinants.

Independent variables	Plasma creatine concentration		Estimated intramuscular creatine concentration	
	Std. β (95% CI)	P-value	Std. β (95% CI)	P-value
Age	0.01 (-0.06; 0.07)	0.8	−0.23 (-0.29; −0.16)	**<0.001**
Male sex	−0.24 (-0.31; −0.17)	**<0.001**	−0.32 (-0.38; −0.25)	**<0.001**
Height	−0.13 (-0.22; −0.03)	**0.007**	−0.07 (-0.16; 0.02)	0.1
Weight	0.04 (-0.03; 0.11)	0.25	0.08 (0.01; 0.15)	**0.02**
Time between baseline and visit*	−0.01 (-0.05; 0.03)	0.6	−0.10 (-0.17; −0.03)	**0.004**
Anemia	−0.11 (-0.17; −0.05)	**<0.001**	−0.15 (-0.21; −0.09)	**<0.001**
Diabetes	0.02 (-0.04; 0.09)	0.5	−0.05 (-0.11; 0.02)	0.5
eGFR Creat 2021	0.07 (0.01; 0.13)	**0.030**	0.03 (-0.04; 0.09)	0.4
Urinary protein excretion	0.01 (-0.05; 0.07)	0.7	0.04 (-0.02; 0.10)	0.2
Plasma creatine	–	–	0.16 (0.10; 0.27)	**<0.001**
Estimated intramuscular creatine concentration	0.15 (0.09; 0.21)	**<0.001**	–	–

Plasma creatine concentration is log2-transformed prior to analyses. Abbreviations: eGFR, estimated glomerular filtration rate.
All models included a random intercept for each participant to account for within-participant correlations and are adjusted for time between transplantation and visit, age, sex, height and weight.
Std. β: All betas are presented as standardized betas, reflecting the number of standard deviations change in the dependent variable per standard deviation change in the independent variable.
* Analyses performed for kidney transplant recipients (KTR) with time after transplantation > 6 months.

visit at 1 year after transplantation per KTR), higher plasma creatine concentrations and estimated intramuscular creatine concentration were no longer associated with less motivation problems. On the other hand, analyses after excluding KTR with outliers, KTR with an age >70 years and KTR with an eGFR <25 ml/min/1.73 m^2 and analyses without using repeated measures did not materially change the associations of plasma creatine concentration and estimated intramuscular creatine concentration with the total fatigue score, supporting the robustness of these findings.

4. Discussion

The current study presented a newly validated method for the simultaneous measurement of circulating creatine and creatinine. The NMR-based assay described here has good sensitivity, precision and demonstrated linearity well beyond the observed range in healthy individuals. The creatine and creatinine results generated by the assay were comparable to the commonly used enzymatic/spectroscopic methods. In addition, the NMR assay results are not affected by lipemia, hemolysis, or bilirubin (as assessed by the addition of excess triglycerides, hemoglobin or bilirubin, respectively), making it a potentially desirable method for quantifying these two important analytes. Moreover, the NMR assay involves a simple, automated sample preparation (i.e., dilution of serum/plasma with buffer on board the Vantera)

Table 4
Linear mixed-effects models of plasma creatine concentration with self-reported dimensions of fatigue in kidney transplant recipients (KTR) n = 618 over a total of 1007 visits.

Dependent variables	Plasma creatine concentration		Intramuscular creatine concentration	
	Std. β (95% CI)	P-value	Std. β (95% CI)	P-value
Checklist individual strength (CIS) domains (higher score reflecting more impairment)				
Fatigue severity				
Model 1	−0.06 (-0.10; 0.01)	0.08	−0.09 (-0.15; −0.03)	**0.004**
Model 2	−0.06 (-0.12; 0.01)	0.08	−0.09 (-0.16; −0.03)	**0.002**
Model 3	−0.05 (-0.11; 0.01)	0.1	−0.08 (-0.14; −0.02)	**0.012**
Reduced activity				
Model 1	−0.01 (-0.07; 0.06)	0.7	−0.09 (-0.15; −0.03)	**0.005**
Model 2	−0.01 (-0.08; 0.05)	0.7	−0.11 (-0.17; −0.04)	**<0.001**
Model 3	−0.04 (-0.39; 0.21)	0.8	−0.10 (-0.16; −0.03)	**0.003**
Concentration problems				
Model 1	−0.12 (-0.15; −0.19)	**<0.001**	−0.05 (-0.11; 0.01)	0.1
Model 2	−0.12 (-0.19; −0.06)	**<0.001**	−0.04 (-0.11; 0.02)	0.2
Model 3	−0.12 (-0.19; −0.05)	**<0.001**	−0.04 (-0.10; 0.03)	0.2
Reduced motivation				
Model 1	−0.07 (-0.13; −0.18)	**0.048**	−0.07 (-0.13; −0.01)	**0.033**
Model 2	−0.07 (-0.15; −0.04)	**0.040**	−0.07 (-0.14; −0.01)	**0.023**
Model 3	−0.09 (-0.15; −0.03)	**0.049**	−0.06 (-0.13; −0.01)	**0.046**
Total score				
Model 1	−0.08 (-0.14; −0.02)	**0.014**	−0.09 (-0.15; −0.03)	**0.003**
Model 2	−0.08 (-0.15; −0.02)	**0.013**	−0.10 (-0.16; −0.04)	**0.002**
Model 3	−0.07 (-0.14; −0.01)	**0.023**	−0.08 (-0.14; −0.02)	**0.009**

Plasma creatine concentration is log_2-transformed prior to analyses.
All models included a random intercept for each participant to account for within-participant correlations.
Model 1: Adjusted for time between transplantation and visit, age and sex.
Model 2: As model 1, additionally adjusted length, weight, estimated glomerular filtration rate and urinary protein excretion.
Model 3: As model 2, additionally adjusted for anaemia and diabetes.
A higher CIS score indicates a higher fatigue severity, more concentration problems, reduced motivation and reduced activity.

with good sample-to-result throughput, which makes it ideal for use in the clinical laboratory. Taken together, the newly developed assay offers an alternative and efficient quantification method for generating results for circulating creatine and creatinine for clinical research studies. Furthermore, we demonstrated that both plasma creatine concentrations and estimated intramuscular creatine concentrations are higher in women as compared to men KTR. Plasma creatine concentration was positively associated with eGFR and with the estimated intramuscular creatine concentration and negatively associated with anemia. Estimated intramuscular creatine concentration was negatively associated with age, time since transplantation and anemia.

Importantly, the current study provides evidence supporting the relationship between creatine concentrations and self-reported fatigue. Both higher plasma creatine concentration and higher estimated intramuscular creatine concentration were associated with a lower fatigue burden. Higher plasma creatine concentrations were linked to fewer mental concentration and motivation problems, while higher estimated

Table 5
Sensitivity analyses on the associations of plasma creatine concentration and intramuscular creatine concentration with self-reported dimensions of fatigue in kidney transplant recipients (KTR).

Dependent variables	Plasma creatine concentration		Intramuscular creatine concentration	
	Std. β (95% CI)	P-value	Std. β (95% CI)	P-value
CIS domains (higher score reflecting more impairment)				
Fatigue severity				
Base	−0.05 (-0.11; 0.01)	0.1	−0.08 (-0.14; −0.02)	**0.012**
No outliers*	−0.07 (-0.13; −0.01)	**0.043**	−0.08 (-0.14; −0.02)	**0.014**
Single measurements**	−0.05 (-0.14; 0.03)	0.2	−0.13 (-0.21; −0.05)	**0.002**
Age < 70 years	−0.05 (-0.12; 0.01)	0.1	−0.07 (-0.14; −0.01)	**0.018**
eGFR > 25 ml/min/ 1.73 m^2	−0.05 (-0.12; 0.01)	0.1	−0.08 (-0.14; −0.02)	**0.015**
Reduced activity				
Base	−0.04 (-0.39; 0.21)	0.8	−0.10 (-0.16; −0.03)	**0.003**
No outliers*	−0.04 (-0.10; 0.03)	0.3	−0.10 (-0.17; −0.04)	**0.002**
Single measurements**	−0.02 (-0.10; 0.06)	0.7	−0.17 (-0.25; −0.09)	**<0.001**
Age < 70 years	−0.01 (-0.07; 0.07)	0.9	−0.08 (-0.15; −0.02)	**0.010**
eGFR > 25 ml/min/ 1.73 m^2	−0.02 (-0.09; 0.05)	0.6	−0.09 (-0.16; −0.03)	**0.004**
Concentration problems				
Base	−0.12 (-0.19; −0.05)	**<0.001**	−0.04 (-0.10; 0.03)	0.2
No outliers*	−0.12 (-0.19; −0.05)	**<0.001**	−0.04 (-0.11; 0.02)	0.2
Single measurements**	−0.15 (-0.23; −0.06)	**<0.001**	−0.07 (-0.16; 0.01)	0.1
Age < 70 years	−0.11 (-0.18; −0.05)	**0.001**	−0.02 (-0.09; 0.04)	0.5
eGFR > 25 ml/min/ 1.73 m^2	−0.11 (-0.18; −0.04)	**0.001**	−0.03 (-0.10; 0.03)	0.3
Reduced motivation				
Base	−0.09 (-0.15; −0.03)	**0.049**	−0.06 (-0.13; −0.01)	**0.046**
No outliers*	−0.08 (-0.14; −0.01)	**0.024**	−0.07 (-0.14; −0.01)	**0.029**
Single measurements**	−0.05 (-0.14; 0.03)	0.2	−0.07 (-0.16; 0.01)	0.09
Age < 70 years	−0.05 (-0.12; 0.02)	0.1	−0.06 (-0.12; 0.01)	0.08
eGFR > 25 ml/min/ 1.73 m^2	−0.07 (-0.14; −0.01)	**0.035**	−0.07 (-0.13; −0.01)	**0.038**
Total score				
Base	−0.07 (-0.14; −0.01)	**0.023**	−0.08 (-0.14; −0.02)	**0.009**
No outliers*	−0.09 (-0.16; −0.03)	**0.005**	−0.09 (-0.09; −0.02)	**0.007**
Single measurements**	−0.09 (-0.17; −0.01)	**0.043**	−0.14 (-0.22; −0.05)	**0.002**
Age < 70 years	−0.07 (-0.14; −0.01)	**0.036**	−0.07 (-0.14; −0.01)	**0.025**
eGFR > 25 ml/min/ 1.73 m^2	−0.08 (-0.14; −0.01)	**0.018**	−0.08 (-0.14; −0.02)	**0.013**

Plasma creatine concentration is log_2-transformed prior to analyses.
All models included a random intercept for each participant to account for within-participant correlations and are adjusted for time between transplantation and visit, age, sex, length, weight, eGFR, urinary protein excretion, anaemia and diabetes.
A higher CIS score indicates a higher fatigue severity, more concentration problems, reduced motivation and reduced activity.
* Outliers were defined as 2 times the interquartile range above or below Q1/Q3.
** Single measurement refers to only using the first visit at 1 year after transplantation per KTR.

intramuscular creatine concentration was associated with reduced fatigue severity, increased physical activity and less motivation problems.

Creatine plays a crucial bioenergetic role in adenosine triphosphate turnover and is especially important in tissues with high and fluctuating energetic demands, that is skeletal muscles, brain and heart. Creatine is produced naturally within the human body, but it is also found in diets that contain animal products. The first step of the biochemical synthesis of creatine is facilitated by the enzyme arginine:glycine amidinotransferase (AGAT), which converts arginine and glycine into guanidinoacetate, and it is at this step that regulation of endogenous creatine synthesis occurs [2,8]. In humans, highest AGAT activities are present in kidney, while other tissues, such as brain, pancreas and testes, are believed to express lower activities of AGAT. The high expression of AGAT in the kidneys likely explains the positive association we found between plasma creatine concentration and eGFR. In previous studies, we also found that creatine concentrations are lower in patients with virtually no renal function, i.e. hemodialysis patients, as compared to the general population [32]. Indeed, the creatine concentrations of the KTR in the current study were also lower as compared to those of the general population, which were on average 27 vs 37 μmol/L, respectively [33,34]. The second step of creatine synthesis is facilitated by the liver enzyme guanidinoacetate N-methyltransferase (GAMT), converting guanidinoacetate to creatine by performing a methylation step, which uses up to 40% of all endogenously generated S-adenosylmethionine. The importance of creatine and endogenous creatine synthesis becomes apparent from the rare inherited AGAT and GAMT deficiency syndromes, leading to severe mental retardation, autism, epilepsy movement disorders, hypotonia, and fatigue [35–37]. After being released into the circulation, creatine is transported into tissues by the creatine transporter 1 (CrT1), encoded by the SLC6A8 gene, located on the X-chromosome. Through the years, several sex-based differences in creatine homeostasis have been identified. Rates of endogenous creatine biosynthesis in women have been found to be lower than in men, which is also reflected by lower serum guanidinoacetate concentrations in women than in men. In line with this, the total creatine pool is also lower for women, as evidenced by the urinary creatinine excretion [38]. Generally, dietary creatine intake is also lower in women than in men [39]. Despite the lower production and intake in women, we found significantly higher plasma concentrations of creatine in women, compared to men. Already in 1989 Delanghe et al. reported potential sex-based differences in serum creatine in a small population of 60 healthy adults and also found significantly higher creatine concentrations in women compared to men (on average 50 μmol/L in women and 41 μmol/L in men) [40]. In a large population-based cohort we previously found a similar difference of 12.3 μmol/L higher concentrations in women whereas in a hemodialysis cohort a difference of 14 μmol/L was found [32,33]. In the current study, the difference was also comparable with women having a 12 μmol/L higher plasma creatine concentration. Of note, we also found that the estimated intramuscular creatine concentration was higher in women than in men, albeit with a smaller percentual difference of roughly 10% [41]. It should be noted that this is in alignment with an earlier human study that found 10% higher creatine concentrations in women as compared to men in the vastus lateralis muscle using biopsies [41]. Furthermore, the gradient (ratio) between intramuscular and circulating creatine was nearly 1000, but was higher in men as compared to women.

Analyses into potential determinants of plasma creatine concentration and estimated intracellular creatine concentration highlighted certain similarities and differences. Both were inversely associated with male sex and anemia, while they were positively associated with each other. In contrast, only the estimated intramuscular creatine concentration was inversely associated with age and time since transplantation, hinting that creatine stores are depleted over time.

Fatigue is an important patient reported outcome in many populations, including KTRs [42]. Unfortunately, fatigue is often underestimated and underrecognized, as demonstrated by a previous study

that found that fatigue was found in 59% of KTRs, while only 13% had this symptom documented in medical records [42]. The same study found that fatigue in KTRs was in the same range as chronically ill patients, with reduced activity, and reduced motivation levels approaching those observed in chronic fatigue syndrome. The current study demonstrated that low plasma creatine concentrations as well as low estimated intramuscular creatine concentrations were associated with higher overall fatigue, as evidenced by the CIS total score. Of interest, the estimated intramuscular creatine concentration was associated most strongly with the reduced activity, whereas plasma creatine concentration was most strongly associated with concentration problems. Creatine supplementation has been demonstrated to increase both the plasma creatine concentration and intracellular creatine concentration [43], but whether or not creatine supplementation is able to decrease fatigue in KTR is yet to be investigated.

Some limitations should be considered when interpreting these findings. First, the cross-sectional and observational nature of the study limits the ability to establish causality. Longitudinal studies and intervention trials would be valuable in determining the causal relationship between creatine concentrations and fatigue outcomes. Additionally, our study was focused on estimated intramuscular creatine concentration calculated using a combination of laboratory and bioimpedance data, which could not precisely reflect actual intramuscular creatine stores. However, it should be noted that the daily creatine degradation to creatinine has been shown to be constant, and vary little within individuals, supporting the use of this method.

In conclusion, we presented a validated method for the simultaneous measurement of circulating creatine and creatinine. This assay offers the potential for accurate and efficient quantification, reducing technician time and instrument costs. Both higher plasma creatine concentration and higher estimated intramuscular creatine concentration were associated with a lower total fatigue score, reflecting more fatigue. Higher plasma creatine concentration was linked to fewer concentration problems and motivation problems, while higher estimated intramuscular creatine concentration was associated with reduced fatigue severity, increased physical activity and motivation problems. These findings suggest that creatine may play a beneficial role in mitigating fatigue and enhancing cognitive and physical performance. Further research is needed to elucidate the underlying mechanisms and to explore the potential therapeutic applications of creatine supplementation in kidney transplantation-associated fatigue management.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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