REVIEW

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D_3-creatine dilution for skeletal muscle mass measurement: historical development and current status

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Abstract

The French chemist Michel Eugène Chevreul discovered creatine in meat two centuries ago. Extensive biochemical and physiological studies of this organic molecule followed with confirmation that creatine is found within the cytoplasm and mitochondria of human skeletal muscles. Two groups of investigators exploited these relationships five decades ago by first estimating the creatine pool size *in vivo* with ^{14}C and ^{15}N labelled isotopes. Skeletal muscle mass (kg) was then calculated by dividing the creatine pool size (g) by muscle creatine concentration (g/kg) measured on a single muscle biopsy or estimated from the literature. This approach for quantifying skeletal muscle mass is generating renewed interest with the recent introduction of a practical stable isotope (creatine-(*methyl*-d_3)) dilution method for estimating the creatine pool size across the full human lifespan. The need for a muscle biopsy has been eliminated by assuming a constant value for whole-body skeletal muscle creatine concentration of 4.3 g/kg wet weight. The current single compartment model of estimating creatine pool size and skeletal muscle mass rests on four main assumptions: tracer absorption is complete; tracer is all retained; tracer is distributed solely in skeletal muscle; and skeletal muscle creatine concentration is known and constant. Three of these assumptions are false to varying degrees. Not all tracer is retained with urinary isotope losses ranging from 0% to 9%; an empirical equation requiring further validation is used to correct for spillage. Not all tracer is distributed in skeletal muscle with non-muscle creatine sources ranging from 2% to 10% with a definitive value lacking. Lastly, skeletal muscle creatine concentration is not constant and varies between muscles (e.g. 3.89–4.62 g/kg), with diets (e.g. vegetarian and omnivore), across age groups (e.g. middle-age, ~4.5 g/kg; old-age, 4.0 g/kg), activity levels (e.g. athletes, ~5 g/kg) and in disease states (e.g. muscular dystrophies, <3 g/kg). Some of the variability in skeletal muscle creatine concentrations can be attributed to heterogeneity in the proportions of wet skeletal muscle as myofibres, connective tissues, and fat. These observations raise serious concerns regarding the accuracy of the deuterated-creatine dilution method for estimating total body skeletal muscle mass as now defined by cadaver analyses of whole wet tissues and *in vivo* approaches such as magnetic resonance imaging. A new framework is needed in thinking about how this potentially valuable method for measuring the creatine pool size *in vivo* can be used in the future to study skeletal muscle biology in health and disease.

Keywords body composition; isotope dilution; malnutrition; nutritional assessment

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Introduction

Skeletal muscle is the largest component of the human body except in people with obesity in whom adipose tissue predominates.[1] Despite such a large relative mass and clinical relevance, the total amount of skeletal muscle present in humans has been difficult to accurately quantify.[2] Direct assessment of total body skeletal muscle mass in human cadavers, of which only 51 are reported since the 19th century,[3] is a tedious process that is fraught with measurement errors.[4] The knowledge base on the total body skeletal muscle mass component in humans is thus formulated around indirect measurement methods applied *in vivo* across the full lifespan.[2] An early approach for estimating total body skeletal muscle mass, dilution of labelled creatine,[4–6] is now gaining fresh interest.[7,8] Here, we review the historical evolution and recent developments of this method. A timeline for major milestones in the development of creatine dilution methods for estimating total body skeletal muscle mass is shown in *Figure* 1.

Historical development

Biochemistry and physiology

Creatine, an organic compound, was discovered in meat extracts by the French chemist Michel Eugène Chevreul in 1832.[9] Many studies in the early and mid-20th century clarified the biochemical and physiological properties of the creatine molecule that are relevant to an understanding of the creatine-muscle mass method. Myers and Fine in 1913 and others of that era confirmed that creatine is present in vertebrate skeletal muscles and other tissues.[10] The Eggletons at Cambridge and Fiske and Subbarow at Harvard separately discovered in 1927 that creatine *in vivo* exists in two forms, free and phosphorylated (phosphocreatine).[11,12] Studies over the next several decades established that creatine can be derived *in vivo* from two sources, the pre-formed molecule from foods containing meat and fish and endogenous synthesis. Endogenous creatine is synthesized from three amino acids, arginine, glycine, and *S*-adenosyl-methionine by the liver, kidney, and to a less extent pancreas.[13,14] Both ingested and synthesized creatine are then actively transported across cell membranes into target cells via a sodium (+) and chloride (−) dependent mechanism where creatine is then phosphorylated in the cytosol and mitochondria by the enzyme creatine kinase.[13,15] Diet and endogenous synthesis in omnivorous adults each contribute about 1 g/day to the total creatine pool of about 100–150 g, approximately 40% of which is free creatine and 60% is phosphocreatine.[14] Creatine and phosphocreatine serve as part of the intracellular processes that provide the energy needed primarily for myofibrillar contraction.[14]

Creatine needs to be replenished by the diet and endogenous synthesis because the molecule undergoes non-enzymatic conversion to creatinine at a rate of about 1.7% or about 1.5–2.0 g/day[16] secondary to the combined catabolism of free creatine and phosphocreatine at respective rates of 1.1% and 2.64% per day.[17] The resulting creatinine diffuses out of the cell and is excreted unchanged in urine through glomerular filtration and to a less extent tubular secretion. Under steady state conditions, urinary creatinine is thus a good marker of a person's creatine pool size. Myers and Fine in 1913 recognized this linkage, stating that 'a constant relation exists between the total creatine of the body and the daily creatinine elimination'.[10] Burger took this observation one step further in 1919, reporting that 1 g of

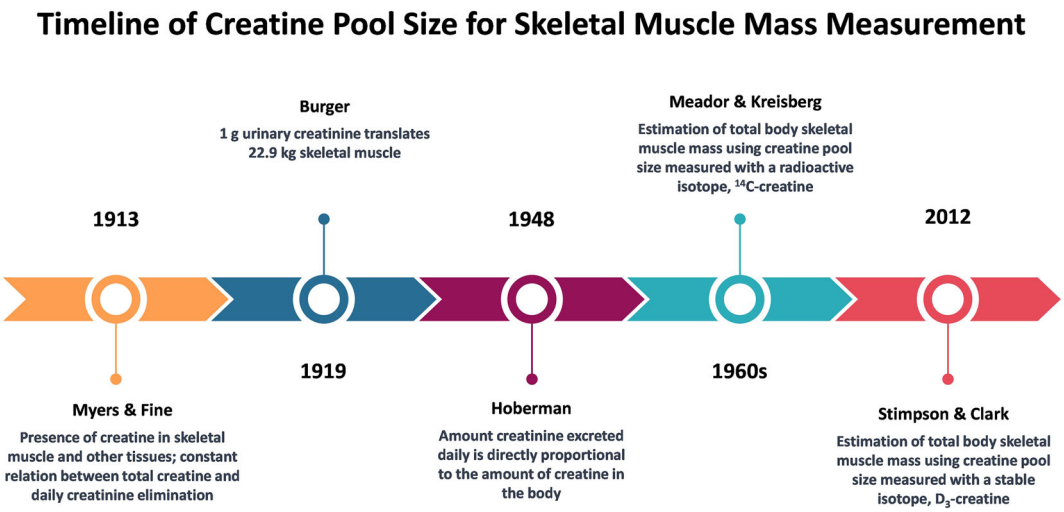

Figure 1 Timeline of milestones in the evolution of the creatine pool size estimation method for skeletal muscle mass.

urinary creatinine per day translates to 22.9 kg of whole wet skeletal muscle in adults.[18] Conducting elegant ^{15}N-labelled creatine studies in 1948, Hoberman and his colleagues stated that 'the amount of creatinine excreted daily was shown to be directly proportional to the amount of creatine in the body'.[16] Chinn, in his 1966 body composition study, showed a strong linear correlation between 24 h urinary creatinine and total-body creatine in full-fed and restricted-fed albino rats (*Figure* S1).[19] Reeds *et al*. similarly showed a good linear correlation between 24 h urinary creatinine and in Jamaican children before and following recovery from protein-energy malnutrition (*Figure* S1).[20]

Early methods

With the rapidly increasing understanding of creatine biology and physiology, Clifton K. Meador and his colleagues working at the University of Alabama in Birmingham[4] in the late 1960s recognized the possibility of estimating total body skeletal muscle mass by directly measuring the creatine pool size with a radiolabelled isotope. The investigators tested their approach in a rat model using creatine-1-^{14}C as the tracer. The investigators administered an intravenous dose of the isotope and the main route of radiolabel loss over the following 12 days was via urinary excretion; no isotope appeared in faeces. Most of the remaining ^{14}C was present in muscle with a small amount distributed across multiple other organs and tissues. These results led Meador *et al*. to assume that all creatine was present in muscle and that the average ^{14}C concentration present in four representative muscles reflected that of all muscles. Total body muscle mass was then calculated as

$$\frac{\text{total body creatine} - {}^{14}\text{C}}{\text{creatine-}{}^{14}\text{C concentration in muscle.}} \quad (1)$$

To test the accuracy of their estimates, the authors compared their results to the wet fat-free muscle mass acquired by dissection and chemical analysis of 24 rats. Calculated and anatomic muscle mass were significantly correlated (r = 0.941, $P < 0.001$) with a non-significant difference between mean values.

The Birmingham group, now led by Robert A. Kriesberg, extended their approach several years later to four adult humans.[5] Two of the participants were 'normal' and the other two had endocrinopathies. An intravenous dose of creatine-1-^{14}C was administered to the participants and daily urine collections were then analysed for ^{14}C. Urine ^{14}C losses were subtracted from the administered dose to derive the amount remaining in the participant's body. The ^{14}C concentration was next measured in a biopsied quadriceps muscle on day 8 and total wet fat-free muscle mass was calculated as in equation 1. The authors recognized that the required muscle biopsy would limit the applicability of their method outside of clinical research facilities. They therefore devised a simplified approach. Although details of their analyses are limited, Kriesberg *et al*. used the time-curve of radioactivity remaining in the person's body graphed at daily intervals to derive creatine half-life, turnover, and pool size. Rather than a muscle biopsy, they estimated wet skeletal muscle creatine concentration (4.3 g/kg) from limited data published in three earlier papers.[21–23] Total body wet fat-free skeletal muscle mass (kg) was then calculated as creatine pool size (g)/skeletal muscle creatine concentration (g/kg), the latter the purported literature value of 4.3 g/kg.[5] The skeletal muscle estimates by the two approaches in the four subjects were close with slightly higher values using the creatine pool size method.

Several years later, in 1976, Picou and colleagues applied a hybrid of Meador's method to estimate total body skeletal muscle mass in Jamaican infants and children recovering from protein-energy malnutrition.[6] Protein-energy malnutrition was a global concern during the 1970s much as the added concern with obesity is today. The group replaced ^{14}C labelled creatine with its stable ^{15}N counterpart, but retained intravenous dosing, pool size estimation, and the muscle biopsy. Their approach generated new knowledge on skeletal muscle growth in children, notably how the structure and selected biochemical features of muscle recover with refeeding following starvation.[20]

At the same time as Meador, Kriesberg, Picou, and their colleagues were developing and reporting their creatine dilution muscle mass methods, Godfrey Hounsfield was constructing the first computerized axial tomography (CT) scanner that was clinically evaluated in 1975.[24] Two years later, in 1977, Raymond Damadian conducted the first full-body magnetic resonance imaging (MRI) scan.[25] Richard Mazess was also developing dual photon absorptiometry systems during the 1970s that later were transitioned to dual-energy X-ray absorptiometry (DXA).[26] All three of these methods provided estimates of regional and whole-body skeletal muscle mass that soon swept into academic research programmes and even clinical practice. The isotopic creatine dilution methods for measuring muscle mass soon fell into obscurity given the radiation exposure with ^{14}C-creatine, use of an invasive biopsy, and laboratory facilities needed to implement studies using radiolabelled compounds and stable isotopes. Rather, the study of creatine biology during the 1970s turned to the question if supplementation has beneficial effects on physical performance and cardiovascular and cognitive function.[13,27–29]

Creatine-(*methyl*-d_3) dilution method

Several decades passed before introduction of another creatine dilution method for measuring total body skeletal mass

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by Stimpson *et al*. in 2012.[8] By then, disorders associated with loss in skeletal muscle mass and function such as sarcopenia, frailty, and cachexia were on the rise.[30] Stimpson's study in rats was followed 2 years later by the report of Clark *et al*. that included adult human evaluations.[7] The Stimpson–Clark method is based on the measurement of the whole-body creatine pool size by stable isotope dilution and the conversion of the estimated creatine pool size to skeletal muscle mass, a strategy like that of Kriesberg *et al*.[5]

The foundation of the Stimpson–Clark method is based on the previously reviewed biochemistry and physiology of creatine along with several key assumptions: that an oral dose of creatine-(*methyl*-d_3) (D_3-creatine), a stable isotope, is fully absorbed with no urinary or faecal isotope losses; that the absorbed labelled creatine enters the circulation and is transferred to the whole-body creatine pool located almost entirely in skeletal muscle; that the absorbed labelled skeletal muscle creatine concentrations are consistent within and between muscles; that the labelled creatine within skeletal muscle is slowly catabolized to creatinine that preserves deuterium abundance; and the D_3-labelled creatinine is cleared by the kidney and excreted in urine. The ratio of urinary D_3-creatinine to total creatinine is measured at a specified time point and whole-body creatine pool size is calculated using the dilution principle. Total body skeletal muscle mass can then be derived assuming the concentration of creatine in muscle tissue is known and stable across people.

This biochemical and physiological sequence as formulated lends itself to a single compartment model (*Figure* 2). Intracellular creatine phosphate is a small energy source and can only sustain large muscle group contractions for about 10 seconds; creatine re-phosphorylation occurs through interaction with adenosine triphosphate via the enzyme creatine kinase. The inherent dephosphorylation – phosphorylation cycle thus keeps the creatine pool well mixed. The only route of creatine catabolism is non-enzymatic stable conversion to creatinine; the ratio of urine D_3-creatinine to unlabelled creatinine thus preserves the D_3-creatine to total creatine ratio in muscle.

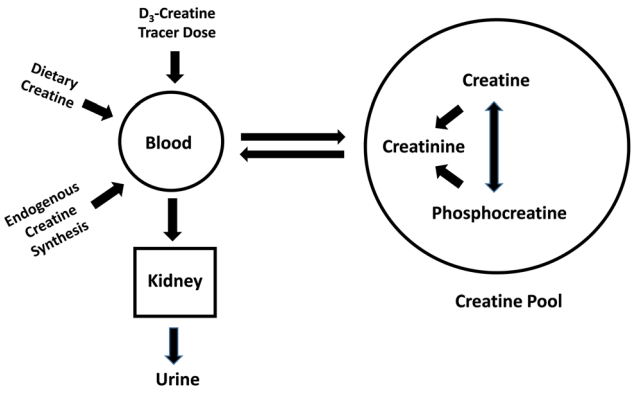

Figure 2 Single-pool model describing the D_3-creatine dilution method for estimating the total body creatine pool size.

The enrichment of D_3-creatinine in urine can thus be used as a measure of D_3-creatine enrichment. Because the rate of catabolism of creatine is slower than the distribution of D_3-creatine to skeletal muscle and the clearance of creatinine, the system mathematically can be treated as a single compartment in non-growing mammals. The creatine pool size can then be calculated according to the dilution principal that can be expressed mathematically using material balance as:

$$\text{tracee pool size (moles)} = \frac{\text{dose of tracer (moles)}}{\text{tracer concentration in tracee pool}} \quad (2)$$

where the tracer is D_3-creatine and the tracer concentration (enrichment) in the tracee pool (D_3-creatinine/total creatinine, both in molar units) is measured after the administered isotope is equally distributed in the creatine pool. The mean steady-state ratio of D_3-creatinine to total creatinine is measured in urine at a specified time point after the dose and pool size calculated according to the 'plateau' method.[31] The applied form of equation 2 is:

$$\text{creatine pool size (g)} = \frac{(131.1/134.1)\ \text{x}\ [D_3\ -\ \text{creatine dose (g)} – D_3\ -\ \text{creatine excreted (g)}]}{(\text{urine } D_3\text{-creatinine/total creatinine})} \quad (3)$$

In adults, the appearance of D_3-creatinine in urine requires just over 2 days to reach equilibrium after the dose and then its disappearance rate is slow with a half-life of 5 to 7 weeks. Equation 3 includes adjustment for unlabelled creatine and D_3-creatine molecular weights and correction for D_3-creatine dose spillage (excretion) in urine following its ingestion at baseline.

Other creatine models are also reported, as for example, Persky and Brazeau's[13] pharmacologic model includes multiple compartments with associated rate constants. The Persky–Brazeau model is useful for creatine dosing in clinical contexts.

Assumptions

The D_3-creatine dilution method for measuring skeletal muscle mass is dependent on the aforementioned assumptions, that if only partially true, will decrease the accuracy of pool size determination. The D_3-creatine dilution method is founded on assumptions like those of the traditional 24 h urinary creatinine excretion method of estimating skeletal muscle mass[32] with additional assumptions similar to other isotope dilution methods.[31]

Tracer absorption is complete

The first assumption is that 100% of the tracer dose is delivered to the creatine pool. In the case of the D_3-creatine method, this requires that the D_3-labelled creatine is fully absorbed when given orally. This assumption has been shown to be accurate by comparing the areas under the excretion

curves of D_3-creatine after oral and intravenous administration in small laboratory animals and finding they are equal.[8] Thus, orally administered isotope losses in faeces do not reduce the accuracy of the pool size measurement.

Tracer is all retained

The second assumption is that once absorbed, none of the tracer is lost from the system before it equilibrates with the creatine pool. This assumption is partially false as some of the D_3-creatine tracer can be lost in urine. This results in an overestimate of the creatine pool size that is proportional to the fraction spilled (i.e. 2% urinary loss = 2% pool size overestimate). In adults, the time required for the tracer to equilibrate across the body is about 40 h.[33] During the 3 days after the dose, the average tracer spillage in 38 adults varying in age was 1% in males with a range of 0 to 5% and 3% in females with a range of 0% to 9%.[33,34] There was no effect of age on spillage.[34] Shankaran and colleagues found that the creatine to creatinine ratio present in a fasting urine sample at baseline is significantly correlated with the cumulative proportion of the D_3-creatine dose excreted,[34] although the association is highly variable. This observation led to the development and validation of a dose spillage correction equation.[33,34] A spillage correction algorithm has not yet been validated in children or people over the age of 81 years.

A corollary of this assumption is that no new creatine enters the muscle creatine pool during the equilibration period, an effect that will lead to an overestimate of the creatine pool size. This is a minor factor in most healthy adults in whom the turnover of creatine is about 1.7% per day and the overestimate is thus about 3.4% for a specimen collected two days after the dose. In neonates, however, muscle increases in mass at a rate averaging about 17% per day and this growth is accompanied by an increase in the creatine pool size.[35] This is likely why the tracer enrichment (D_3-creatinine to total creatinine ratio in urine) does not reach a plateau in neonates. The plateau method is therefore not appropriate for estimating enrichment, as equilibration of the tracer cannot be identified by a flat (unchanging) period in the enrichment vs. time graph. An alternative model to the plateau method, such as the slope-intercept method, is thus required when evaluating growing infants. The influence of other examples of rapid growth or major replacement of damaged muscle mass such as severe soft tissue trauma and burn injuries have not been studied following D_3-creatine administration. More information is needed on the method when applied in patients with evolving weight loss and severe emaciation secondary to cancer and other forms of cachexia.

Tracer is distributed solely in skeletal muscle

This assumption is false. Creatine is distributed across multiple organs and tissues ranging in concentration from low to high (*Table* 1).[14] Skeletal muscle is a typical high concentration tissue at about 4–4.5 g creatine/kg fat-free wet weight[10,36–38] and has a total mass of about 17 kg and 28 kg in Reference Woman and Man, respectively.[39,40] The combination of high creatine concentration and large mass makes skeletal muscle the largest contributor to the total creatine pool. Heart has a lower creatine concentration (~2 g/kg)[41] and a smaller mass (~0.24–0.33 kg).[39] Brain has a creatine concentration of ~1.2–1.8 g/kg[42] and a mass of about 1.2–1.4 kg.[39] Organs and tissues outside of skeletal muscle thus add to the total creatine pool with estimates of muscle's contribution ranging from a low of about 90%[5,6,15,43,44] to a high of 98%[8,17,18,45] with an estimate of 95% the most frequent.[27,33] Notably, these are rough estimates as no studies are available for setting an exact value on the average proportion of the creatine pool residing in skeletal muscle. Meador administered creatine-^{14}C in his rat model[4] and recovered (X ± SE) 93.36 ± 0.49% of the isotope in skeletal muscle, although he was concerned with this estimate as some muscle tissue was lost during the dissection process. The total creatine pool size is thus 2% to 10% larger than the skeletal muscle tissue creatine pool size with the likely percentage between these two extremes.

Skeletal muscle creatine concentration is constant

This assumption is false. Total body skeletal muscle mass is calculated in the final step in the D_3-creatine method by dividing the measured pool size by an assumed 'constant' value for creatine concentration in skeletal muscle tissue. Most of the recent published studies using the creatine dilution method to estimate skeletal muscle mass used Kriesberg's approximation of 4.3 g per kg wet fat-free muscle.[5] There are many reports of skeletal muscle creatine concentrations over the past century, and several conclusions emerge from these studies.

First, estimates vary depending on how creatine concentrations are expressed with reference bases including whole-wet muscle, fat-free wet muscle, dry muscle, non-connective tissue muscle nitrogen (i.e. protein), alkali-soluble protein, and per unit deoxyribonucleic acid.[46] Kriesberg provided three citations for his estimate,[21–23] and these reports collectively only included five 'normal' adults whose creatine concentration in unspecified muscles ranged from 2.96 to 4.90 g/kg wet (not fat-free) weight. The origin of the widely used[34,35,47–52] 4.3 g/kg 'constant' is unclear.

Second, creatine concentration values are usually derived on specific muscles that are accessible to biopsy[21] and individual muscles vary in their creatine concentration. Pectoral, abdominal, leg, and psoas muscles are reported to have creatine concentrations of 4.62, 3.96, 3.91, and 3.89 g/kg, respectively.[53] Some muscles, such as the intercostals, sternocleidomastoids, tongue, and diaphragm have low creatine concentrations primarily because they are rich in connective tissue that 'dilutes' the myofibre mass.[36] That is why some investigators express creatine per unit non-collagen ni-

Table 1 Varying creatine concentrations (g/kg wet weight) in different organs and tissues

Low		Intermediate		High	
Lung	0.71 ± 0.07 GP53 0.76 ± 0.04 Mo53 0.71 ± 0.15 R53	Brain	1.23*‡‡ H40 1.76*‡‡ H40 1.19 H49 1.04 H49 0.33 ± 0.28†† H51 1.38 ± 0.16†† H51 1.29 D46 1.29 R46 1.32 ± 0.06 GP53 1.57 ± 0.18 Mo53 1.56 ± 0.18 R53	Skeletal Muscle	Normal*‡ 3.1–4.8 H48 Pathology** 1.33–7.0 H35 4.22–4.62* H54 4.75 ± 0.65 H58 3.91 H20 2.70‡‡‡ H20 2.97‡‡‡ H20 3.77–4.70 D45 4.3 Mk46 4.7 Mk46 4.24 R46 5.34 R46 2.87 ± 0.17 GP53 2.88 ± 0.12 Mo53 2.96 ± 0.15 R53
Kidney	0.14 D45 0.33 Mk46 0.26 R46 0.66 ± 0.08 GP53 1.06 ± 0.07 Mo53 0.90 ± 0.11 R53	Brown Adipose Tissue	0.48 ± 0.07 R47	Heart	3.55 ± 0.42 H55 2.16 ± 0.78† H55 1.5–2.2* H54 1.99 Mk46 3.26 D46 1.92 R46 1.69 ± 0.01 GP53 1.67 ± 0.05 Mo53 1.65 ± 0.18 R53
Spleen	0.15 R46 0.27 D46 0.19 Mk46	Intestine	0.67 R46	Seminal Vesicle Fluid	2.99 ± 0.40 Mo59 1.70 ± 0.20 R59
Liver	0.40 Mk46 0.06 R46 0.12–0.41 D45 0.98 ± 0.06 GP53 0.89 ± 0.11 Mo53 0.64 ± 0.16 R53 0.03 R50	Seminal Vesicles	1.9 ± 0.56 Mo59 0.7 ± 0.14 R59	Testes	1.81 D45 2.97 R46 1.64 ± 0.10 Mo59 1.47 ± 0.38 R59
White adipose tissue	0.05 ± 0.01 R47	Depending on publication, values are mean, mean ± SD, or min–max range.			
Blood cells	0.04 ± 0.01 HM52	D, dog; GP, guinea pig; H, human; HM, human male; HF, human female;			
Serum	0.05 ± 0.01 HF52	Mk, monkey; Mo, mouse; R, rat.			
Plasma	0.005 ± 0.002 HM52 0.006 ± 0.003 HF52 0.004 ± 0.001 HM56 0.003 ± 0.001 HF56 0.004 ± 0.002 HM57 0.005 ± 0.002 HF57				

*The data collected was from humans at the time of post-mortem exam; unless otherwise noted, death was not due to disease.
**Pathologies of 74 autopsied humans include: uraemia with creatinine retention (n = 8), bronchopneumonia (n = 19), tuberculosis (n = 9), malignancy (n = 6), acute inflammation (n = 9), circulatory involvement (n = 13), uraemia and heart failure (n = 4) and miscellaneous (n = 6).
‡This range is from the lowest concentration, found in the diaphragm to the highest concentration, found in the psoas and deltoid.
‡‡Pathologies of seven humans include: myocarditis, arsenic poisoning, stabbing followed by acute peritonitis, fractured skull (n = 3), and pneumonia.
‡‡‡These values are taken from biopsies of subjects with hypokalaemia and hyperkalaemia.
†These values are from cardiac patients with various pathologies that include: cardiac amyloidosis (n = 2), hypertensive heart disease (n = 4), valvular disease (n = 2), hypertrophic cardiomyopathy (n = 2), dilated cardiomyopathy (n = 1), and post-operative atrial septal defect (n = 1).
††These values are from the lowest mean brain creatine concentration, found in the hypophysis, and the highest mean, found in the cerebellar cortex.

trogen, a surrogate measure of contractile protein and myofibrillar mass.[54] Creatine phosphate and total creatine levels are also higher in type 2 than type 1 muscle fibres which explains why the vastus lateralis (59% type 2; creatine phosphate/total creatine, [X ± SD] 82.1 ± 6.0/133.9 ± 7.7 mmol/kg muscle) has a significantly ($P < 0.05$–0.01) higher phosphocreatine and total creatine level than the soleus muscle (35% type 2; 76.1 ± 6.7/100.6 ± 8.8).[27,55] There is also a likelihood that individual muscle proportions, and thus whole-body creatine concentration in skeletal muscle, varies with voluntary weight loss, body-building exercises, and muscle-wasting conditions. The creatine concentration of a single biopsied skeletal muscle thus cannot reliably serve as

representative of the average creatine concentration of total body skeletal muscle mass.

Third, skeletal muscle creatine concentrations are moderated by a person's health status, age, activity level, diet, and pregnancy status. Denis in 1916 and Corsaro in 1940 were among the first to show that creatine concentrations in skeletal muscles were markedly lowered in patients with severe emaciation.[36,37] This effect was attributed to relative increases in atrophied muscle fat and connective tissue with reductions in creatine-rich contractile fibres. Denis and Corsaro's hypothesis was confirmed in a classic 1946 study of Fischer and Ramsey who examined the chemical composition of experimental muscle atrophy in rabbit models.[54] Atrophied muscles had marked increases in collagen and reductions in non-collagen proteins, including myosin, an effect shown in patients with severe emaciation and cancer whose psoas muscle composition is graphically portrayed in *Figure* 3.[56] Active creatine-containing myofibre mass is thus relatively reduced in atrophied muscles. Similarly, diseases associated with muscle atrophy, such as Duchenne muscular dystrophy, are well known to be accompanied by low muscle creatine concentrations (<3 g/kg).[14,23,57–59] Increased plasma membrane fragility and creatine leakage from myofibrils due to dystrophin deficiency also contributes to the low skeletal muscle creatine levels present in patients with Duchenne muscular dystrophy.[14]

Three rare creatine deficiency syndromes are recognized that have variable effects on creatine-creatinine metabolism, two of which are creatine biosynthesis disorders (guanidinoacetate methyltransferase deficiency and L-arginine: glycine amidinotransferase deficiency), and one is an X-linked transmembrane creatine transporter deficiency.[60] All three of these inherited conditions are characterized by low brain creatine concentrations that are accompanied by developmental delays, intellectual disability, neurological manifestations, and behavioural disturbances. Low skeletal muscle creatine concentrations, hypotonia, and weakness are recognized in patients and comparable knockout mouse models with these syndromes.[15,60]

The first and second steps in endogenous creatine synthesis are catalysed by enzymes present mainly in the kidney (L-arginine : glycine amidinotransferase) and liver (guanidinoacetate *N*-methyltransferase), respectively. Disturbances in creatine metabolism and deficits in total body creatine are recognized in animal models of experimental uraemia and in patients with chronic kidney diseases.[15] Creatine deficiency is compounded by low protein diets for uraemia management and chronic dialysis treatments that can wash out creatine. Similarly, endogenous creatine synthesis is reduced in patients with chronic liver disease who also can be prescribed low protein diets for management of hepatic encephalopathy.[61] Cachexia is often present in patients with end-stage kidney and liver diseases who frequently have low skeletal muscle creatine concentrations.[37]

Age at both ends of the life spectrum has a marked effect on skeletal muscle creatine concentration. Studies in rabbits, chickens, and cats show a rise in muscle creatine concentration in the early postnatal period.[10,62] A shift in muscle composition towards a relative loss of myofibre mass and increase in connective tissue is well-established with aging and

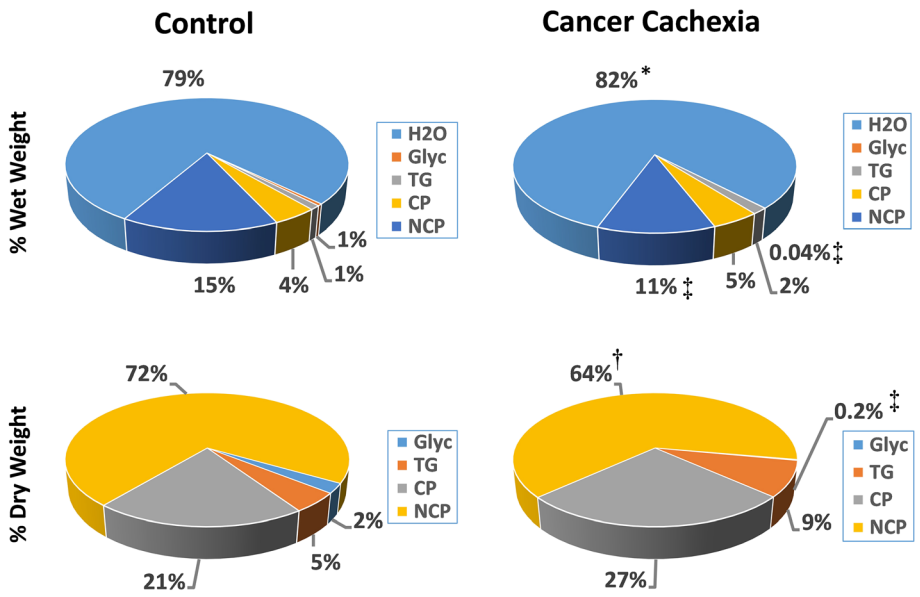

Figure 3 Skeletal muscle composition in controls (n = 11) and cancer patients (n = 14) with severe emaciation.[56] Atrophied psoas muscles in the cancer patients had increased water (H_2O) content, connective tissue protein (CP), and triglyceride (TG) while non-collagen protein (NCP) and glycogen (Glyc) were markedly reduced. Non-collagen protein is a surrogate marker of myofibre mass, the contractile portion of skeletal muscles. Tissue composition is expressed as a percent (%) of wet (upper) and dry (lower) weight. *P*-values: *0.10; †0.05; ‡0.01.

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conditions such as sarcopenia and frailty.[46,56,63] Forsberg and colleagues observed a lower total creatine concentration per kg DNA in older adults (age >61 years) compared with their younger counterparts.[46] In Clark's D_3-creatine study,[7] the ratio of creatine pool size to total body skeletal muscle mass measured with MRI was 4.5 and 4.6 g/kg in young men and middle-aged women (mean age 23 and 57 years), higher concentrations than observed in older men and women (mean ages 75 and 76 years; both 4.0 g/kg).

Level of physical activity and related body composition also moderates skeletal muscle creatine concentration. Sagayama *et al*. reported that young Japanese athletes had a creatine concentration of (X ± SD) 5.0 ± 0.5 g/kg MRI-measured total body skeletal muscle mass.[64] Morris-Paterson *et al*. evaluated the D_3-creatine method in a sample of British national-level male and female kayakers.[53] The observed mean creatine concentration was 5.4 and 5.3 g per kg MRI-measured total body skeletal muscle mass, respectively. High activity levels may lead to myofibre hypertrophy, a shift in muscle fibre type, stimulate creatine uptake via increased blood flow, or promote muscle membrane creatine translocation.[13] A related question is use of the D_3-creatine dilution method in exercise trials aimed at inducing anabolic effects on skeletal muscle. Can the method be used during the dynamic stage of the intervention?

Diet has long been known to moderate creatine physiology.[65,66] Vegetarians and vegans have lower tissue creatine and phosphocreatine concentrations than omnivores.[15,67] Supplementation with creatine can increase skeletal muscle creatine concentrations[13] and is one of the most widely used ergogenic aids for enhancing muscular strength and power among athletes and participants in fitness programmes.[29] For example, Balsom *et al*. in their review[27] reported that total creatine levels increased in the vastus lateralis muscle of adults with supplementation from about 120 mmol/kg to an upper limit of 160 mmol/kg dry muscle.

Lastly, Forsum and colleagues examined creatine concentrations in the quadriceps femoris muscle in pregnant women and non-pregnant controls. Creatine concentrations expressed per kg DNA and per kg alkali-soluble protein increased significantly ($P < 0.05$–0.01) at pregnancy weeks 18 and 36.[68]

The variability in the creatine pool size to total body skeletal muscle mass ratio is depicted by the graph shown in *Figure* 4. Mean group values for creatine pool size are plotted against MRI-measured total body skeletal muscle mass from the published literature[7,33,53,64] with corresponding slope (creatine mass/skeletal muscle mass) lines ranging from 3 to 6 g/kg. The data points fall between slopes of about 4 to 5.5 g/kg; the current assumed value for creatine skeletal muscle concentration is 4.3 g/kg.

The D_3-creatine dilution method thus has several sources of error that range in magnitude (*Table* 2). Assuming the creatine pool size represents solely skeletal muscle leads to an overestimate of uncertain size in the muscle compartment. This error can be estimated in experimental investigations and corrected for in future studies. The empirical spillage correction[33] also includes prediction error of uncertain magnitude and the developed model is not validated in children and elderly adults, another potential future task. Lastly, the assumed skeletal muscle creatine concentration used to derive total body muscle mass from pool size (4.3 g/kg) can at best now be referred to as a rough estimate. There thus exists serious limitations of the creatine dilution method as now applied, particularly as other more accessible approaches for evaluating regional and whole-body skeletal muscle mass are available in most research and clinical settings.[69,70] The optimum participant protocol and conditions for measuring the creatine pool size based on our aforementioned discussion have yet to be established. The main positive and negative features of the D_3-creatine pool size measurement method are summarized in *Table* 3. In the next section, we examine potential ways to overcome some of the concerns with the D_3-creatine dilution method as identified in our in-depth review.

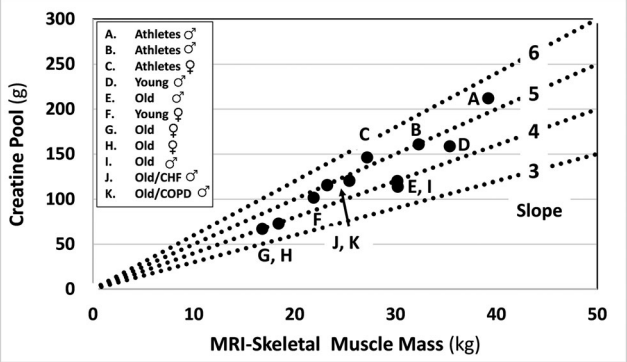

Figure 4 Association between measured mean creatine pool size and MRI-measured total body skeletal muscle mass as reported for groups in recent publications.[7,33,53,64] Hypothetical slopes (creatine concentration in skeletal muscle; creatine pool size/skeletal muscle mass) are shown in the figure overlaid on actual data points. This plot shows how, in the evaluated samples, whole-body creatine concentration of skeletal muscle varies from a low of about 3.8 to a high of 5.4 g/kg. Creatine pool size in one study was back-calculated from the reported creatine-muscle mass estimates.[33] CHF, congestive heart failure; COPD, chronic obstructive pulmonary disease. (A) Male athletes[53]; (B) male athletes[64]; (C) female athletes[53]; (D) young males[7]; (E) old males[7]; (F) young females[7]; (G) old females[7]; (H) old females[33]; (I) old males[33]; (J) old males with CHF[33]; (K) old males with COPD.[33]

Future potential approaches

Here, we examine four general approaches for improving the D_3-creatine dilution method for estimating total body skeletal muscle mass and its contractile tissue component. These comments are intended to stimulate new ideas on how to capture valuable aspects of the creatine dilution method

Table 2 Assumptions and potential sources of error in the D_3-creatine dilution method for measuring skeletal muscle mass

Assumption	Potential error sources
An oral dose of D_3-creatine is fully absorbed and retained	**Partially false**: although D_3-creatine appears to be fully absorbed, some isotope can be lost in urine, leading to pool size overestimation (mean values 1% in males and 3% in females): → ***overestimation of skeletal muscle mass***. Empirical spillage correction formulas associated with error at the individual level and have not been validated in all age groups.
The whole-body D_3-creatine pool is distributed only in skeletal muscle	**False:** Although skeletal muscle is the largest contributor (high creatine concentration; large mass), other organs and tissues contribute to pool size with reported ranges from 2% to 10% (mean 5%): → ***overestimation of skeletal muscle mass***.
The labelled intra-muscular D_3-creatine is slowly catabolized to creatinine	**True**: The only route of D_3-creatine catabolism is non-enzymatic conversion to D_3-creatinine.
D_3-creatinine is cleared by the kidney and excreted in urine	**True**: D_3-creatinine is cleared by the kidney and excreted in urine.
Skeletal muscle creatine concentrations are consistent (4.3 g/kg wet fat-free muscle) within and between all muscles	**False:** Creatine concentration in muscles ranges from ~3 to 5 g/kg, is not constant across all muscles, and varies according to the amount and type of muscle fibres, age, level of physical activity, and diet: → ***under or overestimation of skeletal muscle mass***.

Table 3 Main positive and negative features of the D_3-creatine dilution method for measuring creatine pool size *in vivo*

Positive	Negative
Safe, little subject burden and relatively easy to carry out	Feasibility and relative cost
Can be used in remote settings and bedside assessments	Specialized laboratory facilities needed for D_3-creatine measurement
Indirect method for measuring skeletal muscle contractile (myofibre) mass	Time-delay in obtaining results
Reported good associations with muscle function and clinical outcomes	Not a direct measure of total skeletal muscle mass: possible errors in estimation due to inaccuracy of current model assumptions
Whole-body pool size estimate	Cannot discern regional muscles that can have functional impairments

while moving away from the flawed approach as now applied.

Statistical model

First, because total body muscle creatine concentration varies with age, sex, activity level, diet, and other unspecified factors, it should be possible to eventually create skeletal muscle prediction models with covariates including creatine pool size and other definable factors. This approach would require the evaluation of pool size in a large and diverse sample along with skeletal muscle mass measured with a reference method such as CT or MRI. Skeletal muscle mass prediction models could then be developed and validated in this and other samples. However, it is hard to see the value of such a time-consuming approach when other methods that apply a similar strategy in estimating skeletal muscle mass[2,71] are so widely available and practical to apply.

Absolute pool size and indices

Second, rather than convert creatine pool size to muscle mass, this suggested approach would solely use absolute pool size as a muscle characteristic in research studies and clinical practice. An additional step would be to adjust pool size for between individual differences in body size, for example as an index such as creatine pool size/height^2. The height power of 2 is reasonable given that skeletal muscle mass in adults scales to height with a power of about 2.[72] A basis for this approach is suggested by the multiple studies that report good associations between D_3-creatine muscle mass estimates and various clinical 'outcomes'.[52,73–76] Here, the rationale is that the creatine pool size better reflects muscle 'function' than total wet or fat-free muscle mass that includes non-contractile elements such as connective tissue and fat. In effect, this approach makes the critical distinction between total skeletal muscle 'mass' and muscle 'contractile' mass. Another potential advantage of this approach is that it does not assume a constant proportionality between the creatine pool size and whole-wet skeletal muscle mass independent of body size as is now the common practice. If a plot of creatine pool size versus total body skeletal muscle mass has a non-zero intercept, then the assumed 'constant' (e.g. 4.3 g/kg) will vary as a function of muscle mass.

Another variation of this suggested approach is to convert creatine pool size to a body composition component such as

skeletal muscle cell protein. Baldwin reported that 0.0221 g of muscle creatine is present for each g of intracellular non-collagen protein.[21,51] A similar value (0.0235 g/g) was reported by Picou *et al*.[6] in Jamaican infants who had recovered from malnutrition. Forsberg *et al*. found strong correlations across men and women (R, 0.94 and 0.89) between total quadriceps femoris muscle creatine and alkali-soluble protein, a measure of myofibre protein mass, in adults.[46] Creatine concentrations were relatively stable (~0.0262 g/kg or 1 kg alkali-soluble protein = 0.038 g creatine) across age groups, although levels were about 10% higher in women. Thus, myofibre protein mass (kg) is roughly equal to 0.04 kg/g × creatine pool size (g). This type of model is conceptually like Moore's body cell mass (kg) model derived as 0.0092 kg/mmol × total body potassium (mmol)[77]; potassium, like creatine, is almost entirely found within the intracellular compartment.[21] The advantage of this approach is that muscle cell protein, in addition to being linked with contractile function, is an established component at the molecular level that lends itself to the development of more advanced body composition models.[19,78,79]

Combination models

Skeletal muscle is a complex tissue that contains both contractile and structural elements. Muscle function and 'quality' relates to both anatomic features. Notably, the extracellular matrix plays an important role in skeletal muscle's mechanical and structural properties.[80] As noted earlier, the proportion of skeletal muscle as contractile mass decreases and extracellular elements increase in people who are emaciated, with aging and inactivity, and in some muscle diseases. Thus, there is more information regarding muscle function and quality than provided solely by an estimate of myofibre mass. Proof-of concept for this hypothesis was recently established by Rush and Bourgeois and their colleagues[81,82] in a series of studies demonstrating improved strength predictions when DXA regional lean mass (i.e. a surrogate of whole-muscle) measures were combined with impedance-derived fluid distribution estimates. Even though they overlap, skeletal muscle mass (e.g. as measured with MRI or other imaging methods) and muscle myofibre mass (i.e. creatine pool size) thus contain different types of valuable information. These methods may be complementary rather than simply measuring the same thing. Multiple ways of formulating these relationships should be examined in future studies.

Combining creatine dilution space estimates with reference measurements of skeletal muscle mass by CT or MRI can advance fields well beyond those reviewed in this report. Our exploration of literature on chronic conditions associated with cancer and organ failure associated with cachexia revealed scattered and often conflicting findings on skeletal muscle creatine concentrations and related cellular metabolism. The availability of a stable isotope method of measuring the total body creatine pool size and the increasingly automated analysis of whole-body CT and MRI scans for skeletal muscle mass[83] presents a new opportunity to advance the non-invasive study of creatine biology in multiple disease states.

Conclusions

The aging population with emergence of the non-communicable disease epidemic is leading to a resurgence of interest in quantifying regional and total body skeletal muscle mass as part of diagnosing and studying the pathophysiology of senescence and associated diseases. The D_3-creatine dilution method, one-century in its making, enters the armamentarium of tools available for quantifying components of skeletal muscle and builds upon basic biochemistry discoveries and isotope dilution approaches introduced several decades ago. Our critical analysis of the D_3-creatine dilution method as now applied shows multiple potential sources of measurement error that suggest a need for deep scrutiny before undertaking major research programmes without *a priori* consideration of these concerns. Specifically, advancing the D_3-creatine dilution method as a reference standard for quantifying 'whole-body skeletal muscle mass' has a very limited physiological foundation when applied across a broad range of humans who are healthy or who have acute and chronic diseases. We also lay out ideas on how to improve and further develop the method and emphasize a shift in thinking about D_3-creatine dilution as a way of quantifying skeletal muscle 'mass' to its muscle fibre component. This frame shift would improve how D_3-creatine dilution mechanistically fits along with the multiple other methods of measuring regional and total body skeletal muscle mass.[84]

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Conflicts of interest

Dr. Heymsfield is on the Medical Advisory Board of Tanita Corporation and he serves as an Amazon Scholar. The other authors and their close relatives and their professional associates have no financial interests in the study outcome, nor do they serve as an officer, director, member, owner, trustee, or employee of an organization with a financial interest in the outcome or as an expert witness, advisor, consultant, or public advocate on behalf of an organization with a financial interest in the study outcome. The opinions or assertions

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Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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