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Original Article

Stable isotope approaches to study muscle mass outcomes in clinical populations

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ABSTRACT

Both low muscle mass and muscle loss are associated with reduced physical function, mobility, independence, and quality of life, and are characteristic of a number of clinical conditions including diabetes, cardiovascular disease (CVD), chronic obstructive pulmonary disease (COPD), and critical illness. The accurate measurement of muscle mass is critical to assess the efficacy of an intervention or therapy. Stable isotope amino acid approaches can be used to quantify specific aspects of whole-body and muscle protein turnover, including synthesis and breakdown, which play distinctive roles in muscle mass maintenance in direct response to therapies. This review aims to elucidate whether acute responses measured using stable isotope amino acid tracers relate to changes in muscle mass in vulnerable clinical populations. Experimental studies quantifying whole-body protein synthesis and breakdown rates in clinical populations have been conducted to determine the response to nutritional interventions or to compare disease with health; however, these studies show limited potential to translate to expected muscle mass outcomes. In addition, clinical studies that have assessed both muscle mass and acute changes in whole-body or muscle protein turnover are lacking. We argue that the assessment of both muscle protein synthesis and breakdown rates, or simply limb net balance, obtains the most complete picture in relation to muscle-specific outcomes. While stable isotope amino acid tracer experiments provide meaningful mechanistic insight into the acute response to clinical interventions, they should be

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combined with, and/or followed-up by, longer-term studies incorporating measurements of muscle mass to ascertain the impact of an intervention on muscle mass maintenance in clinical populations.

1. The relevance of muscle mass to clinical outcomes

Skeletal muscle is one of the largest groups of tissues in the human body, and is highly adaptive to external stimuli such as nutrition and physical (in)activity [1–3]. Low muscle mass, irrespective of body size, is prevalent in a number of metabolic conditions including type two diabetes mellitus [4], cancer [5], and COPD [6], and is associated with poor clinical outcomes in hospitalised patients, including longer hospital length of stay (LOS), more postoperative complications, worsened disease prognosis, and increased mortality [7–13]. Similarly, acute muscle loss, such as that observed during hospitalisation for critical illness [14–16], is also associated with poor outcomes including increased intensive care unit (ICU) LOS and reduced physical function that persists after hospital discharge [17]. Correspondingly, the maintenance of muscle mass in hospitalised patients is associated with improved survival [18]. Given the importance of muscle mass for clinical outcomes, the ability to quantify the response to interventions aimed to attenuate muscle loss or increase muscle mass over time is critical, both for the patient and the healthcare system.

A number of techniques exist that enable the quantification of muscle mass in a clinical setting. Magnetic resonance imaging (MRI) and computed tomography (CT; including of the 3rd lumbar region) are considered the gold standard imaging methods and, along with dual energy x-ray absorptiometry (DXA), are typically used in clinical research to measure skeletal muscle (or lean) mass [19]. Ultrasonography and bio-electrical impedance (BIA) have gained momentum as cost-effective bedside alternatives to determine muscle thickness/cross-sectional area (CSA) or fat free mass, respectively [14,20,21]. However, techniques used to measure muscle mass in patients often rely on estimations, and can be influenced by differences in methodology [22] and patient variations ('noise') that are difficult to control in a clinical setting, such as physical activity level, nutritional intake, and fluid status [23]. Furthermore, studies comparing different techniques have often reported poor levels of agreement for measuring muscle mass [14,24]. For example, over seven days of ICU admission Puthucheary et al. showed greater degrees of atrophy when measured using the ratio of protein to DNA (30% loss) when compared to muscle fibre CSA (18% loss) and ultrasound-derived *m. rectus femoris* CSA (10% loss) [14]. Therefore, it is important to recognise that the observed degree of muscle loss depends on the quantification technique used. While changes in muscle mass are related to clinical outcomes and provide insight into the longer-term response to interventions, acute responses may be used to test the efficacy and response to an intervention and gain understanding of the underlying mechanisms as well as the expected magnitude of change in muscle mass. Our review aims to elucidate whether acute responses measured using stable isotope amino acid tracers relate to changes in muscle mass in vulnerable clinical populations. It is envisaged that this will support researchers and clinicians in their interpretation of findings from these acute tracer studies and expected impact on clinical outcomes.

2. Stable isotope tracer techniques to measure protein turnover and net balance

The continuous turnover of muscle proteins is required to maintain muscle quality, and the net balance between synthesis and breakdown determines whether muscle protein is lost, maintained, or gained. In clinical populations experiencing muscle loss, muscle protein breakdown (MPB) must exceed muscle protein synthesis (MPS). This may be due to an increase in breakdown, a reduction in synthesis, or a combination of both, thereby resulting in a negative net balance. Decades of research

and continuous advances in the field of muscle protein metabolism have resulted in various techniques to measure MPS and MPB in humans. Although acute measurements of MPS or MPB rates provide insight into the mechanism and physiological response to external stimuli such as nutrition and exercise, it has been argued that the determination of net balance on a whole-body or skeletal muscle level provides a more complete account of protein turnover and, as such, relates to muscle mass outcomes [25].

There are several approaches to quantify net balance on a whole-body or skeletal muscle tissue level, with the most commonly used methods included in Table 1. Although there are other approaches that can measure MPS or MPB alone (e.g. 3-methylhistidine, with or without stable isotope tracers, for MPB [26]), for conciseness we have only included methods that allow the quantification of net balance. A commonly used approach, due to its applicability in a clinical setting, is the nitrogen balance technique. There are limitations to this technique in that factors such as losses via sweat and respiration affect the accuracy of absolute daily nitrogen balance (i.e. this technique under-estimates losses and over-estimates intake) [27] and muscle-specific nitrogen balance is difficult to obtain (Table 1), yet this technique can provide useful insight into whole-body nitrogen balance patterns and can assess the response to an intervention over multiple days to weeks. In a more detailed, yet also more complex and costly way, intravenous infusion of stable isotope amino acids combined with repeated blood samples can be used to provide insight into the turnover of specific amino acids on a whole-body level. However, factors such as inflammation or disease state may influence organ protein turnover. As muscle protein turnover represents only ~25–30% of whole-body protein turnover [28], whole-body protein turnover does not accurately reflect the response of skeletal muscle [29]. Therefore, muscle-specific measurements are required to determine the effect of specific interventions on muscle. The most commonly used approach to measure net balance *in vivo* in humans is the arteriovenous balance (AV) approach across an arm or leg, which uses simultaneous sampling from an artery (or arterialised hand vein) and a vein (e.g. femoral or antecubital), combined with arterial blood flow measurements of the arm or leg (e.g. via Doppler ultrasound or indocyanine green infusion). This relatively simple approach results in the net balance of amino acids, as well as other metabolites if required [30], which is the most direct method to determine changes in net balance, and may be used to predict the effect of a longer-term intervention on muscle mass. An advantage of this method is that only amino acid concentrations and blood flow measurements are required for the calculation of net balance. However, when combined with a stable isotope amino acid tracer infusion this approach provides a more complete description of protein turnover [31], especially when muscle biopsy collection is also included [32]. While it can be assumed that with correct cannula placement AV balance represents net balance of muscle tissue (and not other tissues such as skin and adipose tissue [33,34]), an alternative approach is to calculate muscle net balance directly. Specifically, this can be achieved via the combination of two different stable isotope amino acid tracers (using the same amino acid with a different label, e.g. ^{13}C-Phe for fractional synthesis rate; FSR and ^{15}N-Phe for fractional breakdown rate; FBR) with repeated blood and muscle tissue sampling, thereby measuring the FSR via the direct incorporation of labelled amino acids in muscle tissue [35] as well as the FBR via the dilution of the muscle intracellular free pool [36]. Given the relative burden of repeated muscle biopsies, the limited evidence available applying the assessment of postprandial responses with the dilution technique [37], and the technical challenges associated with the FBR calculations, there is a paucity of data applying this method in human intervention studies [38,39]. The invasiveness of this approach also limits its feasibility in clinical populations. Moreover, a downside of this method is that to date it cannot provide net balance of specific muscle fractions such as the myofibrillar (i.e. contractile) fractions, as FBR is measured from mixed muscle protein. An increasingly used, logistically feasible approach that can provide crucial insight in the integrative FSR and FBR of specific proteins of interest is oral ingestion of deuterium oxide ($^{2}H_2O/D_2O$). Unfortunately, there is a difference in required body water and muscle protein bound enrichments to measure MPS and MPB using deuterium oxide [40]. As a result, the timeframes for performing these measurements do not align, which precludes FSR and FBR to be measured in parallel. Of note, it is possible to use D_2O-derived MPS rates and a measurement of muscle mass to estimate MPB (e.g. Ref. [41]), which might provide information on the impact of a nutritional intervention in clinical practice. Altogether, although oral deuterium oxide consumption is a powerful method that will further increase our insight into human muscle protein turnover under free-living

Table 1
Overview of methods for the quantification of whole-body and skeletal muscle protein balance, with the rows in grey highlighting the recommended approaches to quantify net balance in humans.

Level	Method	Outcome and calculation	Strengths	Limitations	References
Whole-body	Nitrogen balance	Whole-body nitrogen balance = nitrogen ingested - nitrogen excreted	• Simple • Non-invasive • Allows for longer-term measurements (including multiple meals)	• Cannot measure muscle-specific changes • Overestimates intake and underestimates excretion	[80]
	Stable isotope tracer kinetics	Whole-body protein net balance = whole-body protein synthesis − endogenous Ra	• Relatively non-invasive	• Cannot measure muscle-specific changes • Validity of non-steady state postprandial measurements is subject to discussion [81]	[31]
Skeletal muscle	AV balance	Limb net balance = $(C_A - C_V) * BF$	• Direct measurement of limb net balance • Relatively non-invasive. Can be performed by using arterialised instead of arterial samples. • Can be combined with stable isotope tracer infusion for 2-pool calculations, and muscle biopsies for 3-pool calculations	• Measurement is potentially influenced by non-muscle tissues, although this can be minimized by correct cannulate placement [33]	[32]
	Stable isotope tracer kinetics	Muscle net balance = FSR - FBR	• Direct measurement of skeletal muscle protein net balance • Sensitive • Can assess changes over short time periods	• Relatively invasive due to requirement for repeated muscle biopsies and intravenous infusions. • Can only measure NB of mixed muscle protein • Limited time frame for FBR measurement prevents NB measurement over entire postprandial period	[35,36]

AV: arteriovenous; BF: blood flow; C_A: arterial(ised) concentration; C_V: venous concentration; FBR: fractional breakdown rate; FSR: fractional synthesis rate; NB: net balance; Ra: rate of appearance.

conditions, this method cannot be used to calculate net balance in the way that other discussed methods (Table 1) can. In the next section we will further discuss the feasibility of these acute stable isotope techniques in clinical populations and explore studies that have used these techniques in relation to measures of muscle mass.

3. Studies applying stable isotope techniques in clinical populations to assess the potential relationship with muscle mass

3.1. Acute tracer studies and changes in muscle mass in healthy populations

In healthy populations, acute MPS rates following a single bout of resistance exercise increase by 50–100% and align with more modest MPS responses observed over a prolonged period (i.e. increases of ~20–35% in daily MPS rates during several days using deuterium oxide) [40,42–44]. While these increases in MPS often occur in parallel with muscle hypertrophy following weeks to months of exercise training in young and older individuals, the acute increase in MPS may not quantitatively predict the degree of associated changes in muscle mass [45,46]. In line, the acute MPS response to protein ingestion does not always translate to protein supplementation-induced changes in muscle mass over the longer term [47–50]. While these acute studies provide relevant data on the magnitude and impact of nutrition and exercise interventions on muscle mass changes in health when conducted in a fairly controlled setting, there is a paucity of studies that have applied acute stable isotope tracer measurements in clinical populations and assessed their relation to changes in muscle mass.

3.2. Whole-body protein net balance studies

Acute stable isotope tracer studies have assessed whole-body protein turnover rates (i.e. protein synthesis and breakdown, and the resulting net balance) in a broad variety of clinical populations. This includes studies in patients following surgery [51,52], undergoing dialysis [53,54], with COPD [55,56], liver cirrhosis [57], or cancer cachexia [58,59], and critically ill and burns patients [60–62]. These studies report an overall negative whole-body protein net balance, with rates of breakdown exceeding synthesis in patients when compared to healthy controls, which reflects the catabolic state observed in these clinical conditions. In response to nutritional interventions, such as hyperaminoacidaemia [51,54,58,61] or protein supplementation [53,59,63,64], whole-body protein balance becomes positive, primarily due to increases in whole-body protein synthesis of up to 50%, while whole-body protein breakdown remains unchanged or slightly decreased. This reflects a comparable anabolic response to a healthy population, and as such, provides insight into the efficacy of interventional strategies that over time may result in the attenuation of muscle mass loss in clinical populations. In line, two studies in ICU patients have assessed the effect of both enteral and parenteral nutrition on whole-body protein kinetics (via primed intravenous infusions of L-[ring-$^{2}H_5$]-phenylalanine and L-[3,3-$^{2}H_2$]-tyrosine and the uptake of amino acids via L-[1-^{13}C]-phenylalanine added to ongoing nutrition) in the acute phase (24 h [65]) and over several days of ICU admission [66]. These studies show that amino acid administration increases whole-body protein synthesis rates to levels above rates of breakdown (which remained unchanged), leading to improved whole-body protein net balance and suggesting a potential anabolic effect of amino acid administration during critical illness. However, whole-body protein turnover does not necessarily align with the response in skeletal muscle protein turnover and skeletal muscle protein turnover has been shown to be much slower than the turnover of splanchnic tissues [67], so that whole-body protein turnover may overestimate the muscle protein anabolic response. In addition, as the impact of organ turnover and disease state likely affects whole-body protein turnover rates in clinical populations [29,68], acute measurements of whole-body protein net balance are less accurate to predict changes in muscle. As such, although whole-body protein turnover can provide valuable insight into the whole-body response to an intervention it does not necessarily reflect changes in muscle mass, and muscle-specific measurements are required to assess the effect on skeletal muscle tissue.

3.3. Muscle net balance studies

Both the AV-balance methodology (e.g. forearm or leg net balance), as well as combined FSR and FBR measurements, provide greater insight into skeletal muscle amino acid kinetics than whole-body measurements, and have been applied in clinical populations to assess alterations in muscle protein metabolism [62,69–74]. For example, basal whole-body and leg protein synthesis and breakdown rates

have been shown to be higher in severely burned patients when compared to healthy controls [62]; however, since absolute breakdown rates were higher (+80% versus controls) than synthesis rates (+50% versus controls), this resulted in a more negative net protein balance in burns patients. In critically ill patients, leg protein breakdown has been shown to be significantly elevated compared with leg protein synthesis, resulting in negative muscle protein net balance over the first 1–3 weeks of the ICU stay [14,73,74]. Similarly, a study that assessed FSR and FBR of mixed-muscle proteins in septic patients following burn injury observed a reduced muscle protein net balance as a result of a 2.4 fold increased FBR and no effect on FSR when compared with non-septic burned patients [71]. Thus, the negative muscle protein net balance is reflective of the significant muscle wasting observed during critical illness, which is predominantly driven by accelerated muscle protein breakdown. The anabolic response to a nutritional intervention assessed by muscle protein net balance has been studied acutely [70,72] and over more prolonged periods [74,75] in patient populations. As an example, a study in chronic kidney disease (CKD) patients assessed the anabolic response to different protein diets over 6 weeks using repeated measurements of forearm perfusion combined with infusions of L-[ring-$^{2}H_{5}$]-phenylalanine. It was demonstrated that the diet higher in protein did not alter forearm phenylalanine rate of disappearance (i.e. a proxy for MPS) but lowered whole-body and forearm phenylalanine rate of appearance (i.e. MPB), resulting in a 40% higher (yet still negative) forearm protein net balance [75]. However, the lack of muscle mass measurements preclude any conclusions on the relationship between improved (forearm and whole-body) net balance and changes in absolute muscle mass. A recent study by Davies et al., shows a reduced acute forearm branched chain amino acid (BCAA) net balance following ingestion of a mixed meal in patients with Crohn's disease when compared with healthy controls [76]. Interestingly, the lower postprandial BCAA response (suggestive of anabolic resistance, i.e. a reduced anabolic response to protein ingestion) was associated with a lower baseline appendicular lean mass in the patient group. Although no stable isotope tracers were applied to gain insight into amino acid kinetics, this study illustrates that forearm net balance can be used to measure protein net balance following a nutritional intervention and corresponds with absolute muscle mass. In support, a continuous net release of phenylalanine and total amino acids from the leg has been observed in the first 2 weeks of ICU stay (despite continuous enteral and parenteral feeding) [77], which corresponds with the substantial muscle loss generally observed in these patients. Consequently, in a catabolic state, protein net balance being negative over a substantial timeframe can likely, for a large part, be attributed to an increase in the breakdown of muscle proteins. Therefore, the assessment of protein net balance arguably provides a more valid estimation of long-term muscle mass changes in clinical populations with acute muscle wasting than isolated measures of MPS alone.

3.4. Predictive changes in muscle mass in clinical conditions

With data available on FSR and FBR in healthy conditions, and; therefore, net balance, we can calculate theoretically expected changes in muscle mass in clinical populations. As an example, a change of 10% in basal (i.e. 0.003–0.007%/h) or postprandial (i.e. 0.004–0.005%/h) MPS or MPB rates would result in a ~0.5–1% decrease in muscle protein turnover over 1 week (assuming 12 h in basal or postprandial state). Such a 1% decrease in turnover represents 450 g of lean mass (assuming a body weight of 75 kg and 60% lean mass), or 250 g of appendicular lean mass. If both the postprandial rise in MPS and suppression of MPB were affected, changes in postprandial protein handling as small as 5% could result in a ~1 kg loss of muscle mass per week. While such values of muscle protein turnover are realistic, they do not always align with the degree of muscle mass loss observed. A reason for this might be that muscle loss is heavily influenced by other factors such as disease state and medication use, dietary intake, and physical (in)activity levels. Moreover, the sensitivity of both mass spectrometry analyses for stable isotope enrichments and muscle mass measurements affect the potential to predict the impact of an intervention on long-term muscle mass from acute tracer data. This might imply that although an intervention strategy is able to stimulate net balance acutely, this may not translate into a sufficiently large increase in muscle mass to exceed the 'noise' of the measurement (i.e. timeframe, sensitivity, and method used). Additionally, any acute increase in protein net balance in response to interventional strategies will need to be of a significant magnitude, and applied repeatedly over an extended timeframe, to eventually result in muscle protein accretion.

3.5. Studies combining muscle-specifc measurements of protein turnover and muscle mass assessments

The inclusion of both measurements of muscle protein turnover rates using acute stable isotope tracer methodology combined with the assessment of muscle mass in clinical populations has only been assessed in a handful of studies [14,62,74,78]. Firstly, Biolo et al. combined the AV-balance technique, skeletal muscle biopsies, and stable isotope amino acid tracers [62] to assess whole-body and leg protein synthesis and breakdown, and MPS rates in patients with burns compared to healthy controls. Both whole-body and leg protein synthesis and breakdown rates were increased when compared with healthy controls, with breakdown rates exceeding protein synthesis rates, resulting in a negative protein net balance. Fractional MPS rates were 50% higher in patients than in healthy controls; however, no measures of MPB were included and with the absence of muscle mass measurements it can only be assumed that the more negative protein net balance on a whole-body and leg level is representative of the muscle wasting observed in burns patients. Gamrin-Gripenberg et al. [74] assessed both whole-body and muscle protein net balance (applying the 2- and 3-pool AV model, with 3-methylhistidine for myofibrillar breakdown) in 20 ICU patients, and observed an improved (yet still negative) protein net balance over the course of ICU stay from both the leg (2-pool AV) and muscle (3-pool AV) data, primarily due to an increase in rates of MPS without any change in MPB. These data align with the greater degree of muscle loss that occurs during the early days of ICU stay [14,79], and as such, the assessments of muscle protein net balance using either AV-balance methods with or without muscle biopsies can be an informative indicator of changes in muscle mass during critical illness. To the best of our knowledge, only two studies have directly combined acute measurements of muscle protein turnover with the assessment of muscle mass changes over time in a clinical cohort. A study conducted in colorectal cancer patients planned for surgery showed a significant inverse correlation between the loss of lean leg mass after surgery (−27%) and postprandial FSR [78]. Moreover, they demonstrated that the evident recovery in postprandial MPS rates following the provision of amino acids is accompanied by a decrease in leg protein breakdown (measured as phenylalanine rate of appearance) post-surgery. Although this is suggestive of an increase in muscle protein net balance post-surgery, these data were unfortunately not included. This study does however, demonstrate that changes in muscle protein turnover align with absolute changes in muscle mass. Similarly, Puthucheary et al. used leg AV-balance, primed constant stable isotope amino acid infusions, and muscle biopsies for the measurement of whole-body and muscle protein kinetics in critically ill patients. Muscle mass was assessed over 7 days via ultrasound measurements of the *m. rectus femoris* CSA [14]. Acute FSR increased by >100% over the study period, which, if this had been the only measurement made, would suggest a highly anabolic state. However, although both leg protein synthesis and breakdown rates increased (with nutrition provision as per standard care), overall leg protein net balance remained negative due to higher absolute breakdown rates (29% and 14% higher than leg protein synthesis on days 1 and 7, respectively), these acute changes were accompanied by a severe decline in muscle mass (−13% in *m. rectus femoris* CSA and −18% in muscle fibre CSA) [14]. This demonstrates again that while the increase in leg protein synthesis and FSR provides insightful data on muscle protein turnover in clinical conditions, the overall net balance provides a more complete picture of muscle mass changes. The fact that net balance was similarly negative on days 1 and 7, and muscle loss was linear, suggests that net balance represents a valuable indication of the changes in skeletal muscle mass in a clinical setting.

4. Conclusion

The development of effective (nutritional) interventions to preserve muscle mass in clinical populations is important to improve patient outcomes. Stable isotope amino acid approaches can be conducted in smaller cohorts of patients to obtain insight into the dynamic and mechanistic responses to an intervention that are not directly possible from the quantification of muscle mass alone. These approaches provide insight into acute responses that occur prior to detectable changes in muscle mass and may be used prior to embarking on a large-scale clinical trial focussed on improving muscle mass as the primary outcome. However, in this review we have provided evidence that the standard practice of solely quantifying MPS is insufficient to predict the longer-term effect on muscle mass in clinical populations. Since increases in MPB are often accelerated in illness when compared to healthy

individuals, we argue that measurements of protein net balance provide greater insight into predicted changes in muscle mass in a clinical setting. While whole-body measures of protein synthesis, breakdown, and net balance are fairly common in clinical research, measures across a limb or muscle-specific measurements are preferred since they are less likely to be influenced by changes in organ protein turnover. We argue that the preferred methods are 2- or 3-pool AV-balance, or the calculation of net protein balance from measurements of both MPS and MPB. While these acute measurements of net protein balance may not quantitatively predict the change in muscle mass within a long-term interventional trial, they are more likely to reflect the potential of a (nutritional) intervention to result in longer-term benefits on muscle mass than isolated measurements of MPS or MPB alone. In addition, acute changes in net balance may not directly align with the associated change in absolute muscle mass, due to a variety of anabolic and catabolic factors in clinical practice including disease state, medical therapy, inflammation, nutritional intake, physical activity, sleep, hormonal fluctuations, or fluid status that contribute to muscle mass regulation. We conclude that stable amino acid tracers provide valuable mechanistic insight into the acute change in net balance in response to clinical interventions yet these must be combined with, or followed by, a measurement of muscle mass to determine the true influence of an intervention on clinically-relevant outcomes prior to implementation into clinical practice.

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Author contribution

LSC, MLD, and IWKK were responsible for conceptualisation, data curation, and original draft writing and review and editing of the final manuscript.

Declaration of interests

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.

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