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Critical Review

Assessing Muscle Protein Synthesis Rates In Vivo in Humans: The Deuterated Water (^2H_2O) Method

Andrew M Holwerda [1], Philip J Atherton [2], Kenneth Smith [2], Daniel J Wilkinson [2], Stuart M Phillips [3], Luc JC van Loon [1,*]

[1] *Department of Human Biology, NUTRIM Institute of Nutrition and Translational Research in Metabolism, Maastricht University, Maastricht, The Netherlands;* [2] *MRC/ARUK Centre for Musculoskeletal Ageing Research and National Institute of Health Research, Biomedical Research Centre, School of Medicine, University of Nottingham, Derby, United Kingdom;* [3] *Department of Kinesiology, McMaster University, Hamilton, Ontario, Canada*

A B S T R A C T

Skeletal muscle tissue is in a constant state of turnover, with muscle tissue protein synthesis and breakdown rates ranging between 1% and 2% across the day in vivo in humans. Muscle tissue remodeling is largely controlled by the up- and down-regulation of muscle tissue protein synthesis rates. Research studies generally apply stable isotope-labeled amino acids to assess muscle protein synthesis rates in vivo in humans. Following labeled amino acid administration in a laboratory setting, muscle tissue samples are collected over several hours to assess the incorporation rate of these labeled amino acids in muscle tissue protein. To allow quantification of bulk muscle protein synthesis rates over more prolonged periods, the use of deuterated water methodology has regained much interest. Ingestion of daily boluses of deuterium oxide results in ^2H enrichment of the body water pool. The available ^2H-atoms become incorporated into endogenously synthesized alanine primarily through transamination of pyruvate in the liver. With ^2H-alanine widely available to all tissues, it becomes incorporated into de novo synthesized tissue proteins. Assessing the increase in tissue protein-bound ^2H-alanine enrichment in muscle biopsy samples over time allows for the calculation of muscle protein synthesis rates over several days or even weeks. As the deuterated water method allows for the assessment of muscle tissue protein synthesis rates under free-living conditions in nonlaboratory settings, there is an increasing interest in its application. This manuscript describes the theoretical background of the deuterated water method and offers a comprehensive tutorial to correctly apply the method to determine bulk muscle protein synthesis rates in vivo in humans.

Keywords: muscle atrophy, muscle hypertrophy, dietary protein, exercise, aging

Introduction

Skeletal muscle tissue is in a constant state of turnover, with muscle protein synthesis and breakdown rates ranging between 1% and 2% per day in vivo in humans [1,2]. Muscle tissue remodeling is largely controlled by the up- and down-regulation of muscle tissue protein synthesis rates. Research studies generally apply stable isotope-labeled amino acids to assess the impact of various anabolic or catabolic stimuli on muscle protein synthesis rates in vivo in humans. The most applied approach is the primed, continuous intravenous infusion of a stable isotope labeled amino acid [e.g., L-(*ring*-$^{13}C_6$)-phenylalanine] for several hours, during which blood and muscle tissue samples are collected. Mass spectrometry analysis is subsequently applied to measure labeled amino acid enrichments in blood samples and protein-bound labeled amino acid enrichment levels in hydrolyzed protein extracted from the muscle tissue samples. From these data, the muscle protein synthesis rate during the infusion period can be calculated (Figure 1). Over the last 3 decades, the use of primed, continuous, stable isotope-labeled amino acids has advanced our understanding of skeletal muscle protein metabolism, with factors such as physical (in)activity, food ingestion, aging, and disease strongly affecting muscle protein synthesis rates [3–8].

Though primed, continuous stable isotope infusion experiments can help to understand the impact of a single stimulus

Abbreviations: FSR, fractional synthetic rate; GC-P-IRMS, gas chromatograph isotope ratio mass spectrometer equipped with a pyrolysis oven; IRMS, isotope ratio mass spectrometer; MPE, mole percent excess; ^2H2O, deuterium oxide.

* Corresponding author. *E-mail address:* l.vanloon@maastrichtuniversity.nl (L.J.C. van Loon).

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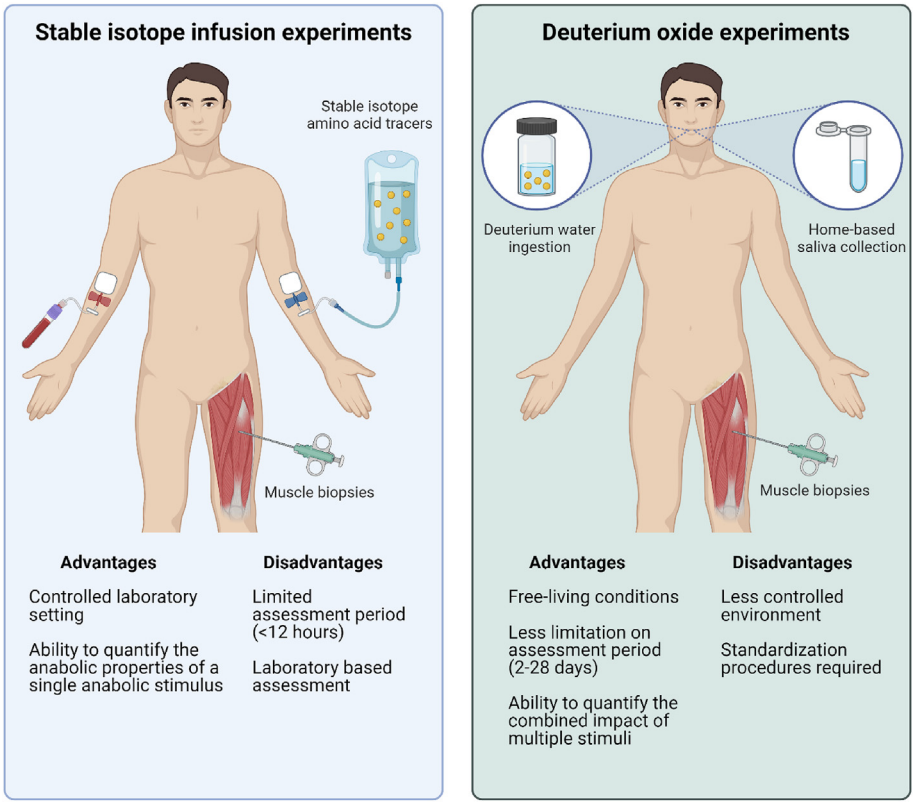

FIGURE 1. Graphical comparison between the experimental parameters of a stable isotope labeled amino acid infusion experiment compared with a deuterium oxide experiment. For an infusion experiment, participants undergo a primed constant intravenous infusion of 1 or more stable isotope-labeled amino acid tracers throughout a test day lasting between 4 and 12 h. Blood samples and muscle biopsies are collected throughout the test day and used to assess fractional muscle protein synthesis rates. Stable isotope infusion experiments require specialized and dedicated laboratory space. The highly controlled nature of stable isotope infusion experiments results in high sensitivity to assess the anabolic properties of a single stimulus. For deuterium oxide experiments, test participants drink small amounts of deuterium oxide throughout the experimental test period, which can last several days to weeks. Instead of blood sampling, participants may carry out home-based saliva sample collection. Dedicated laboratory space is only required for muscle biopsy collection. Overall, deuterium oxide experiments allow participants to remain in their habitual or clinical setting(s) while partaking in the experimental intervention. Although less controlled relative to intravenous stable isotope infusion experiments, the "free-living" conditions that are available in study designs applying deuterium oxide allow the integration of all anabolic and catabolic factors into the assessment of daily muscle protein synthesis rates.

within a short timeframe (~2–12 h) under controlled laboratory conditions; they do not provide insight into more real-life settings where 1 or more stimuli are modulating fluctuations in muscle protein synthesis rates over a more prolonged period (days or weeks). Furthermore, more holistic evaluations are required to assess the impact of changes in lifestyle or the development and progression of disease on skeletal muscle tissue remodeling. Assessing the impact of one or more factors on muscle protein synthesis rates during more prolonged periods under free-living conditions requires a different approach. Over the last few decades, the application of deuterium oxide (^2H_2O, deuterated water, heavy water) ingestion has re-emerged as a means to assess changes in muscle protein synthesis rates in vivo in humans over several days to weeks under nonlaboratory, free-living conditions [9–11]. The application of ^2H_2O to measure in vivo muscle protein synthesis rates requires the ingestion of relatively small amounts of ^2H_2O throughout an assessment period where study participants or patients are subjected to pharmaceutical, nutritional, and/or physical (in)activity interventions [1,9,12–14]. The ingestion of ^2H_2O increases the ^2H-enrichment of the body water pool, allowing ^2H-atoms to become incorporated into numerous metabolic substrates, including different amino acids. For the measurement of bulk muscle protein synthesis rates, alanine is the preferred amino acid. Alanine becomes rapidly labeled with ^2H through its endogenous synthesis by way of transamination of pyruvate in the liver [11]. With ^2H-alanine rapidly becoming available to all tissues, it is utilized as a precursor amino acid for protein synthesis and thus becomes incorporated into all newly synthesized tissue proteins [1, 6–8]. Prior to and after an assessment period, skeletal muscle biopsy samples can be collected to measure the amount of ^2H-alanine that has been incorporated into muscle protein. With this approach, the impact of the experimental intervention on bulk muscle protein synthesis rates can be assessed under nonlaboratory, real-life conditions over an extended period. Consequently, the impact of the experimental intervention can be determined through integration with various concurrent factors that are generally not controlled for in laboratory-based studies. Therefore, this approach allows us to better integrate real-life conditions and

extends our capacity to assess the impact of intervention strategies to modulate bulk muscle protein synthesis rates beyond the basic insights gained from acute tracer infusion protocols (~2–12 h). Given this advantage, it is not surprising that the ^2H_2O method is now rapidly becoming established as a key methodology for evaluating muscle protein synthesis rates over more prolonged experimental periods. In fact, within the last 10 y, nearly 50 studies have applied ^2H_2O to measure bulk muscle protein synthesis rates in vivo in humans (Supplemental Table 1). Without the need for procedures or facilities required to perform primed, continuous intravenous infusions with pharmaceutical-grade labeled amino acids prepared under sterile conditions, the use of deuterated water is a method that is more readily available to numerous research groups. Consequently, we receive many questions regarding the ^2H_2O method and the preferred study design and research protocol to evaluate the anabolic properties of various interventions over several days or even weeks. To assist researchers in applying the ^2H_2O approach to accurately assess daily protein synthesis rates of bulk muscle protein fractions (i.e., mixed muscle protein, myofibrillar, collagen, etc.), we herein describe the theoretical background of the deuterated water methodology and offer a practical, comprehensive tutorial for researchers to correctly apply this method to determine muscle protein synthesis rates in vivo in humans.

Theoretical aspects involved in conducting deuterated water experiments

The use of stable isotopes to measure protein synthesis rates

To measure muscle protein synthesis rates, stable isotope administration is combined with skeletal muscle biopsy sampling. With this experimental approach, the *precursor-product* method can be applied to calculate fractional muscle tissue protein synthesis rates. The basic principle is that free amino acids residing in the plasma are transported into the muscle fibers, where they mix with the intracellular free amino acid pool. Within the intracellular space, free amino acids become acylated to aminoacyl-tRNA (the true precursor pool for protein synthesis) before being incorporated into proteins (i.e., protein synthesis). Free amino acids and aminoacyl-tRNAs are referred to as the *precursors,* and muscle protein is referred to as the *product*. Administering a stable isotope such as ^2H_2O introduces a heavy label to the precursor amino acids, allowing researchers to track the amount of free amino acids that have been incorporated into synthesized proteins. The degree of stable isotope presence in the free amino acid and tissue protein pools is termed *enrichment*, which is expressed as an isotope ratio (i.e., the ratio between the heavy amino acid isotope and the lighter, more abundant amino acid isotope). For ^2H_2O, the heavy isotope is ^2H, and the lighter (more abundant) isotope is ^1H. Enrichment values can be expressed in several ways, but most commonly as a tracer-to-tracee ratio, mole percent excess (MPE), or atom percent (AP) excess. Briefly, AP excess represents the enrichment of a labeled isotope at the atomic level (used to express deuterium enrichment in body water), whereas MPE represents the enrichment at the molecular level (used to express deuterium-labeled amino acid enrichment). After determining both precursor and product enrichments, the muscle protein fractional synthetic rate (FSR) can be calculated (*described below*). An FSR is the fraction of the entire protein pool (i.e., muscle protein) that is synthesized over a set timeframe and is generally expressed as a percentage per hour or per day (i.e., %/h or %/d). The precursor enrichment defines the maximum label incorporation that can theoretically be reached in the protein pool. Theoretically, equivalent enrichments of the precursor and muscle protein pools would occur once complete renewal (i.e., 100% synthesis) of the entire protein pool is reached. With an FSR of 1%–2%/d [1], a large portion of the muscle tissue protein pool will undergo complete renewal over a 2–3 mo period. Overall, the precursor-product method allows the key advantage of assessing protein synthesis rates directly in muscle tissue in vivo in humans.

Amino acid deuterium labeling properties

The application of ^2H_2O to measure in vivo muscle protein synthesis rates requires the ingestion of relatively small amounts of ^2H_2O throughout an assessment period. The subsequent increase in ^2H-enrichment of the body water pool allows ^2H-atoms to become incorporated into endogenously de novo synthesized alanine primarily through transamination of pyruvate in the liver, with minor contributions from synthesis in the kidney and muscle tissues [15] (Figure 2). Although several nonessential amino acids become labeled with ^2H, alanine is the preferred amino acid target for measuring muscle protein synthesis rates because of its rapid, high, and predictable labeling pattern relative to body water ^2H enrichment. Deuterium can be incorporated into alanine in place of the hydrogens on either the central carbon position (i.e., α-carbon) or side-chain carbon (i.e., β-carbon) position [11] (Figure 2). The mixture of ^2H incorporation at these 4 positions results in single labeling of alanine (i.e., alanine mass+1, or "M+1") that is ~3.7-fold greater than body water deuterium enrichment in humans [1,11,12]. More than 1 deuterium atom may be incorporated into a single alanine molecule. However, in humans undergoing daily deuterium dosing, single deuterium labeling constitutes 97% of the sum of all deuterium labeling of alanine (i.e., the sum of M+1, M+2, M+3, and M+4) and remains constant over several days of labeling (Supplemental Table 2). Therefore, when applying deuterated water to measure muscle protein synthesis rates in vivo in humans, single deuterium labeling of alanine (i.e., M+1) has become the preferred analytical target.

Importantly, free ^2H-alanine enrichment rapidly equilibrates (within 1 h) with body water ^2H enrichments following deuterated water administration [16]. The rapid and continuous rate of endogenous alanine labeling contributes to the stability of free ^2H-alanine labeling over time under various settings of altered metabolism, including during exercise, during postexercise recovery, and following meal ingestion (Table 1) [16]. Lastly, the turnover of body water is relatively slow, which contributes to a long half-life of deuterium in the body (10–14 d) and sustained endogenous labeling of ^2H-alanine [12]. The constant relationship of ^2H-labeled body water and alanine is important for the accuracy of muscle protein synthesis measurements and for incorporating fluctuating habitual factors or interventions (i.e., diet, exercise, and physical activity) into the measurement. In summary, the rapid, stable, and high degree of deuterium labeling combined with the slow elimination rate of deuterium from the body water pool allows plasma-free ^2H-alanine enrichments to remain elevated and in near steady-state conditions

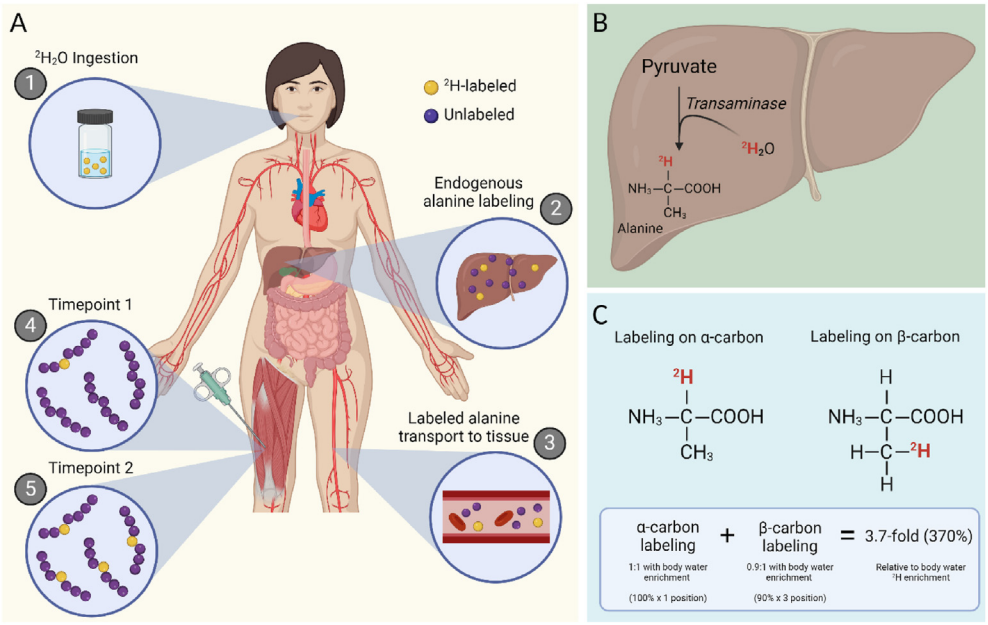

FIGURE 2. Graphical representation of the experimental model of deuterium oxide (^2H_2O) ingestion (A) and endogenous alanine labeling in vivo in humans (B, C). Study participants ingest ^2H_2O (70%), thereby increasing ^2H enrichment levels in the body water pool (step 1). The available ^2H atoms are incorporated into endogenously synthesized alanine through transamination of pyruvate in the liver (step 2, B). Deuterium is incorporated into alanine (C) either on the central α-carbon at a 1:1 (or 100%) ratio relative to body water ^2H enrichment or on 1 of the 3 β-carbons at a 0.9:1 (or 90%) ratio relative to body water ^2H enrichment. Labeling at these 4 positions occurs in parallel and culminates as a single-labeled ^2H-alanine (M+1) enrichment that is 3.7-fold greater than body water ^2H deuterium enrichments. ^2H-alanine is transported along with unlabeled alanine in the circulation and reaches all tissues (step 3). Once free ^2H-alanine is available in the tissue, it becomes incorporated into tissue proteins through protein synthesis. Throughout a ^2H_2O experiment, the amount (or enrichment) of ^2H-alanine increases in muscle (and other tissue) proteins.

following daily dosing over experimental protocols that last for days to weeks [1,9].

Practical aspects when applying deuterated water to assess bulk protein synthesis

Required materials

The materials required to apply deuterated water in study participants include 70 or 99 AP ^2H_2O (e.g., for 70% ^2H_2O: DLM-2259-70-1L; Cambridge Isotopes Laboratories, Inc), sealable 100 mL dosing bottles (e.g., 414004-123; VWR), blood sample collection tubes (e.g., vacutainer EDTA tubes, 367863; BD), blood collection needles (e.g., eclipse venipuncture needle with vacutainer holder, 368650; BD), other blood collection materials (i.e., tourniquet, gauze, and disinfectant), cotton dental rolls for saliva collection [e.g., celluron nr. 2 (40 x 10 mm), 431302; Hartmann], dental roll storage tubes (e.g., eppendorf tubes 5.0 mL with snap cap, 0030119401; Eppendorf), syringes for saliva extraction (e.g., 5 mL BD Emerald syringes, 63132; BD), muscle biopsy collection materials (e.g., Bergström biopsy needles or conchotome, vacuum syringe, scalpels, disinfectant, lidocaine, and sterile gauze), sample tubes for –80°C freezer storage (i.e., muscle, blood, and saliva). Study participants should also be provided with a deuterated water dosing and saliva collection schedule that indicates dates and times of dosing and collection.

Dosing protocols

There have been 2 main variations of deuterated water dosing protocols that have been applied to measure daily muscle protein synthesis rates over the last 10 y. The "single dose" protocol constitutes the ingestion of a single 100–150 mL dose at the start of the protocol, and the "dose-maintenance" protocol constitutes deuterated water dosing over a single "loading" day followed by maintenance dosing during the subsequent experimental period (Supplemental Table 1). The "dose-maintenance" protocol is the currently preferred approach since steady-state ^2H enrichment can be better maintained in the precursor pools [1,17,18], resulting in greater stability of free alanine labeling in the precursor pools to assess muscle protein synthesis rates over several days in vivo in human participants (Figure 3).

After screening and including a study participant, the experimental period must begin with a baseline saliva and/or blood sample to establish the background deuterium enrichment in body water and/or free alanine pools, respectively. Following sample collection, participants begin with ^2H_2O loading. The loading days involve the ingestion of 200 mL of 70% deuterated water. The deuterated water can be ingested over a shorter time span in healthy individuals (e.g., 2 x 100 mL, consumed 2 h apart), whereas dosing should be spread out over a longer period (e.g., 4 x 50 mL, consumed 1 h apart) in more compromised populations (e.g., frail older or clinically compromised populations). Depending on the experimental setting, a muscle sample can be collected after the loading dose and before starting the intervention (*described in detail below*). During the experimental intervention, several smaller deuterated water doses are ingested to maintain a steady state of precursor enrichment. Ideally, the maintenance doses on subsequent days are 20 mL of

TABLE 1
An overview of the associated assumptions of the application of stable isotope tracers to assess muscle protein synthesis rates and considerations and advice when applying deuterated water over several days.

	Description of assumption	Consideration(s) for measurement periods over several days	Advice for ^2H_2O applications
1	The rate of incorporation of unlabeled (tracee) amino acids is constant over the period of incorporation of tracer.	Measurement using ^2H_2O over several days is intended to capture fluctuations in the rate of tracer incorporation over the experimental period to represent an "integrated" protein synthetic response to the intervention. Therefore, the duration of the measurement should be selected to incorporate expected fluctuations in incorporation to reflect "real-life" conditions and/or more prolonged interventions.	A period of 2–28 d should suffice for most purposes as diurnal fluctuations in hormones, habitual physical activity, and dietary intake are captured along with the acute responses to repeated bouts of physical activity (and recovery) and short-term muscle disuse.
2	None of the tracer incorporated into the protein reappears because of protein breakdown during the time course of tracer administration.	Re-appearance of ^2H-alanine from breakdown will be at a very low enrichment and will not disturb precursor enrichment as ^2H-alanine is held at equilibrium with body water ^2H enrichment.	No major consequence of re-appearing ^2H-alanine on FSR calculation over shorter measurements (<28 d).
3	The unlabeled pool size of the bound protein is constant throughout the experiment.	The protein pool size changes during resistance exercise training or muscle disuse. FSR measurements for a prolonged period during these interventions may result in inaccuracy. In humans, disuse atrophy occurs at ~0.5%/d, which may cause FSR to be overestimated by ~3% when measured over a 7-d period.	Inaccuracies can be avoided with shorter experimental protocols (3–7 d). This is more relevant for disuse studies since disuse atrophy occurs much more rapidly than training-induced muscle hypertrophy.
4	There is no significant time delay in the incorporation of amino acids into protein.	Equilibration of precursor pools occurs within 2 h of initial dosing, which does not impact accuracy over prolonged measurement periods (i.e., >1 d).	The collection of a muscle biopsy 1 d after dosing is appropriate. In place of a muscle biopsy, a baseline (unlabeled) plasma sample may be used to establish baseline protein enrichment.
5	The measured precursor pool enrichments reflect the true precursor pool.	Precursors may include body water ^2H enrichments, plasma ^2H-alanine enrichments, and intracellular ^2H-alanine enrichments.	Plasma and saliva (with 3.7-fold correction) are preferred to best characterize the precursor pool enrichment as opposed to measuring muscle acyl-tRNA enrichments.

Abbreviations: FSR, fractional synthetic rate; tRNA, transfer ribonucleic acid; ^2H_2O, deuterium oxide.

70% deuterated water ingested daily (typically ingested in the evening) to account for ~10% body water replacement per day [12,19]. This dosing protocol results in a ^2H body water enrichment of 0.2%–0.5%, which remains steady over a 21 d experimental protocol (Figure 4A) [20]. The experimental period concludes once a muscle tissue biopsy sample is collected. Using this protocol, we have observed myofibrillar protein synthesis rates of 1.37% ± 0.31%/d in healthy young males [20]. This is in agreement with prior work in which myofibrillar protein synthesis rates of ~1.30%/d were reported when applying initial dosing protocols, providing larger volumes of deuterated water in the same population under comparable conditions [21–24]. Although daily maintenance dosing provides an ideal precursor steady-state condition, this is not always feasible in specific populations or experimental settings. Over longer assessment periods (i.e., 14–28 d), maintenance dosing may be provided on a weekly basis [25,26]. Under these conditions, a maintenance dose of ~100 mL can be provided every 7 d, with saliva samples collected to characterize the precursor environment (*described in greater detail below*). Overall, the dose-maintenance protocol should be considered the preferred dosing protocol since tissue ^2H-alanine enrichments are generally well above detection limits of mass spectrometry instrumentation, and steady-state conditions are well maintained, which allows accurate measurements of bulk muscle protein synthesis rates over periods ranging between 2 and 28 d.

Some investigators have opted to modify the "dose-maintenance" protocol and administer deuterated water relative to lean body mass. Dosing relative to lean body mass is intended to reduce intersubject variability in precursor labeling following the dosing day. However, intersubject variability is accounted for by measuring both the precursor and product labeling over the period of the experiment when calculating FSR. Dosing relative to lean body mass does not necessarily result in more stable precursor labeling over the duration of an experiment in comparison to the described fixed-amount dosing protocol. Although both fixed and relative dosing approaches are valid, a researcher may consider dosing relative to lean body mass when comparing populations with greater differences in body mass and/or body composition (e.g., obese, frail). For relative dosing, the amount of ^2H_2O ingested on the dosing day should be 2.5 mL/kg and 0.25 mL/kg on maintenance days. In the case of weekly maintenance doses, 1.25 mL/kg should be ingested every 7 d.

Although the purpose of the deuterated water method is generally to perform longer measurements, investigators should be aware of potential inaccuracies that could result. For instance, large changes in protein (*product*) pool size (i.e., muscle atrophy or hypertrophy) during longer measurement periods may impact the accuracy of FSR values. However, changes in protein pool size are generally limited in most human intervention studies. For example, muscle disuse atrophy with limb immobilization occurs at ~0.5%/d [27], which may cause FSR to be overestimated by merely ~3% when measured over a 7-d period. Furthermore, the greater part of the muscle protein pool will have turned over in about 50–60 d, meaning that labeling of muscle protein will become equivalent to precursor labeling around this point. Continuing a measurement while protein pool

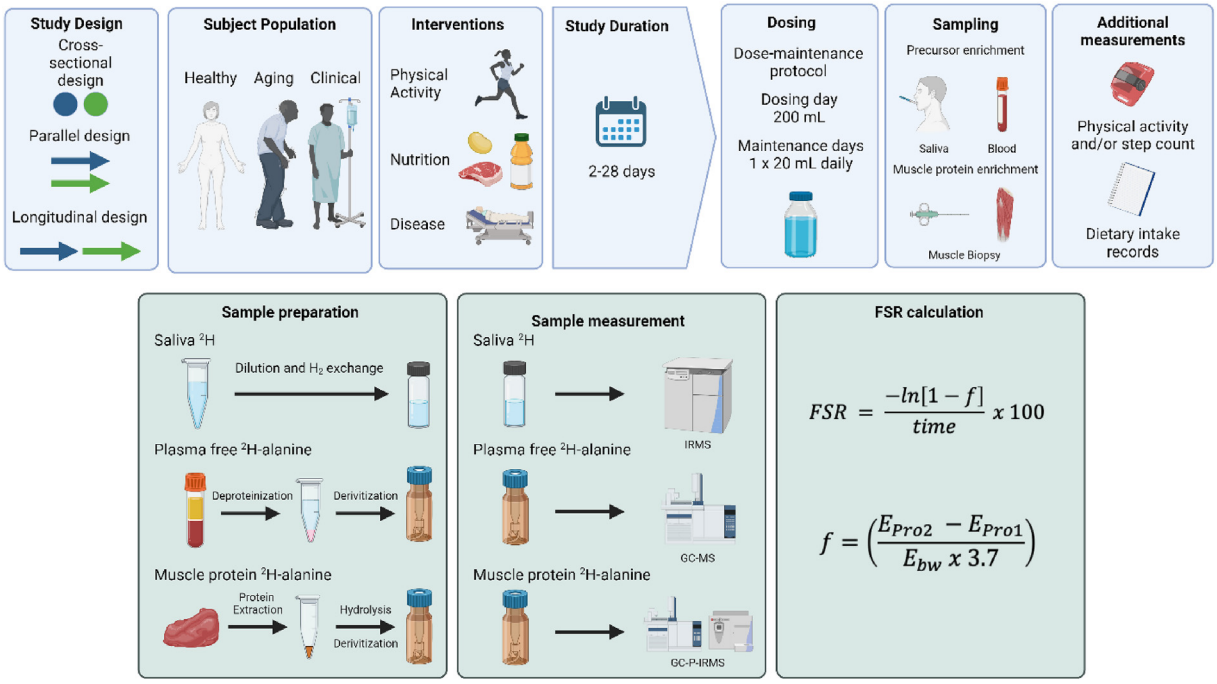

FIGURE 3. A graphical representation of our proposed protocol recommendations for applying deuterated water to measure muscle tissue protein synthesis rates in vivo in humans was assessed over several days. Appropriate deuterium oxide study designs include cross-sectional, parallel, and longitudinal designs. Deuterium oxide can be applied in nearly all healthy and clinical populations. Deuterium oxide is well-suited to detect how modulations of major anabolic or catabolic stimuli (e.g., diet and physical activity) impact muscle protein synthesis rates. A study duration of 2–28 d is sufficient to assess the impact of an intervention under free-living conditions. The "dose-maintenance" protocol achieves a sufficient body water ^{2}H enrichment (between 0.2% and 0.5%). Saliva or blood sampling is most appropriate for measuring body water ^{2}H or free ^{2}H-alanine precursor pools, respectively. Muscle tissue sampling (25–80 mg) is required to determine the increase in ^{2}H-alanine enrichment in tissue protein throughout the experiment. Additional measurements should include physical activity tracking (e.g., ActiGraph) and dietary intake recording (e.g., dietary logs). Saliva samples are measured using an isotope ratio mass spectrometer (IRMS). Blood samples are deproteinized before being derivatized before ^{2}H-alanine enrichments are measured on a GC-MS. Protein is extracted from muscle samples before being hydrolyzed and derivatized, and ^{2}H-alanine enrichments are measured using a GC-P-IRMS. Once data are available, fractional tissue protein synthetic rates can be calculated using an exponential equation. FSR, fractional synthetic rate; GC-MS, gas chromatography-mass spectrometry.

labeling is equal to precursor labeling does not allow any further increase in protein labeling, resulting in artificially low FSR values [28,29]. To avoid inaccurate FSR data, we recommend that total $^{2}H_2O$ dosing periods remain well below 28 d, depending on the timeline required to allow the assessment of the impact of a certain exercise, nutritional, and/or pharmacological intervention (Table 1).

Finally, recent studies have shown that administering larger volumes of deuterated water (i.e., 400 mL) on the dosing day combined with tissue enrichment analysis on a highly sensitive mass spectrometer [i.e., gas chromatograph isotope ratio mass spectrometer equipped with a pyrolysis oven (GC-P-IRMS)] enables the measurement of muscle protein synthesis rates over several hours on the following day [30]. Although this acute approach has not yet been widely applied, there may be potential for applying $^{2}H_2O$ for shorter-term measurements as well.

Side effects of $^{2}H_2O$ administration

$^{2}H_2O$ is safe for human consumption in the dosing protocols described [31,32]. Given the ongoing rates of body water and metabolic substrate turnover, the presence of deuterium will gradually decline over time and be eliminated from the body by ~10 wk after the last dose. Notably, a small fraction of subjects have reported mild vertigo or dizziness during deuterated water dosing [31,33]. The onset of vertigo following ingestion of a single large bolus $^{2}H_2O$ can be mitigated by the ingestion of smaller loading doses staggered over time, as described above. As more clinically compromised populations may be more prone to experiencing dizziness, we propose that such populations ingest smaller $^{2}H_2O$ doses over a slightly longer dosing period (i.e., 50 mL/h for 4 h). The feeling of vertigo seems to be due to a rapid increase in body water ^{2}H enrichment, which causes disturbances to the vestibular system in the inner ear. In this sense, it is remarkable that the tiny vestibular system in the inner ear has a sensitivity that seems equivalent to the expensive, high-end mass spectrometry equipment we have available in our laboratories. Overall, $^{2}H_2O$ administration is generally well-tolerated, with no side effects, allowing application in various clinical and healthy populations.

Sample collection

An overview of the relevant tissue samples and corresponding isotopic analytes, enrichment ranges, and preferred measurement instruments for measurement of muscle protein synthesis rates using $^{2}H_2O$ dosing are displayed in Table 2.

Sample collection must be planned to characterize precursor pool labeling during the experimental intervention and to determine the incorporation of ^{2}H-alanine into muscle protein over the course of the experiment. As described above, the immediate precursor for protein synthesis is aminoacyl-tRNA.

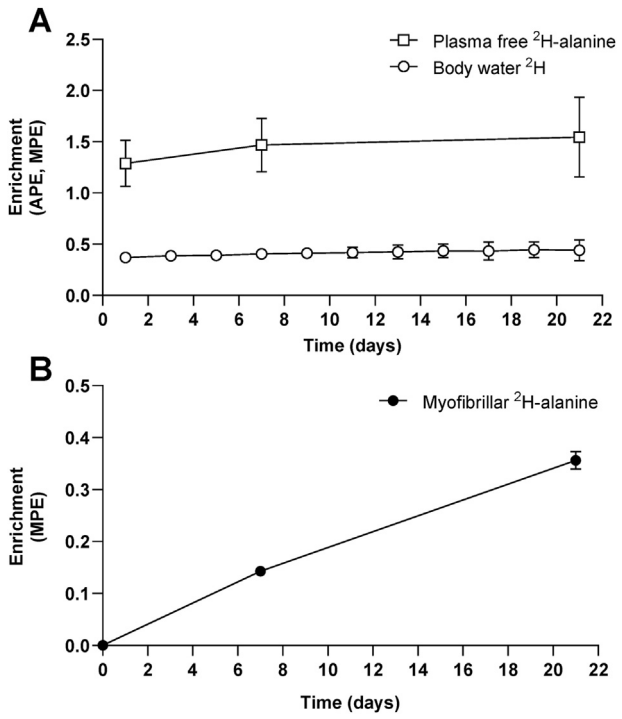

FIGURE 4. Mean plasma-free ^{2}H-alanine (A), body water ^{2}H enrichments (A), and myofibrillar ^{2}H-alanine enrichment (B) over 21 d of deuterium oxide dosing (dose-maintenance protocol). Data are derived from Weijzen et al. [20] and the myofibrillar ^{2}H-alanine data are derived from the nonimmobilized leg. Data are expressed as means ± SEM. APE, atom percent excess; MPE, mole percent excess; SEM, standard error of the mean.

Although it would be ideal to use ^{2}H-alanyl-tRNA enrichment to characterize the ^{2}H-alanine precursor environment, accurate analysis is technically difficult to perform and would require muscle tissue sample sizes that would be too large to collect in vivo in humans [34,35]. Alternatively, precursor enrichments can be estimated by measuring ^{2}H-alanine enrichment in either the muscle or plasma-free amino acid pools or by assessing ^{2}H enrichment in the body water pool through saliva or urine samples. Although the muscle-free amino acid pool may most accurately reflect ^{2}H-alanyl-tRNA labeling, the infrequent collection of muscle samples may lead to inaccurate precursor characterization over the duration of a ^2H_2O study. More accurate characterization can be achieved through more frequent blood and saliva sampling. Although we have observed higher absolute FSR values using muscle ^{2}H-alanine enrichments compared to plasma enrichments, their corresponding FSR values are strongly correlated [1]. Furthermore, using body water ^{2}H enrichments, corrected to estimate plasma-free ^{2}H-alanine enrichments (as discussed below), results in FSR values that fully align with those calculated using plasma-free ^{2}H-alanine enrichments. Therefore, plasma-free ^{2}H-alanine or body water ^{2}H enrichments should be used to determine precursor labeling (Table 1, Figure 3).

Determining mean plasma-free ^{2}H-alanine enrichment requires the collection of sequential venous blood samples of ≥50 μL plasma per time point, with a recommendation of 450 μL plasma in total per time point to accommodate re-measurement and potential other plasma analyses relevant to addressing the research aims (i.e., amino acid profile, fatty acid profile, hormone concentrations, etc.).

One drawback of blood sampling is that trained personnel and blood sampling materials are required for sample collection at the laboratory or on location at the participant/patient's home. As an alternative, body water ^{2}H enrichment can be utilized to characterize precursor labeling. As discussed, plasma precursor labeling is estimated using a correction factor from body water ^{2}H enrichments, which could introduce some inaccuracies in the precursor values when compared to directly measuring plasma-free ^{2}H-alanine enrichments. Saliva sampling is a convenient approach for measuring body water ^{2}H enrichment as it reflects the body water enrichment at the time of collection and can be easily collected by test participants/patients themselves in the comfort of their own homes. The accurate measurement of body water ^{2}H enrichment requires ≥100 μL saliva per time point. To best characterize an mean precursor enrichment over the course of days or weeks, subjects should be instructed to collect saliva samples at the same time every day, preferably in the morning upon waking or 12 h after ingestion of the daily deuterated water dose (Figure 3). When weekly maintenance doses are provided, then saliva should be collected before ingesting the maintenance dose and again 2 h following the dose to determine the peak enrichment for the subsequent week. For longer study protocols, investigators can analyze fewer saliva samples (e.g., every 3 d or before/after weekly maintenance doses) without sacrificing accuracy in characterizing precursor pool enrichments.

Home-based saliva sampling aims to collect a representative sample in a sanitary manner. Although subjects can simply transfer saliva directly from their mouths to the sample storage container, the more sanitary approach is to instruct subjects to light chew on cotton dental swabs to absorb saliva for 2 min and place the saturated swab in the air-tight sample container (described in the Materials section). The saliva samples can be collected and stored in the fridge at the subjects' homes and brought all together to the investigator at the end of the experiment. To reduce sampling error, we advise investigators to provide subjects with a clearly labeled sample storage container (e.g., a daily medicine organizer) and printed sampling instructions with a sample checklist indicating the date, time, and corresponding sample label (e.g., t = day 3) of each sample

TABLE 2
Overview of relevant tissue samples and corresponding isotopic analytes, enrichment ranges, and preferred measurement instruments for measurement of muscle protein synthesis rates using deuterium oxide dosing.

Sample fluid/tissue	Isotopic analyte	Enrichment range	Preferred measurement instrument
Saliva	^2H_2O	0.2–0.5 APE[1]	IRMS
Blood plasma	free ^{2}H-alanine	0.75–1.85 MPE[1]	GC-MS
Muscle	protein-bound ^{2}H-alanine	0.05–0.56 MPE[1]	GC-P-IRMS

Abbreviations: GC-MS, gas chromatography-mass spectrometry; GC-P-IRMS, gas chromatography isotope ratio mass spectrometer outfitted with a pyrolysis oven; IRMS, isotope ratio mass spectrometer; MPE, atom percent excess; ^2H_2O, deuterium oxide.

[1] Enrichment ranges that have been observed over a 7-d period using the preferred deuterium oxide protocol described herein: dosing day: 200 mL 70% ^2H_2O; Maintenance days: 20 mL 70% ^2H_2O.

collection point. For the best representation of body water ^{2}H enrichments, food and drink ingestion should be avoided for ≥60 min prior to sample collection. After determining body water ^{2}H enrichment, the well-established correction factor (*described below*) can be applied to estimate precursor ^{2}H-alanine enrichments [1,12,36].

Determining the increase in ^{2}H-alanine enrichment in bulk muscle protein over the experiment requires the collection of skeletal muscle biopsy samples. The collection of muscle biopsy samples is commonly performed in the *Musculusvastus lateralis* under local anesthetic using a Bergström needle modified for vacuum suction (Figure 3). Alternative muscle tissue collection methods (e.g., conchotome or BARD gun needle) may also be applied if enough tissue is collected. The detection of ^{2}H-alanine enrichment in the various bulk protein fractions within muscle (i.e., mixed muscle, myofibrillar, and mitochondrial) requires 25–80 mg muscle tissue. We recommend that muscle biopsy samples are collected prior to and after the experimental intervention [1]. However, in certain populations (e.g., clinical populations), it is more appropriate to collect a single muscle biopsy only at the end of the measurement period (e.g., during a planned surgical procedure). With this approach, a baseline (unlabeled) blood sample can be collected prior to $^{2}H_2O$ ingestion as a reference [37,38]. Albumin (or whole blood protein) ^{2}H-alanine enrichment in this blood sample will be used to reflect baseline ^{2}H-alanine enrichments of body tissues [37–39].

Analytical approaches to measure ^{2}H enrichment in precursor pools

As mentioned above, the 2 most practical and suitable precursor pools are plasma ^{2}H-alanine and ^{2}H body water enrichments. Determining plasma-free ^{2}H-alanine enrichment requires plasma-free amino acid isolation and subsequent analysis using a GC-MS. Details on the analytical protocol have been provided in 1 of our recently published studies applying the deuterated water method [1]. The measurement of body water ^{2}H enrichment can be carried out using either an isotope ratio mass spectrometer (IRMS), a GC-MS, or a ring-down cavity spectrometer [1,40,41]. Measurement using an IRMS is more sensitive and less variable in comparison to the acetone-exchange method using GC-MS but seems to be equivalent to the ring-down cavity spectrometer [42,43]. As such, measurement of ^{2}H saliva enrichments on IRMS should be considered as the preferred approach (Table 2, Figure 3). However, with the described deuterated water dosing protocols, saliva samples will require dilution to lower ^{2}H enrichment to be within the detection range of the chosen instrument [1]. Overall, saliva sampling may be the preferred choice to determine precursor ^{2}H enrichment due to the ease of collecting several samples away from the laboratory and the greater variety of analytical options.

Analytical approaches to measure ^{2}H-alanine enrichment in protein pools

The measurement of muscle protein-bound ^{2}H-alanine enrichment requires protein isolation from muscle tissue biopsy samples. Aside from mixed muscle protein, bulk protein fractions, such as myofibrillar [5,23], mitochondrial [44], or connective proteins [45,46], can be isolated to provide more detailed information into the muscle adaptive response to an intervention. After isolation, the protein is hydrolyzed into amino acids, which are further purified prior to measurement. The measurement of muscle ^{2}H-alanine enrichments can be conducted on a variety of different instrumental configurations. A GC-P-IRMS has been most often utilized by our research groups (see Supplemental Table 1). The GC-P-IRMS is a suitable measurement instrument as it possesses very high sensitivity, which allows the detection of muscle protein-bound ^{2}H-alanine enrichments that are attained using the dosing protocol described above (Table 2, Figure 3).

Though it is beyond the focus of the present tutorial, technological advances in dynamic proteome profiling have been applied to assess in vivo synthesis rates of (a selection of) individual proteins in skeletal muscle tissue in both rodents [47–52] and human studies [53–58]. For this approach, different analytical approaches (e.g., liquid chromatograph tandem mass spectrometer peptide sequencing and mass isotopomer distribution analysis) [59] are required to determine kinetic proteomics. More development will be required to allow the detection of low enrichment levels following deuterated water administration in vivo in humans and to extend the fraction of proteins that are typically identified and the subsequent fraction of proteins in which the ^{2}H enrichment level can be estimated. Simultaneous application of dynamic proteome profiling and assessment of bulk mixed muscle, myofibrillar, and mitochondrial protein FSR in the same tissue will likely provide more detailed insights into the impact of various interventions on more protein-specific synthesis rates.

Calculation of FSR over several days or weeks

Once precursor and protein ^{2}H-alanine enrichments have been measured, protein synthesis rates can be calculated. In a steady-state precursor environment, the pattern of labeled amino acid incorporation into muscle protein approaches a "plateau" over the labeling period that is theoretically equivalent to the precursor enrichment. This "rise-to-plateau" pattern over several weeks of $^{2}H_2O$ dosing is illustrated in Figure 4, with myofibrillar protein ^{2}H-alanine enrichment increasing more rapidly in the first week when compared to the later 2 wk. To account for the exponential pattern of tracer incorporation, a single exponential equation is suitable for calculating FSR (Figure 3). The equation determines muscle protein FSR based on the increase in muscle protein-bound enrichment relative to the mean precursor enrichments throughout the experiment. Consequently, the average precursor enrichment is designated as the theoretical maximum that the muscle protein-bound enrichment can reach (i.e., saturation or 100% synthesis). The resulting FSR values are, therefore, expressed as a percentage (%) of the entire protein pool (i.e., mixed muscle, myofibrillar, sarcoplasmic, and/or mitochondrial muscle protein) that is synthesized during a set amount of time (i.e., days to weeks).

$$FSR\ (\% \cdot \text{day}^{-1}) = k = \frac{-\ln(1-f)}{t}\ x\ 100 \quad (1)$$

$$f = \frac{E_{PRO2} - E_{PRO1}}{E_{bw}\ x\ 3.7} \quad (2)$$

In Equation *1*, k represents the turnover rate of the protein pool (analogous to FSR). The component f (Equation *2*) represents the change in muscle protein-bound enrichment divided by the mean precursor enrichment over the experimental period

(i.e., $E_{protein2}$-$E_{protein1}/E_{precursor}$). The value for $E_{precursor}$ can be attained either by using ^2H body water enrichment (multiplied by 3.7) or plasma ^2H-alanine enrichment. The values for the protein and precursor enrichments must be expressed in the same unit for enrichment (e.g., MPE). The component *t* is the duration of the experimental period, which is the time between 2 sequential biopsies or the intake of the first dose of ^2H_2O and the first muscle biopsy sample. An example FSR calculation using representative values over a 7-d experiment is displayed in Figure 5.

In some tissues, various parallel pools may exist with large differences in their turnover rate (e.g., such as pools of collagen in the Achilles tendon) [60,61]. The present manuscript focuses on the measurement of muscle protein synthesis rates in humans. Considering that collagen in human muscle tissue represents 1%–10% of the total protein fraction [62–64], the impact of incomplete turnover would only contribute a small error to FSR values for mixed muscle protein.

Study design

The deuterated water method can be applied within cross-sectional, longitudinal, or parallel study designs (Figure 3). A cross-sectional study design simply involves 2 (or more) groups of participants who display differences in a parameter or condition. A cross-sectional study design is best suited for studying the impact of aging, disease, and other settings that cannot be easily manipulated within a single study protocol. For cross-sectional study designs, we recommend a general approach of muscle biopsy sample collection before and after a measurement period of 2–28 d and daily saliva (or blood) sample collection. However, the experimental period duration and sample collection may be modified to accommodate the limitations of the participants and/or experimental setting. As discussed, a baseline blood sample (and isolated albumin protein) can be used in place of the muscle biopsy sample. This baseline blood sample should be collected before the dosing and measurement period. This approach is preferable in clinical settings where muscle (or other organ) tissue collection may be limited to a single time point [39].

A longitudinal study design involves 2 (or more) sequential measurement phases within a single group of participants. One of the measurement phases must be a baseline or control measurement, and 1 (or more) of the other phases includes the intervention(s). Interventions for a longitudinal study design may include an increase or decrease in physical activity (e.g., resistance training, immobilization, and bed rest), a dietary intervention (e.g., increase or decrease in food/protein), and/or administration of pharmacological agents (e.g., hormone analogs). When examining >1 intervention period in a longitudinal design, it is most appropriate to order the interventions in a way to avoid the carry-over effects of prior interventions. For example, we have recently evaluated the impact of bed rest and sedentary behavior by conducting a 7-d measurement period of habitual behavior followed by a 7-d measurement of either bed rest [21] or step reduction [18]. A point of concern is the impact of 1 intervention on the other condition, which may require a cross-over study design with an intervention "wash-out" period in between. Therefore, the preferred study design is strongly dependent on the population and the expected impact of the intervention/control condition on muscle protein metabolism.

Longitudinal study designs can also be applied to examine the impact of a more long-term intervention, such as resistance-type exercise training, on daily muscle protein synthesis rates. This study design requires 2 separate ^2H_2O measurement periods separated by several weeks or months of deuterium "wash-out" during the intervention period. The first dosing period will increase protein-bound ^2H-alanine enrichment, which can remain elevated until the second dosing period. It remains to be shown whether an elevated protein-bound ^2H-alanine enrichment at the start of the postintervention measurement period contributes to

A

Parameter description	Abbreviation	Value
Experimental time period	*t*	7 days
Average body water ^2H enrichment	E_{bw}	0.406 APE
Change in ^2H-alanine enrichment	E_{PRO2}-E_{PRO1}	0.167 MPE

B

$$f = \frac{0.167}{0.406\ x\ 3.7} = 0.111$$

$$FSR\ (\% \cdot day^{-1}) = k = \frac{-\ln(1-0.111)}{7}\ x\ 100$$

$$FSR\ (\% \cdot day^{-1}) = k = 1.68\ \% \cdot day^{-1}$$

C

	A	B	C	D	E	F	G	H	I	J	K	L
1	Variable	E_{PRO1}	E_{PRO2}	E_{PRO2}-E_{PRO1}	E_{bw}	bw corr.	E_{bw} x corr.	*t*		*f*	1-*f*	*k*
2	Unit	MPE	MPE	MPE	APE		MPE	days				%/day
3												
4	Value	0	0.167	0.167	0.406	3.7	1.502	7		0.111	0.889	1.68
5												
6	Equation			=C4-B4			=E4*F4			=D4/G4	=1-J4	=(-LN(K4)/H4)*100

FIGURE 5. An example FSR calculation (B) using representative values expected over a 7-d experiment (A). An example of data organization in Microsoft Excel, along with calculations, is depicted in (C). FSR, fractional synthetic rate.

inaccurate (e.g., artificially lower) muscle protein synthesis rates. A further concern with longitudinal studies is that proteins that rapidly turnover may become fully labeled throughout an experiment. This results in differing levels of labeling between mixed muscle proteins analyzed early compared with late biopsy samples. Over extended periods, rapidly synthesizing proteins may approach full labeling, causing measurements of mixed muscle protein synthesis rates to skew more toward the synthesis rates of more slowly synthesizing proteins. Consequently, bulk muscle protein synthesis rates may appear artificially lower [28,29]. If a longitudinal design is essential for the research question, this issue may be partially mitigated by limiting the protocol length (e.g., to <28 d) and/or isolating the myofibrillar protein fraction for analysis.

A parallel study design is an alternative approach to assess the impact of a long-term intervention on muscle protein synthesis rates. A parallel study design involves a control group and ≥1 intervention group [13,17]. The inclusion of ≥2 separate groups avoids any potential impact from residual muscle protein-bound ^{2}H-alanine enrichment from a prior dosing period, as described above. However, a key drawback to a parallel design is that the inclusion of different subjects introduces more (intersubject) variability due to, among other factors, differences in lifestyle and/or living conditions between participants. One solution to reduce intersubject variability while examining the impact of physical (in)activity on daily muscle protein synthesis rates is to apply a unilateral model within the same subjects. For example, several studies have applied unilateral exercise [1,12,25,26,65] or limb immobilization [20,66] in a randomly assigned leg while using the contralateral leg as a nonintervention control condition. In these studies, accurate measurement of muscle protein synthesis rates requires a baseline muscle biopsy (or unlabeled plasma albumin) sample and a muscle biopsy collected from each leg following the experimental period. In studies combining the unilateral model with a longitudinal design (e.g., an immobilization period followed by a recovery period), a biopsy must be collected from each leg between experimental periods to determine their respective muscle protein ^{2}H-alanine enrichments prior to the second intervention period.

An important consideration with prolonged exposure to isotope tracers is "tracer recycling." Labeled amino acids that are broken down can be recycled, either impacting precursor enrichments or becoming utilized again for protein synthesis. In contrast to acute infusion studies [67], tracer recycling does not appear to have a significant impact in $^{2}H_2O$ studies. Labeled alanine released from protein breakdown is rapidly transaminated, meaning the label will be lost when exposed to unlabeled water [11]. Importantly, the labeling of alanine is closely equilibrated with the deuterium enrichment of body water, resulting in a stable precursor enrichment over time [11,16].

Overall, in most cases, a parallel study design avoids potential labeling issues (i.e., carry-over) and, therefore, would be most suitable when attempting to measure the impact of a long-term intervention on daily muscle protein synthesis rates.

Study populations

Most commonly, studies have applied $^{2}H_2O$ to assess the impact of physical (in)activity and dietary interventions in healthy young and older populations (Supplemental Table 1). The ability to measure under "free-living" conditions provides the opportunity to measure muscle protein synthesis rates while reducing burden in nearly all patient groups. Conducting the measurement over several consecutive days or weeks is more reflective of the clinical setting, integrating the impacts of disease progression and treatment along with disruptions in physical activity and diet. Although there are no limitations to the selection of adult population groups from which to recruit test participants, considerations must be taken to minimize the impact of an intervention or disease/treatment on the stability of body water ^{2}H enrichments. One notable example is chronic kidney disease patients, who undergo dialysis therapy. Dialysis filters remove fluid from the patient, which will result in the loss of deuterium from the body water pool [11]. As ^{2}H-alanine labeling is tightly regulated by body water ^{2}H enrichment, a rapid loss in body water ^{2}H during dialysis combined with the intake of unlabeled water will rapidly reduce ^{2}H-alanine enrichment from the precursor pools and interfere with tracer incorporation into muscle protein. Along with the frequent collection of saliva samples to accurately characterize body water ^{2}H fluctuations, the loss of ^{2}H from the body water pool may be counteracted by re-dosing with deuterated water to rapidly re-label alanine precursor pools for the remainder of the experiment. Overall, virtually any population can be included in a deuterated water study, though the dosing protocol may require modification in certain populations to ensure adequate labeling or assessment of possible fluctuations in precursor pool labeling (Figure 3).

Complimentary lifestyle measurements

Investigators applying $^{2}H_2O$ are encouraged to include complimentary lifestyle measurements to evaluate the subjects' habitual behaviors. Dietary intake and physical activity are of particular interest, considering that they are the 2 most potent stimuli that modulate daily muscle protein synthesis rates (Figure 3). Physical activity (i.e., step count) can be measured using a validated 3-axis accelerometer, such as an ActiGraph wGT3X-BT. Such accelerometers are often worn on the hip in ambulatory settings and on the wrist or ankle in bed rest settings. Activities other than walking (e.g., cycling and swimming) should be accounted for by cross-reference to a physical activity log in which test participants fill in their daily activities along with corresponding intensities and durations. Dietary intake is best evaluated using dietary intake logs in which test participants fill in their food intake as accurately as possible. To minimize inaccuracies, test participants should be provided with food weigh scales and dietary logs should be evaluated for completeness by the investigator together with the test participant. In shorter experiments (<7 d), habitual factors can be fully standardized (e.g., provision of prepackaged meals and snacks and/or targeted number of daily steps and exercise bouts) and/or evaluated daily over the entire course of the experiment. In experiments with longer interventions or intervention studies, including measurements during several phases, lifestyle factors should best be evaluated for ≥3 d during each phase. Although the complete diet is important to standardize or monitor, particular attention should be given to total energy and protein intake as these factors have been shown to impact muscle protein synthesis rates [68,69].

In conclusion, stable isotope-labeled amino acids are typically applied to determine muscle tissue protein synthesis rates in vivo in humans. Deuterated water is increasingly being applied to study the impact of various nutritional, physical (in)activity,

and/or pharmaceutical interventions on daily muscle protein synthesis rates. The method allows the assessment of muscle protein synthesis rates over several days to weeks under non-laboratory, free-living conditions in vivo in humans. So far, the method has been able to confirm the impact of various established anabolic and catabolic stimuli on daily muscle protein synthesis rates when applied in a real-life setting. Consequently, we invite researchers to use the method and apply similar dosing protocols as provided above. Application of our proposed protocol recommendations will facilitate better comparisons of data sets between studies performed in different populations, under different conditions, and by different research groups and increase our understanding of the advantages and disadvantages of using deuterated water dosing to assess in vivo bulk protein synthesis rates. Such a concerted effort will help us all to further increase our understanding of the regulation of muscle protein metabolism in vivo in humans.

Author contributions

The authors' responsibilities were as follows– AMH, PJA, KS, DJW, SMP, LJCvL: wrote the manuscript; Figures were created with Biorender.com; All authors agreed to be accountable for all aspects of the work; and all authors: edited, read, and approved the final manuscript.

Conflict of interest

The authors report no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tjnut.2024.09.012.

References

[1] A.M. Holwerda, K.J. Paulussen, M. Overkamp, J.S. Smeets, A.P. Gijsen, J.P. Goessens, et al., Daily resistance-type exercise stimulates muscle protein synthesis in vivo in young men, J Appl. Physiol. (1985) 124 (1) (2019) 66–75.

[2] B.T. Wall, S.H. Gorissen, B. Pennings, R. Koopman, B.B. Groen, L.B. Verdijk, et al., Aging is accompanied by a blunted muscle protein synthetic response to protein ingestion, PLOS One 10 (11) (2015) e0140903.

[3] N.A. Burd, D.W. West, D.R. Moore, P.J. Atherton, A.W. Staples, T. Prior, et al., Enhanced amino acid sensitivity of myofibrillar protein synthesis persists for up to 24 h after resistance exercise in young men, J Nutr 141 (4) (2011) 568–573.

[4] B.T. Wall, M.L. Dirks, T. Snijders, J.W. van Dijk, M. Fritsch, L.B. Verdijk, et al., Short-term muscle disuse lowers myofibrillar protein synthesis rates and induces anabolic resistance to protein ingestion, Am. J Physiol. Endocrinol. Metab. 310 (2) (2016) E137–E147.

[5] A.M. Holwerda, K.J. Paulussen, M. Overkamp, J.P. Goessens, I.F. Kramer, W.K. Wodzig, et al., Dose-dependent increases in whole-body net protein balance and dietary protein-derived amino acid incorporation into myofibrillar protein during recovery from resistance exercise in older men, J Nutr 149 (2) (2019) 221–230.

[6] D.J. Wilkinson, S.S. Bukhari, B.E. Phillips, M.C. Limb, J. Cegielski, M.S. Brook, et al., Effects of leucine-enriched essential amino acid and whey protein bolus dosing upon skeletal muscle protein synthesis at rest and after exercise in older women, Clin. Nutr. 37 (6 Pt A) (2018) 2011–2021.

[7] P.J. Atherton, V. Kumar, A.L. Selby, D. Rankin, W. Hildebrandt, B.E. Phillips, et al., Enriching a protein drink with leucine augments muscle protein synthesis after resistance exercise in young and older men, Clin. Nutr. 36 (3) (2017) 888–895.

[8] W.K. Mitchell, B.E. Phillips, I. Hill, P. Greenhaff, J.N. Lund, J.P. Williams, et al., Human skeletal muscle is refractory to the anabolic effects of leucine during the postprandial muscle-full period in older men, Clin. Sci. (Lond.). 131 (21) (2017) 2643–2653.

[9] M.M. Robinson, S.M. Turner, M.K. Hellerstein, K.L. Hamilton, B.F. Miller, Long-term synthesis rates of skeletal muscle DNA and protein are higher during aerobic training in older humans than in sedentary young subjects but are not altered by protein supplementation, FASEB J. 25 (9) (2011) 3240–3249.

[10] B.F. Miller, J.J. Reid, J.C. Price, H.L. Lin, P.J. Atherton, K. Smith, CORP: The use of deuterated water for the measurement of protein synthesis, J Appl. Physiol. (1985) 128 (5) (2020) 1163–1176.

[11] S.F. Previs, R. Fatica, V. Chandramouli, J.C. Alexander, H. Brunengraber, B.R. Landau, Quantifying rates of protein synthesis in humans by use of 2H2O: application to patients with end-stage renal disease, Am. J Physiol. Endocrinol. Metab. 286 (4) (2004) E665–E672.

[12] D.J. Wilkinson, M.V. Franchi, M.S. Brook, M.V. Narici, J.P. Williams, W.K. Mitchell, et al., A validation of the application of D_2O stable isotope tracer techniques for monitoring day-to-day changes in muscle protein subfraction synthesis in humans, Am. J Physiol. Endocrinol. Metab. 306 (5) (2014) E571–E579.

[13] J. McKendry, C.V. Lowisz, A. Nanthakumar, M. MacDonald, C. Lim, B.S. Currier, et al., The effects of whey, pea, and collagen protein supplementation beyond the recommended dietary allowance on integrated myofibrillar protein synthetic rates in older males: a randomized controlled trial, Am. J Clin. Nutr. 120 (1) (2024) 34–46.

[14] M.J. Lee, N.J. Caruana, N.J. Saner, J. Kuang, T. Stokes, J.C. McLeod, et al., Resistance-only and concurrent exercise induce similar myofibrillar protein synthesis rates and associated molecular responses in moderately active men before and after training, FASEB J 38 (1) (2024) e23392.

[15] N. Kamoda, Y. Minatogawa, M. Nakamura, J. Nakanishi, E. Okuno, R. Kido, The organ distribution of human alanine-2-oxoglutarate aminotransferase and alanine-glyoxylate aminotransferase, Biochem. Med 23 (1) (1980) 25–34.

[16] D.A. Dufner, I.R. Bederman, D.Z. Brunengraber, N. Rachdaoui, F. Ismail-Beigi, B.A. Siegfried, et al., Using 2H2O to study the influence of feeding on protein synthesis: effect of isotope equilibration in vivo vs. in cell culture, Am. J Physiol. Endocrinol. Metab. 288 (6) (2005) E1277–E1283.

[17] A.M. Holwerda, M. Overkamp, K.J. Paulussen, J.S. Smeets, J. van Kranenburg, E.M. Backx, et al., Protein supplementation after exercise and before sleep does not further augment muscle mass and strength gains during resistance exercise training in active older men, J Nutr 148 (11) (2018) 1723–1732.

[18] B.J. Shad, J.L. Thompson, A.M. Holwerda, B. Stocks, Y.S. Elhassan, A. Philp, et al., One week of step reduction lowers myofibrillar protein synthesis rates in young men, Med. Sci. Sports Exerc. 51 (10) (2019) 2125–2134.

[19] Y. Yamada, X. Zhang, M.E. Henderson, H. Sagayama, H. Pontzer, D. Watanabe, et al., Variation in human water turnover associated with environmental and lifestyle factors, Science 378 (6622) (2022) 909–915.

[20] M.E. Weijzen, A.M. Holwerda, G.H. Jetten, L.H. Houben, A. Kerr, H. Davis, et al., Vicia faba Peptide Network Supplementation Does Not Differ from Milk Protein in Modulating Changes in Muscle Size during Short-Term Immobilization and Subsequent Remobilization, but

Increases Muscle Protein Synthesis Rates during Remobilization in Healthy Young Men, J Nutr 153 (6) (2023) 1718–1729.
[21] M.L. Dirks, J.S. Smeets, A.M. Holwerda, I.W. Kouw, G.N. Marzuca-Nassr, A.P. Gijsen, et al., Dietary feeding pattern does not modulate the loss of muscle mass or the decline in metabolic health during short-term bed rest, Am. J Physiol. Endocrinol. Metab. 316 (3) (2019) E536–E545.
[22] A.R. Konopka, J.L. Laurin, R.V. Musci, C.A. Wolff, J.J. Reid, L.M. Biela, et al., Influence of Nrf2 activators on subcellular skeletal muscle protein and DNA synthesis rates after 6 weeks of milk protein feeding in older adults, GeroScience 39 (2) (2017) 175–186.
[23] S.Y. Oikawa, M.J. Kamal, E.K. Webb, C. McGlory, S.K. Baker, S.M. Phillips, Whey protein but not collagen peptides stimulate acute and longer-term muscle protein synthesis with and without resistance exercise in healthy older women: a randomized controlled trial, Am. J Clin. Nutr. 111 (3) (2020) 708–718.
[24] S.Y. Oikawa, R. Bahniwal, T.M. Holloway, C. Lim, J.C. McLeod, C. McGlory, et al., Potato protein isolate stimulates muscle protein synthesis at rest and with resistance exercise in young women, Nutrients 12 (5) (2020).
[25] M.S. Brook, D.J. Wilkinson, W.K. Mitchell, J.N. Lund, B.E. Phillips, N.J. Szewczyk, et al., Synchronous deficits in cumulative muscle protein synthesis and ribosomal biogenesis underlie age-related anabolic resistance to exercise in humans, J Physiol. 594 (24) (2016) 7399–7417.
[26] M.S. Brook, D.J. Wilkinson, W.K. Mitchell, J.L. Lund, B.E. Phillips, N.J. Szewczyk, et al., A novel D $_2$ O tracer method to quantify RNA turnover as a biomarker of de novo ribosomal biogenesis, in vitro, in animal models, and in human skeletal muscle, Am, J Physiol. Endocrinol. Metab 313 (6) (2017) E681–E689.
[27] C. Suetta, L.G. Hvid, L. Justesen, U. Christensen, K. Neergaard, L. Simonsen, et al., Effects of aging on human skeletal muscle after immobilization and retraining, J Appl. Physiol. (1985) 107 (4) (2009) 1172–1180.
[28] H.G. Gasier, S.E. Riechman, M.P. Wiggs, S.F. Previs, J.D. Fluckey, A comparison of 2H2O and phenylalanine flooding dose to investigate muscle protein synthesis with acute exercise in rats, Am. J Physiol. Endocrinol. Metab. 297 (1) (2009) E252–E259.
[29] C.L. O'Reilly, S.C. Bodine, B.F. Miller, Current limitations and future opportunities of tracer studies of muscle ageing [Internet], J Physiol. (2023) [cited Jan 25, 2024]. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1113/JP285616.
[30] D.J. Wilkinson, J. Cegielski, B.E. Phillips, C. Boereboom, J.N. Lund, P.J. Atherton, et al., Internal comparison between deuterium oxide (D_2O) and L – [*ring* - $^{13}C_6$] phenylalanine for acute measurement of muscle protein synthesis in humans, Physiol. Rep. 3 (7) (2015) e12433.
[31] P.J. Jones, S.T. Leatherdale, Stable isotopes in clinical research: safety reaffirmed, Clin. Sci. (Lond.). 80 (4) (1991) 277–280.
[32] J.F. Thomson, Physiological effects of D20 in mammals, Ann. N Y Acad, Sci. 84 (1960) 736–744.
[33] B.R. Landau, J. Wahren, V. Chandramouli, W.C. Schumann, K. Ekberg, S.C. Kalhan, Contributions of gluconeogenesis to glucose production in the fasted state [Internet], American Society for Clinical Investigation, 1996 [cited November 15, 2023]. Available from: https://www.jci.org/articles/view/118803/pdf.
[34] P.W. Watt, Y. Lindsay, C.M. Scrimgeour, P.A. Chien, J.N. Gibson, D.J. Taylor, et al., Isolation of aminoacyl-tRNA and its labeling with stable-isotope tracers: use in studies of human tissue protein synthesis, Proc. Natl. Acad. Sci. U S A. 88 (13) (1991) 5892–5896.
[35] R.R. Wolfe, J. Song, J. Sun, X.J. Zhang, Total aminoacyl-transfer RNA pool is greater in liver than muscle in rabbits, J Nutr 137 (11) (2007) 2333–2338.
[36] S.P. Kilroe, J. Fulford, A.M. Holwerda, S.R. Jackman, B.P. Lee, A.P. Gijsen, et al., Short-term muscle disuse induces a rapid and sustained decline in daily myofibrillar protein synthesis rates, Am. J Physiol. Endocrinol. Metab. 318 (2) (2020) E117–E130.
[37] N.A. Burd, B. Pennings, B.B. Groen, A.P. Gijsen, J.M. Senden, L.J. van Loon, The single biopsy approach is reliable for the measurement of muscle protein synthesis rates in vivo in older men, J Appl. Physiol. (1985) 113 (6) (2012) 896–902.
[38] N.A. Burd, B.B. Groen, M. Beelen, J.M. Senden, A.P. Gijsen, L.J. van Loon, The reliability of using the single-biopsy approach to assess basal muscle protein synthesis rates in vivo in humans, Metabolism 61 (7) (2012) 931–936.
[39] L.H. Houben, T. Tuytten, A.M. Holwerda, E. Wisanto, J. Senden, W.K. Wodzig, et al., A low or high physical activity level does not modulate prostate tumor tissue protein synthesis rates, Med. Sci. Sports Exerc. 56 (4) (2024) 635–643.
[40] K.E. Bell, C. Séguin, G. Parise, S.K. Baker, S.M. Phillips, Day-to-day changes in muscle protein synthesis in recovery from resistance, aerobic, and high-intensity interval exercise in older men, J Gerontol. A Biol. Sci. Med. Sci. 70 (8) (2015) 1024–1029.
[41] A.F. Salvador, C.F. McKenna, K.J. Paulussen, A.R. Keeble, A.T. Askow, H.Y. Fang, et al., Early resistance training-mediated stimulation of daily muscle protein synthetic responses to higher habitual protein intake in middle-aged adults, J Physiol. 599 (18) (2021) 4287–4307.
[42] T. Thorsen, T. Shriver, N. Racine, B.A. Richman, D.A. Schoeller, Doubly labeled water analysis using cavity ring-down spectroscopy, Rapid Commun. Mass Spectrom. 25 (1) (2011) 3–8.
[43] E.L. Melanson, T. Swibas, W.M. Kohrt, V.A. Catenacci, S.A. Creasy, G. Plasqui, et al., Validation of the doubly labeled water method using off-axis integrated cavity output spectroscopy and isotope ratio mass spectrometry, Am. J Physiol. Endocrinol. Metab. 314 (2) (2018) E124–E130.
[44] T.A. Churchward-Venne, P.J. Pinckaers, J.S. Smeets, W.M. Peeters, A.H. Zorenc, H. Schierbeek, et al., Myofibrillar and mitochondrial protein synthesis rates do not differ in young men following the ingestion of carbohydrate with whey, soy, or leucine-enriched soy protein after concurrent resistance- and endurance-type exercise, 2019, J Nutr 149 (2) (2019) 210–220.
[45] A.M. Holwerda, J. Trommelen, I.W. Kouw, J.M. Senden, J.P. Goessens, J. van Kranenburg, et al., Exercise plus presleep protein ingestion increases overnight muscle connective tissue protein synthesis rates in healthy older men, Int. J Sport Nutr. Exerc. Metab. 31 (3) (2021) 217–226.
[46] J. Trommelen, A.M. Holwerda, J.M. Senden, J.P. Goessens, J. VAN Kranenburg, A.P. Gijsen, et al., Casein ingestion does not increase muscle connective tissue protein synthesis rates, Med. Sci. Sports Exerc. 52 (9) (2020) 1983–1991.
[47] A.M. Holwerda, F.G. Bouwman, M. Nabben, P. Wang, J. van Kranenburg, A.P. Gijsen, et al., Endurance-type exercise increases bulk and individual mitochondrial protein synthesis rates in rats, Int. J Sport Nutr. Exerc. Metab. 30 (2) (2020) 153–164.
[48] M.P. Bubak, A. Davidyan, C.L. O'Reilly, S.A. Mondal, J. Keast, S.M. Doidge, et al., Metformin treatment results in distinctive skeletal muscle mitochondrial remodeling in rats with different intrinsic aerobic capacities, Aging Cell (2024) e14235.
[49] B.F. Miller, G.A. Pharaoh, K.L. Hamilton, F.F. Peelor, J.L. Kirkland, W.M. Freeman, et al., Short-term calorie restriction and 17α-estradiol administration elicit divergent effects on proteostatic processes and protein content in metabolically active tissues, J Gerontol. A Biol. Sci. Med. Sci. 75 (5) (2020) 849–857.
[50] C.A. Wolff, M.M. Lawrence, H. Porter, Q. Zhang, J.J. Reid, J.L. Laurin, et al., Sex differences in changes of protein synthesis with rapamycin treatment are minimized when metformin is added to rapamycin, GeroScience 43 (2) (2021) 809–828.
[51] S.J. Hesketh, H. Sutherland, P.J. Lisboa, J.C. Jarvis, J.G. Burniston, Adaptation of rat fast-twitch muscle to endurance activity is underpinned by changes to protein degradation as well as protein synthesis, FASEB J. 34 (8) (2020) 10398–10417.
[52] C.A. Stead, S.J. Hesketh, S. Bennett, H. Sutherland, J.C. Jarvis, P.J. Lisboa, et al., Fractional synthesis rates of individual proteins in rat soleus and plantaris muscles, Proteomes 8 (2) (2020) 10.
[53] D.M. Camera, J.G. Burniston, M.A. Pogson, W.J. Smiles, J.A. Hawley, Dynamic proteome profiling of individual proteins in human skeletal muscle after a high-fat diet and resistance exercise, FASEB J. 31 (12) (2017) 5478–5494.
[54] M. Shankaran, C.L. King, T.E. Angel, W.E. Holmes, K.W. Li, M. Colangelo, et al., Circulating protein synthesis rates reveal skeletal muscle proteome dynamics, J Clin. Invest. 126 (1) (2016) 288–302.
[55] M. Shankaran, T.W. Shearer, S.A. Stimpson, S.M. Turner, C. King, P.Y. Wong, et al., Proteome-wide muscle protein fractional synthesis rates predict muscle mass gain in response to a selective androgen receptor modulator in rats, Am. J Physiol. Endocrinol. Metab. 310 (6) (2016) E405–E417.
[56] W. Evans, M. Shankaran, E. Nyangau, T. Field, M. Hussein, R. Wolfe, et al., Effects of Fortetropin on the rate of muscle protein synthesis in older men and women: a randomized, double-blinded, placebo-controlled study, J Gerontol. A Biol. Sci. Med. Sci. 76 (1) (2021) 108–114.
[57] K. Srisawat, C.A. Stead, K. Hesketh, M. Pogson, J.A. Strauss, M. Cocks, et al., People with obesity exhibit losses in muscle proteostasis that are partly improved by exercise training, Proteomics 24 (14) (2024) e2300395.

[58] C.H. Murphy, M. Shankaran, T.A. Churchward-Venne, C.J. Mitchell, N.M. Kolar, L.M. Burke, et al., Effect of resistance training and protein intake pattern on myofibrillar protein synthesis and proteome kinetics in older men in energy restriction, J Physiol. 596 (11) (2018) 2091–2120.
[59] M.K. Hellerstein, R.A. Neese, Mass isotopomer distribution analysis: a technique for measuring biosynthesis and turnover of polymers, Am. J Physiol 263 (5 Pt 1) (1992) E988–E1001.
[60] K.M. Heinemeier, P. Schjerling, J. Heinemeier, S.P. Magnusson, M. Kjaer, Lack of tissue renewal in human adult Achilles tendon is revealed by nuclear bomb (14)C, FASEB J. 27 (5) (2013) 2074–2079.
[61] C.B. Abbott, M.M. Lawrence, K.A. Kobak, E.B. Lopes, F.F. Peelor, E.J. Donald, et al., A novel stable isotope approach demonstrates surprising degree of age-related decline in skeletal muscle collagen proteostasis, Function (Oxf) 2 (4) (2021) zqab028.
[62] J.M. Haus, J.A. Carrithers, S.W. Trappe, T.A. Trappe, Collagen, cross-linking, and advanced glycation end products in aging human skeletal muscle, J Appl. Physiol. (1985) 103 (6) (2007) 2068–2076.
[63] A.M. Holwerda, M.E. Weijzen, A. Zorenc, J. Senden, G.H. Jetten, L.H. Houben, et al., One week of single-leg immobilization lowers muscle connective protein synthesis rates in healthy, young adults, Med. Sci. Sports Exerc. 56 (4) (2024) 612–622.
[64] J.M. Haus, J.A. Carrithers, C.C. Carroll, P.A. Tesch, T.A. Trappe, Contractile and connective tissue protein content of human skeletal muscle: effects of 35 and 90 days of simulated microgravity and exercise countermeasures, Am. J Physiol. Regul. Integr. Comp. Physiol. 293 (4) (2007) R1722–R1727.
[65] C.H. Murphy, N.I. Saddler, M.C. Devries, C. McGlory, S.K. Baker, S.M. Phillips, Leucine supplementation enhances integrative myofibrillar protein synthesis in free-living older men consuming lower- and higher-protein diets: a parallel-group crossover study, Am. J Clin. Nutr. 104 (6) (2016) 1594–1606.
[66] C.R. Willis, I.J. Gallagher, D.J. Wilkinson, M.S. Brook, J.J. Bass, B.E. Phillips, et al., Transcriptomic links to muscle mass loss and declines in cumulative muscle protein synthesis during short-term disuse in healthy younger humans, FASEB J 35 (9) (2021) e21830.
[67] W.F. Schwenk, E. Tsalikian, B. Beaufrere, M.W. Haymond, Recycling of an amino acid label with prolonged isotope infusion: implications for kinetic studies, Am. J Physiol 248 (4 Pt 1) (1985) E482–E487.
[68] J.L. Areta, L.M. Burke, D.M. Camera, D.W. West, S. Crawshay, D.R. Moore, et al., Reduced resting skeletal muscle protein synthesis is rescued by resistance exercise and protein ingestion following short-term energy deficit, Am. J Physiol. Endocrinol. Metab. 306 (8) (2014) E989–E997.
[69] D.R. Moore, J.E. Tang, N.A. Burd, T. Rerecich, M.A. Tarnopolsky, S.M. Phillips, Differential stimulation of myofibrillar and sarcoplasmic protein synthesis with protein ingestion at rest and after resistance exercise, J Physiol. 587 (4) (2009) 897–904.