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### Original Article

# Understanding the effects of nutrition and post-exercise nutrition on skeletal muscle protein turnover: Insights from stable isotope studies

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#### SUMMARY

Skeletal muscle is the largest organ of the human body and plays a pivotal role in whole-body homeostasis through the maintenance of physical and metabolic health. Establishing strategies aimed at increasing the amount, and minimising loss, of muscle mass are of upmost importance. Muscle mass is primarily dictated by the meal-to-meal fluctuations in muscle protein synthesis (MPS) and muscle protein breakdown (MPB), each of which can be quantified through the use of stable isotopically labelled tracers. Importantly, both MPS and MPB can be influenced by external factors such as nutritional manipulation, specifically protein ingestion, and changes in loading via exercise. To date, research involving stable isotopic tracers has focused on determining the optimal dose, timing surrounding bouts of exercise, distribution and composition of protein to maximally stimulate MPS and inhibit MPB, both at rest and following exercise. In this review we focus on the use of these stable isotopically-labeled tracers to unravel the intricacies of skeletal muscle protein turnover in response to specific nutritional interventions.

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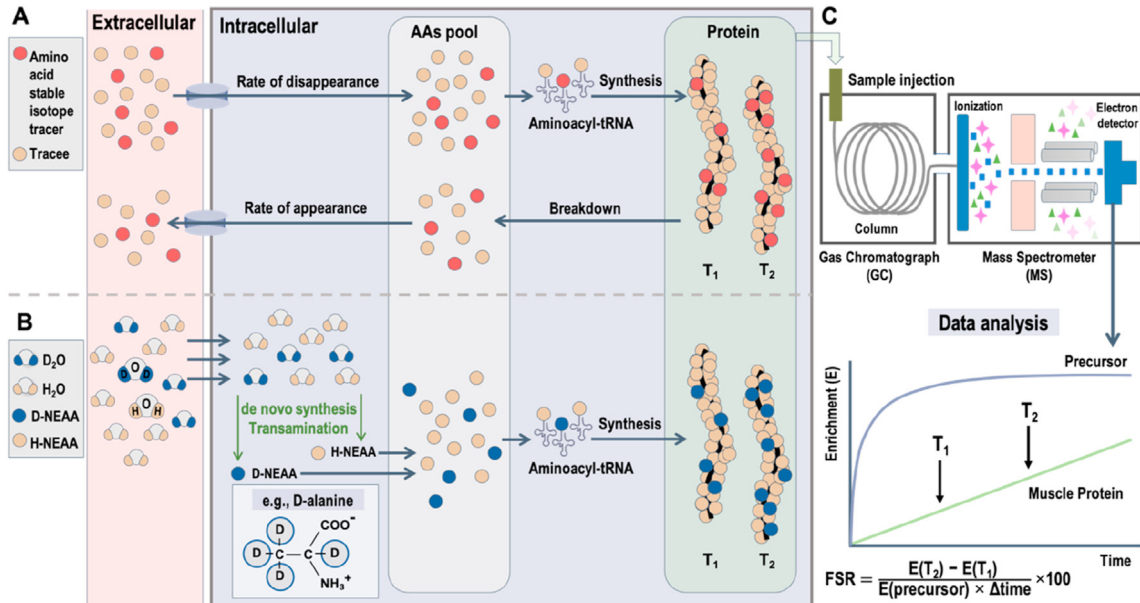
## 1. Introduction

Skeletal muscle is a remarkably plastic tissue that can change its phenotype in response to changes in loading demands. Skeletal muscle also plays an integral role in whole-body metabolism and homeostasis. The rate of muscle protein turnover is dependent on the balance between two opposing, ongoing, but interrelated kinetic processes: muscle protein synthesis (MPS) and muscle protein breakdown (MPB). This continuous turnover of muscle proteins results in efficient repair and renewal of damaged (whether mechanically, via oxidation, misfolding, nitrosylation, or otherwise) proteins and underpins the plasticity of skeletal muscle in response to contractile and nutritional perturbations [1]. In the postabsorptive state, MPB exceeds MPS and muscle proteins are catabolized to supply amino acids (AA) back into the free pool, but most of which are recycled and reused. However, some AA are lost from muscle, mostly as alanine and glutamine (nitrogen carriers) for glucose production via gluconeogenesis, or as a fuel for enterocytes. When MPB exceeds MPS, the net catabolism of skeletal muscle, or a negative net protein balance (NPBAL) is transient, however [2]. Ingestion of a mixed-meal, and the ensuing rise in plasma AA and insulin, stimulates MPS and suppresses MPB leading to net accretion of protein, and a positive muscle NPBAL. In healthy, active adults, assuming adequate intakes of protein, periods of postabsorptive catabolism remain in dynamic equilibrium with periods of postprandial anabolism over a 24hr period and muscle mass is maintained. This is likely true in fully grown adults in their third decade of life and onward into their fourth and possibly fifth decade; however, at a certain point NPBAL begins to shift toward a net negative state and muscle is slowly lost. This slow loss of muscle with aging is termed sarcopenia [3].

Exercise increases muscle protein turnover. Specifically exercise, independent of nutrition, results in an increase in both MPS and MPB, but the increase in MPB outweighs that of MPS and thus resulting in a negative NPBAL. However, the consumption of protein is able to increase the MPS response and drive a positive NPBAL. Resistance exercise (RE) leads to the sensitization of the muscle protein translational machinery to the presence of AA for at least 24–48 h [4], resulting in an additive stimulation of MPS over that due to hyperaminoacidemia alone [5]. The increase in MPS following exercise is dependent on the type of exercise completed. For example, RE is commonly associated with increases in muscle size, whereas endurance exercise (EE) is characterized by remodelling of the muscle towards a more oxidative phenotype. Initially, the stress of RE and EE in untrained adults upregulates myofibrillar and mitochondrial protein synthesis [6]; however, as training progresses the response is refined to be more specific to form, resistance or endurance, of exercise. An acute bout of RE after 10 weeks of resistance training (RT) increased myofibrillar but not mitochondrial protein synthesis [7]. In contrast, acute EE increased mitochondrial protein synthesis after 10 weeks of endurance training, with no detectable effect on the myofibrillar sub-fraction [7]. Post-exercise protein intake supports the synthesis of proteins in these exercise-responsive protein sub-fractions [7]. These data underscore the importance of measuring fraction-specific protein turnover to understand the specificity of skeletal muscle adaptation.

It is now possible to combine stable isotopes with liquid chromatography and mass-spectrometry to investigate the fractional synthesis rate and abundance of hundreds of individual proteins within a given muscle sub-fraction [8,9]. Determining the abundance and synthesis rate of individual proteins also permits the calculation of protein breakdown rates. Once changes in individual protein abundance and FSR are obtained by D<sub>2</sub>O ingestion and alanine labelling, the absolute rate of individual protein breakdown can be calculated by difference. This allows researchers to circumvent issues associated with bulk MPB measurements using the tracer dilution technique (i.e. the need for a physiological steady-state) and multiple biopsies during the dilution of the tracer [10,11].

In this review, we focus on the application of stable isotope tracers to elucidate the impact of protein ingestion and exercise on skeletal muscle protein turnover in humans (Fig. 1). Specifically, we consider the influence of total protein intake, protein source and daily protein distribution on muscle protein synthesis and, where data are available, muscle protein breakdown. Given the breadth of information on this topic, and the consideration of distinct clinical populations in accompanying reviews in this special issue, we limit our discussion primarily to healthy young and older adults without existing clinical comorbidities.



**Fig. 1.** Schematic representation of the use of AA stable isotope tracers and D<sub>2</sub>O for the measurement of muscle protein turnover. **(A)** To measure acute (typically 0–6 h post-exercise and/or feeding) MPS, stable AA isotope tracers are infused intravenously which results in an increased plasma enrichment of the isotope. The AA isotope is taken up by the muscle (rate of disappearance) into the intracellular AA pool. The labeled AA is incorporated into new proteins (MPS). As infusion time progresses the change in incorporated AA stable isotope is proportionate to the rate of total MPS. Alternatively, MPB can be measured through the release of labelled AA stable isotope into the intracellular AA pool or inferred from the rate of appearance of the labelled AA into the venous blood samples. **(B)** To measure integrated (free-living) MPS D<sub>2</sub>O is ingested and results in an increase in the enrichment of the body water pool. Once entering the cell the deuterium becomes incorporated into alanine during the transamination process, these deuterium labelled alanine AA are then incorporated into new muscle proteins and are indicative of MPS. **(C)** For both methods, muscle protein/plasma/saliva enrichment is determined using mass spectrometry following derivatization of AA. To calculate the rate of MPS, the enrichment of one muscle sample (T<sub>1</sub>) is subtracted from the enrichment of a later muscle sample (T<sub>2</sub>) and divided by the mean enrichment of the precursor (plasma enrichment when using stable AA isotope; body water enrichment when using D<sub>2</sub>O) multiplied by the time.

## 2. Protein dose

In young healthy individuals skeletal muscle accounts for ~40% of total body mass and serves as an important hub for dietary protein uptake and utilization, a protein dose normalized for total body mass may seem practical. As such, the current guidelines (or recommended daily allowance (RDA)) suggest the amount of protein required for a healthy adult is equivalent to 0.8 g/kg/d (RDA) [12]. However, these recommendations were established from early nitrogen balance studies, within which the primary focus was the achievement of nitrogen balance and the avoidance of protein deficiency [13]. Thus, current protein intake recommendations are inadequate, particularly for those looking to increase skeletal muscle mass with exercise training, mitigate situations of muscle loss (i.e., limb immobilization or bed rest) or prevent age-related sarcopenic muscle loss. The utilization of stable isotopes, and the and continuous development of the associated analytical techniques, has paved the way for a plethora of investigations within human metabolic research. At the forefront of protein metabolism research, the late Professor Mike Rennie and colleagues pioneered the first investigations, in humans, to demonstrate that the incorporation of a stable isotope tracer (<sup>13</sup>C-Leucine) into skeletal muscle was increased following protein feeding [14] indicating a higher rate of MPS. Subsequently, since the inception of stable isotopic tracers within metabolic research, the influence that nutritional intricacies exert on skeletal muscle protein metabolism has become a niche field that has flourished in recent years.

### 2.1. Protein distribution

MPS is a modifiable process, the magnitude and duration of which can be influenced by a variety of feeding strategies. An early line of enquiry for researchers was to decipher the maximal capacity of the human body to digest, absorb and subsequently utilize the constituent AA for anabolism of contractile and metabolically functional proteins. Fittingly, Mike Rennie's group were amongst the first to demonstrate that MPS was elevated in a dose–response manner to increasing concentrations of circulating (extracellular) AA in resting skeletal muscle [15]. Organ tissues utilize AA derived from dietary protein intake to replace damaged proteins and to synthesize an array of molecules required for normal bodily function. Acute infusion studies using labelled AA <sup>13</sup>C<sub>6</sub>-Phenylalanine, have demonstrated that muscle protein synthesis is maximally stimulated by protein intakes of ~0.24 g/kg and ~0.4 g/kg body mass in healthy young and older adults at rest in a post-prandial state, respectively [16]. Beyond these amounts, oxidation of AA – measured in this case as an increase in breath <sup>13</sup>CO<sub>2</sub> enrichment – is accelerated as is ureagenesis. However, the meal-induced rise in MPS, without the influence of exercise, is transient and returns to basal rates after 2–3 h despite a persistent hyperaminoacidemia and intramuscular anabolic signaling [17]. Together these findings provide a rationale for evenly distributing protein throughout the day such that each meal maximally stimulates MPS, while simultaneously minimizing catabolism. In support of this approach, 20 g protein beverages consumed every 3 h stimulated myofibrillar protein synthesis to a greater degree than a pulsed (8 × 10 g protein every 1.5 h) or bolus dosing strategy (2 × 40 g protein every 6 h) when measured over 12 h in resistance-trained young men [18].

Most adults in Western society consume their daily protein intakes in a skewed manner. A disproportionate amount (~40–50%) of daily protein is consumed at the late-day dinner meal, with the remainder divided amongst breakfast and lunchtime meals. Because of this unequal distribution, <50% of young adults and only ~7.5% of older adults consume the amount of protein at breakfast and lunch that would be required to maximally stimulate MPS [19]. From a muscle-centric perspective, increasing the amount of protein consumed at breakfast and lunch, while simultaneously reducing the amount consumed at dinner should foster an enhanced anabolic environment throughout the day without increasing daily intake. Indeed, evenly distributing protein intake over breakfast, lunch and dinner meals (30 g at each meal) led to a ~25% greater stimulation of 24 h mixed-muscle protein synthesis compared to a skewed intake of the same total amount of protein (10 g, 15 g, and 65 g at breakfast, lunch and dinner) in middle-aged adults [20]. Consuming a protein supplement at breakfast also augmented lean mass accretion after 12 RT in young men compared to when the same supplement was consumed at dinner [21]. Similarly, older adults who increased their protein intake at breakfast and

lunch above 0.4 g/kg via milk protein supplementation demonstrated a significantly greater increase in lean body mass over a 24-week dietary intervention period compared to those given a maltodextrin control [22]. However, the need to evenly distribute protein ingestion has been challenged. When older adults consumed either 0.8 or 1.5 g of protein/kg/day distributed either evenly or skewed (15% breakfast, 20% lunch and 65% dinner) between 3 meals for 4 days, both groups consuming higher protein had greater MPS than the groups consuming less protein, suggesting that protein quantity and not protein distribution affected MPS (Kim et al., 2015). The concept of even protein distribution was further challenged by the same research group when they demonstrated that when older adults consumed 1.1 g of protein/kg/day either distributed evenly or skewed (15% breakfast, 20% lunch and 65% dinner) over 3 meals for 8 weeks no differences in MPS, lean body mass or strength were observed between groups (Kim et al., 2018). Although the protein consumed in this study was of high-quality total protein consumption per day (1.1 g/kg/day) may not be sufficient in maximally stimulating MPS in older individuals and therefore may mask any differences that could exist between protein distribution. Specifically, consuming 1.1 g of protein/kg/day in a balanced manner would result in consuming approximately 0.36 g of protein/kg at each meal which may not be able to maximally stimulate MPS in older adults.

Date supporting the importance of even protein distribution throughout the day is equivocal, therefore there is no concordance on the efficacy of evenly distributed protein the potential relevance and generalizability of the distribution hypothesis to daily nutritional practices [23]. Most notable is the reality that protein is often consumed as part of a mixed-meal containing carbohydrate and lipid rather than as an isolated nutrient. This ‘whole-food matrix’ alters the digestion and absorption kinetics of protein and can influence the subsequent anabolic response. Indeed, the consumption of an intrinsically-labelled whey protein beverage induces a rapid and transient rise in the rate of phenylalanine appearance, which peaks at ~30–60 min and returns to basal levels by ~180–240 min [24]. In contrast, the rate of phenylalanine appearance into plasma is sustained at an elevated level for a longer period of time following the ingestion of labelled casein protein [24]. These distinct absorption profiles probably explain the recent finding that, when whey and casein are co-ingested (i.e. as milk protein), MPS remains elevated throughout the 5hr postprandial period [25]. This observation is in contrast to rapid return of MPS to basal levels 2–3 h after the ingestion of a whey protein isolate and suggest that ingestion of a mixed-meal may protract the anabolic response. These data argue against a strict 3 h spacing window between meals as implied by earlier work that formed the foundation for the muscle-full hypothesis [17,26]. More research is now needed to investigate the dose–response of MPS to whole-food protein sources and the daily eating strategies that maximize anabolism over the course of the day to place these findings into a broader nutritional context.

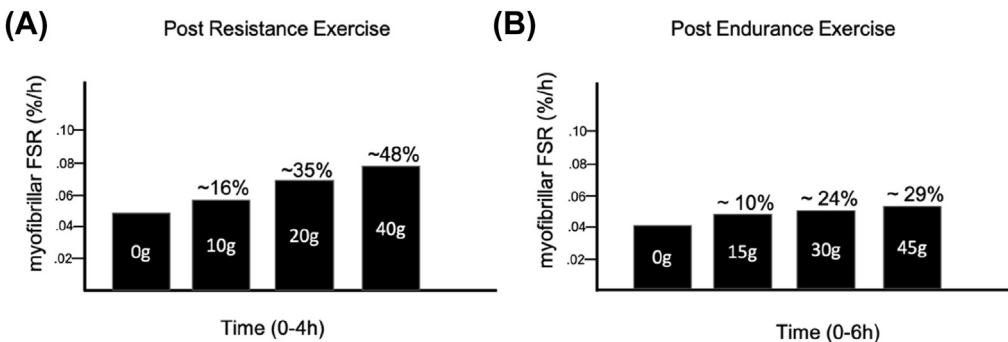
An even rather than a skewed intake of dietary protein may be beneficial during periods of energy restriction (ER). This thesis rests on the observation that ER leads to a reduction in basal and postprandial MPS in young adults despite protein intakes of ~2x RDA [27]. Our laboratory has also shown a reduction in fed-state myofibrillar protein synthesis following a 4-week hypocaloric dietary intervention in older men [28]. However, participants who consumed their daily protein evenly distributed across four daily meals (25% daily protein per meal) demonstrated an attenuated decline in acute myofibrillar protein synthesis relative to participants consuming protein in a skewed manner (7:17:72:4% at breakfast, lunch, dinner and pre-bed snack, respectively) [28]. The effects were subtle, however, and follow up analysis (using biopsy tissue from the same participants) showed that the difference in MPS between groups is no longer significant when measured using D<sub>2</sub>O to capture integrated changes over a 2-week period [9]. Nonetheless, when viewed at the level of individual muscle proteins, balanced and skewed protein intakes differentially regulated the synthesis of proteins belonging to distinct biological pathways. For instance, a balanced intake of dietary protein increased the abundance of proteins involved in myofibrillar assembly to a greater extent compared to skewed protein intake during ER and RT [9]. Taken together, these data suggest that balanced protein consumption may be an ideal strategy for distributing daily protein during periods of energy deficit, particularly when combined with RT, and also underscore the importance of using complimentary tracer techniques to capture nuanced but potentially important changes in the muscle proteome that are diluted when quantified as ‘bulk’ sub-fractional averages.

Finally, protein ingestion before sleep should also be considered when determining ideal daily protein distribution patterns. For most individuals sleep represents the longest period spent in the fasted state, and thus a negative NPBAL. In one of the first proof of principle investigations to assess the effectiveness of pre-sleep protein ingestion on muscle anabolism, Groen and colleagues provided participants with 40 g of casein protein via a nasogastric tube during sleep [29]. The AA contained in the beverage were effectively digested and absorbed, thus increasing plasma AA availability throughout sleep and augmenting MPS. The combination of RE in the evening and pre-sleep protein ingestion had an even greater stimulatory effect on MPS in healthy young [30] and older adults [31] and augmented RT-induced gains in skeletal muscle mass and strength after a 12 week intervention [32]. Therefore, the ingestion of pre-sleep protein may be an effective way to further extend an individual's time spent in a positive NPBAL.

Evenly distributing protein intake throughout the day is a pragmatic strategy to enhance skeletal muscle anabolism at each meal – especially when combined with RT. However, with increasing daily protein intakes, the benefits of an even vs. skewed protein consumption pattern ostensibly become less important. Thus, the benefits observed when balancing protein intake across meals may be secondary to an increase in total daily protein intake and therefore reflect the inadequacy of current protein intake guidelines rather than an effect of protein distribution per se.

2.2. Individual/per-feed protein dose

To extend upon the early work from Bob Wolfe's group, which elegantly demonstrated the sensitization of skeletal muscle to AA provision following RE [5]. Moore and colleagues provided the first evidence that, following an intense acute bout of RE of the leg in healthy previously trained young men, MPS was saturated at a relatively moderate dose – 20 g – of protein [33]. Specifically, ingestion of isolated egg protein stimulated MPS in a dose-dependent manner up to 20 g (equivalent to 8.6 g essential amino acids [EAA]), and when the protein dose was doubled (40 g) the rate of MPS was not further augmented [33]. This dose–response phenomenon was replicated by Witard and colleagues, who showed that RE-induced MPS was maximally stimulated following the provision of 20 g whey protein in young, resistance-trained, men [34]. Taken together, this supports the notion that MPS, in response to resistance exercise and protein feeding, is saturated at a ‘moderate’ dose (~20 g or ~0.25 g protein/kg) of high quality, protein (Fig. 2).



**Fig. 2.** Dose–response of muscle protein synthesis, to protein ingestion following exercise. Protein ingestion after (A) resistance exercise and (B) endurance exercise presented as a percent change from the ingestion of no protein (0 g) results in an increases in myofibrillar FSR. Following RE the consumption of 40 g versus 20 g of whey protein increased myofibrillar FSR only slightly and the increase was statistically significant indicating that 20 g of protein maximally stimulated MPS following RE (A). Following endurance exercise 45 g of carbohydrates was consumed with 0 g, 15 g, 30 g, and 45 g of intrinsically labelled milk protein. Myofibrillar FSR was maximally stimulated with the ingestion of 45 g of carbohydrates and 30 g of protein, the consumption of 15 g of protein did not significantly increase MPS rates above that of the consumption of 0 g of protein. Figure was altered from Stokes and colleagues 2018 [37], data re-drawn from Witard and colleagues 2014 [34] and Churchward-Venne and colleagues 2020 [36].



Although a moderate dose of protein appears sufficient to saturate the RE-induced increase in MPS, others report that following whole-body RE the consumption of 40 g (~0.5 g/kg), compared with 20 g, led to a small but significantly higher (~18%) acute MPS response [35]. The authors propose that the small benefit observed, with double the dose of protein, may be explained by the amount of muscle mass recruited for the whole-body RE, which would likely increase the demand for AA; though this is yet to be confirmed. Importantly, however, two other studies found that when ingesting 40 g of protein the stimulation of MPS was numerically but not statistically greater than that seen with ingestion of 20 g of protein by 10% [34] and 8% [33]; thus, it seems that the statistically significant increase seen by Macnaughton and colleagues is small and likely of little consequence in affecting long-term muscle NPBAL [35]. Following endurance exercise, MPS was observed to be saturated with the ingestion of 30 g (~0.49 g/kg) milk protein and the authors also report that whole-body NPBAL displayed a clear dose–response relationship (i.e., 45 g PRO > 30 g PRO > 15 g PRO) [36] (Fig. 2).

In recent years, a number of developments within isotopic tracer methodologies, has significantly advanced the field and enabled a deeper, yet more wholistic, understanding of skeletal muscle protein metabolism. Following initial attempts [38], in 2009, Professor Luc van Loon, and his research group, extended upon a novel tool for use within muscle protein metabolism research [39]. Specifically, Holstein cows were infused with 1-[1-<sup>13</sup>C]-phenylalanine at the beginning of lactation, which enabled them to produce milk that yielded intrinsically 1-[1-<sup>13</sup>C]phenylalanine-labeled milk proteins when processed. This enabled precise determination of how much of the ingested milk protein was first-pass cleared by splanchnic AA extraction, AA uptake into the muscle, and subsequent MPS within a single *in vivo* experiment [40]. The ingestion of intrinsically labelled proteins allows for the assessment of protein digestion and absorption kinetics, whereas the ingestion of labelled free amino acids does not. Measuring the muscle protein synthetic response to intrinsically labelled foods is less likely to disturb the tracer equilibrium and accounts for the “true” physiology of food more closely relate to the consumption of a ‘regular’ meal, instead of infusing individuals with labelled amino acids. Accordingly, from a single 20 g dose of 1-[1-<sup>13</sup>C]phenylalanine-labelled milk protein ~50% of the constituent AA are extracted by splanchnic tissues (i.e., gut and liver) and the remaining ~50% becomes available within the circulation. It is important to note that digestion and extraction values reported are specific to the use of phenylalanine as an isotopically labeled tracer and vary between amino acids. Perhaps surprisingly, only ~10% of ingested protein (~2.2 g) was utilized for *de novo* myofibrillar protein synthesis; whereas, the remainder of the protein underwent catabolism and ureagenesis or used for production of other proteins [40]. Subsequently, Churchward-Venne and colleagues were the first to demonstrate a protein dose–response of incorporation of ingested AA into *de novo* mitochondrial proteins in response to endurance exercise (i.e., 45 g PRO > 30 g PRO > 15 g PRO) [36]. Thus, by utilizing the intrinsically labelled protein method a number of the intricacies associated with individual protein doses have been teased out.

### 2.3. Total protein dose

Individual protein dose aside, the chief determinant of skeletal muscle growth, and/or maintenance, is total daily protein intake. As previously mentioned, the current guidelines are far from optimal if one were looking to maximize MPS in response to RE. A host of researchers and expert groups have since advocated for increased daily protein intakes particularly for older persons [41–43], many of which rely on important information obtained from stable isotope tracer studies. A recent meta-analysis reported, using a bi-phasic regression analysis of 49 studies and 1863 participants, that total protein intakes beyond ~1.6 g/kg/day (95% confidence interval: 1.0–2.2 g/kg/d) resulted in no further increases in RE-induced gains in fat-free mass [44]. However, following advances in the indicator amino acid oxidation (IAAO) method, whereby one EAA is limiting and the oxidation of the indicator AA is provided in excess to determine a breakpoint where whole-body protein synthesis is maximal have yielded different answers. In one study it was reported that post-exercise whole body anabolism, or NPBAL, was maximized with a protein intake of ~2.0 g/kg/d [45]. It is however important to note that these inferences were made based on whole-body protein metabolism measures and these are not specific to the skeletal muscle. Importantly, the individuals recruited for this particular study, were resistance trained and habitually consumed a higher protein diet. Nevertheless, this study, and the previous meta-



analysis, suggests that a protein intake (~1.6 to as high as 2.2 g/kg/d) far in excess of the current recommendations (0.8 g/kg/d) may be optimal to maximize the daily body protein balance and muscle protein accrual.

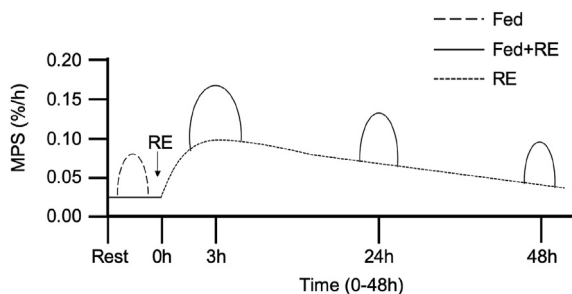
Many years following the development and application of deuterated water (D<sub>2</sub>O) for use in protein metabolism research [46], there has been a resurgence of interest in the use of D<sub>2</sub>O for measuring muscle protein turnover [47]. As a far more convenient and arguably ecologically valid measure of MPS, versus an infusion of labeled AA, D<sub>2</sub>O has provided researchers with a vital tool capable of determining MPS in a free-living setting (Fig. 1). Thus, D<sub>2</sub>O enables the assessment of daily integrated MPS; which may be much more reflective of the anabolic potential of exercise and nutritional interventions. For example, integrated rates of MPS were shown to be greater in young men consuming a higher (~2.35 g/kg/d) compared with a lower (~1.2 g/kg/d) protein diet [48]; during pronounced ER. Importantly, a recent study that employed the D<sub>2</sub>O methodology has also demonstrated that the benefits of a higher protein diet combined with RE extend to a young female population [49]. The utility of D<sub>2</sub>O combined with the recent expansion of proteomics has enabled an increasingly detailed examination of entire protein networks and their regulation during altered muscle protein turnover. Thus, the use of stable isotopic tracers, regardless of methodology (i.e., infusion, IAAO, intrinsically labelled, D<sub>2</sub>O), offer an invaluable tool to the field of muscle protein metabolism.

#### 2.4. Protein timing surrounding resistance exercise

Protein ingestion and RE have a synergistic influence on MPS and muscle growth over time [50,51]. Therefore, peri-workout nutrition has garnered significant research attention, both in athletic populations attempting to maximize their adaptive potential to training and in older individuals with the goal of preserving muscle mass over time. Tipton and colleagues were the first to assess the anabolic effects of pre- vs. post-RE consumption of EAA and carbohydrates (CHO) [52]. The authors demonstrated a superior NPBAL, measured by the rate of appearance and disappearance of labelled L-[ring-<sup>2</sup>H<sub>5</sub>]-Phenylalanine in the blood, in participants who consumed 6 g of EAA + 35 g of CHO prior to RE as opposed to 1hr post-RE [52]. However, when measured directly (tracer incorporation into muscle proteins [53]) as opposed to indirectly (i.e. rate of tracer disappearance from plasma [52]), Fujita and colleagues failed to demonstrate a superior effect of pre-exercise EAA + CHO consumption compared to fasted-state RE on MPS [53]. In contrast, pre-exercise protein and CHO co-ingestion followed by periodic pulses every 15 min *during* a 2 h RE session augmented MPS compared to a CHO placebo [54], which is likely due to enhanced intramuscular anabolic signaling activation [55]. While most of the data regarding pre- and intra-RE protein supplementation demonstrate positive effects on MPS and thus NPBAL, the most popular time to ingest protein is arguably post-RE, when signaling proteins responsive to muscle contraction are activated and the muscle is essentially primed to synthesize new proteins. A meta-analysis showed that consumption of protein in close temporal proximity to RE appeared to positively influence accretion of lean mass, however, no effect of protein timing on muscle hypertrophy was observed when the confounding influence of total protein intake was considered [56]. Thus, total protein intake more so than when it was ingested had the largest effect on muscle hypertrophy [56], which is consistent with a recent meta-analysis from our laboratory [50]. Additionally, skeletal muscle remains sensitized to AA for at least 24 h post-exercise [4] and thus remains highly responsive to post-RE nutrition (Fig. 3). Taken together, peri-workout nutrition plays a minimal role in muscle hypertrophy when total protein intake is sufficient to support muscle hypertrophy.

### 3. Protein quality

In addition to protein dose and timing, protein quality has been the subject of intense scientific focus and is considered to be an important variable that should be incorporated in dietary protein recommendations. The *quantity* of a protein is determined based on the nitrogen content in a sample of isolated protein or food, whereas protein *quality* is dictated by the capacity of the protein to fulfil key metabolic requirements. Therefore, protein quality is organism and physiology-dependent, rather than being limited to a function of chemical or biochemical properties of the protein (i.e. available nitrogen



**Fig. 3.** Muscle protein synthesis is increased following a bout of resistance exercise. Skeletal muscle becomes more sensitized to the ingestion of protein for at least 24 h following resistance exercise. Thus, total protein intake rather than the exact time at which protein is consumed surrounding resistance exercise has the greatest impact on muscle protein synthesis. Figure originally from and has been re-drawn from Churchward-Venne and colleagues [57].

content). It is the EAA content and the proteins inherent digestibility within a particular protein source that are key determinants of the metabolic effects induced by protein ingestion [58–60].

In 1991, the Food and Agriculture Organization (FAO) of the United Nations recommended the use of the protein digestibility-corrected amino acid score (PDCAAS) for the evaluation of protein quality in food products - Protein Quality Evaluation: Report of Joint FAO/WHO Expert Consultation [61]. Briefly, PDCAAS is determined by the AA score of one given crude dietary protein (CP) multiplied by its true faecal nitrogen digestibility. However, PDCAAS had several limitations requiring it to be updated by new, more accurate approaches [60–62]. Since then, an expert Consultation on Protein Evaluation in Human Nutrition report, published in 2013, contained a recommendation to use the digestible indispensable amino acid score (DIAAS); as this method addresses some limitations of the PDCAAS. Specifically, the DIAAS indicates a percentage of the daily requirement for the most limiting EAA in a given protein when an amount equals to the estimated average requirement (EAR) is ingested and it also uses ileal rather than faecal digestibility. Because EAR for different AA varies according to age ranges, the same protein or food protein source can have different DIAAS values based on the requirements applied during the calculation. As opposed to PDCAAS, DIAAS is also not truncated at 1.0 and instead recognized that EAA at levels above those required for growth also needs to be recognized. Interested readers can find a deeper description and discussion on the limitations and advantages of DIAAS or further comparison between DIAAS over PDCAAS elsewhere [58,59,63].

Protein quality has been a known independent variable dictating MPS response. Still, most studies have tested high-quality proteins (i.e. milk-derived proteins, whole-egg protein, etc.) to stimulate MPS in humans [33,64–66]. Therefore, the vast majority of the knowledge in this field is restricted to considered high-quality proteins. A robust protein synthetic response can be stimulated by proteins like whey protein [64]; however, particular characteristics of whey protein as rapid digestibility and high leucine content [24,67], were fundamental to discovering the role of Leucine in MPS activation [24,64,67], as discussed in the following section.

### 3.1. Leucine: the trigger amino acid

More recently, an emphasis has been placed on identifying specific EAA that stimulate MPS, specifically leucine [60]. Altogether, EAA are the main drivers of a sustained MPS response [68], with leucine being the AA that in muscle, acts to activate the process of MPS [69]. Several studies have shown that leucine content, within a protein-containing meal, is the main driver of MPS activation [64,70,71]. Because of this property, a leucine trigger hypothesis has been advocated to better understand how protein ingestion activates MPS [59,72,73]. The hypothesis stipulates that MPS activation is a function of leucine concentration within the intracellular AA pool [59,64]. Currently, it is known that the increase in intercellular concentration of leucine signals to activate MPS through a mechanism involving the regulation of the mechanistic target of rapamycin (mTOR) [74]. The exact mechanism by

which leucine activates mTORC1 pathway has been identified, and it involves proteins of the Sestrin family [74,75]. Specifically, leucine binds to Sestrin 2 changing its phosphorylation state and decreasing its interaction with GAP activity toward the Rag GTPases 2 (GATOR2) [74]. Accordingly, GATOR2 interacts with GATOR1, decreasing its inhibition on mTOR, which in turn leads to an increased protein-synthetic response by the activation of downstream proteins (i.e. ribosomal protein S6 kinase beta-1 – p70S6K1) [74]. Therefore, different protein sources will produce distinct protein synthetic responses; reflective of the leucine and other EAA content.

Due to leucine being the primary AA that activates MPS, it has been used to enhance the anabolic capacity of a given protein meal [70,71,76]. Katsanos and colleagues [77] showed that a mixture of EAA, containing leucine at 1.7 g, stimulated MPS in younger adults, but not in the older adults. However, increased rates of MPS were observed in older subjects when the leucine content was increased to 2.8 g [77]. Thus, it seems total leucine content rather than total protein quantity may be rate limiting in stimulating MPS [64,70]. As a result, modifying the leucine content of a meal/protein source may be an effective strategy to augment the MPS response to a dietary protein-containing meal in situations when a high-quality protein source is unavailable. However, the exact amount of leucine necessary to maximize MPS is not known [78]. For instance, studies have shown that 6 g of whey protein supplemented to contain ~5 g of leucine (4 g as crystalline leucine plus the leucine in 6 g of whey protein) can produce a similar MPS response when compared with 25 g of whey protein [64]. Nevertheless, older subjects require a higher protein and leucine content per meal, to achieve an equivalent MPS response compared with young subjects [16].

Leucine content seems to be an essential variable determining the MPS response in older as well as younger subjects and independent of total daily protein ingestion [76]. For example, the ingestion of 5 g crystalline leucine added to each main meal increased rates of MPS over 3 days, and this effect was independent of whether older subjects consumed 0.8 or 1.2 g protein/kg/d of protein [76]. In addition, Devries and colleagues showed that the ingestion of 10 g of leucine enriched-milk protein (3 g leucine) produced a similar myofibrillar protein synthetic response in comparison to 25 g of whey protein isolate (3 g leucine) [71]. Therefore, MPS is stimulated by leucine in younger and older subjects, and the manipulation of leucine content per meal seems to be a useful strategy to maximally stimulate MPS. However, adjustments in the leucine content per meal based on age may be necessary (at least to ~3 g of available leucine), since older subject require a greater leucine ingestion to stimulate the same anabolic response observed in young subjects [77].

### 3.2. Plant-derived sources

Protein quality relies mostly on AA composition and bioavailability [60]. Most animal-derived proteins (i.e. beef, dairy, eggs, milk) are considered high quality and provide all of the EAA to activate (i.e. leucine trigger effect) and sustain a robust MPS response [60,79–81]. When comparing plant-derived proteins with animal-derived proteins, vegetal-source proteins are usually lower quality due to lower quantities of an EAA. For instance, the first limiting AA in cereals and grains is most commonly lysine, whereas legumes have methionine as their limiting AA [62,82,83]. Even high-quality vegetal proteins like soy isolate (DIAAS = 90–91) are limited in the EAA methionine and cysteine. In contrast, most animal-derived proteins have a DIAAS closer to or greater than 100 [62], meaning that there is no real limiting AA when ingesting such proteins and daily protein recommendations are covered. One relevant exception to this pattern is the animal-derived protein collagen, which has a DIAAS of 0 based on the total absence of tryptophan in its composition [84]. The result of differences in EAA abundance in plant-derived proteins on acute MPS was shown by Wilkinson and colleagues comparing a soy protein-based beverage (~18.2 g protein) versus skim milk after RE [85]. Skim milk ingestion promoted a ~25–30% higher fractional synthetic rate in muscle proteins, in the 3 h post-exercise, when compared to the soy-based beverage [85]. However, this acute difference in MPS seems to be present only when comparing soy-based protein beverages with skim milk [85] or whey protein [86], but not with the ingestion of casein [86].

Because plant-derived proteins are limited in some EAA, the use of different protein blends (animal + plant-derived or different plant-derived) is commonly encouraged [87]. Reidy and colleagues observed a similar MPS response to resistance exercise (following 4 h) after the consumption of

either a 19 g protein blend containing a mixture of 50% sodium caseinate, 25% whey and 25% soy protein isolate, versus ~18 g whey protein in young subjects [88]. Similar findings were observed in middle-aged and older men, despite the use of a greater protein dose [89]. Nevertheless, we lack the knowledge to describe the ability of various mixtures of plant-derived proteins on the MPS response. In this context, Kim and colleagues measured MPS and whole-body protein kinetics, in the fasted state and in response to an egg-based (EGG) or cereal-based (CEREAL) isocaloric (~500 kcal) and iso-nitrogenous (~26 g protein) breakfast followed by lunch (4 h later) containing mainly beef protein (~25 g) [90]. No significant differences were observed in MPS after the ingestion of the different breakfasts.

In addition to the limited amount of methionine or lysine, the content or availability of leucine for some plant-based proteins is also generally lower when compared with animal-based proteins (Table 1), but not at a limiting level [87,91,92]. Therefore, when ingesting plant-based proteins, leucine content, and protein dose are additional relevant variables that have been the focus of some research. Recently, Churchward-Venne and colleagues, determined the MPS response to the ingestion of whey, soy, and leucine-enriched-soy protein beverages (all three providing the equivalent of 20 g of protein) after concurrent resistance and endurance exercise in young subjects [93]. Fractional synthetic rates in myofibrillar and mitochondrial protein fractions were equivalent in the 6 h following exercise and ingestion of the protein beverages [93]. Thus, it seems that if enough leucine and EAA are present, adding additional leucine to plant-based proteins does not further promote increases in MPS, at least in young subjects. Gorissen and colleagues assessed the protein synthetic response to plant-based isolate proteins (i.e. wheat protein and wheat protein hydrolysates) using whey protein or micellar casein as positive controls in older subjects (71 ± 1 yrs) [67]. The authors showed that 35 g of wheat protein hydrolysates produced the same plasma EAA and leucine increases when compared to micellar casein. However, to stimulate myofibrillar MPS, a 60 g wheat protein hydrolysates, containing equivalent amounts of leucine as 35 g of whey protein, was necessary [67].

Ingestion of supplemental plant-based protein isolates can support significant differences in daily MPS [94]. For instance, young women ingesting 50g/day (2 × 25 g) of potato protein isolate (2.5 g of leucine per dose), and increasing total daily protein ingestion up to 1.6 g/kg/day, showed an enhanced myofibrillar protein synthesis (measured by ingestion of D<sub>2</sub>O) during rest and after RE [94]. In addition, research monitoring muscle mass in response to supplementation with plant-based protein has shown promising results when compared to the ingestion of high-quality animal-based protein. Ingestion of either 50g/day pea protein isolate (1.6 g of leucine per dose) or 50 g of whey protein (2.1 g leucine per dose) in young subjects in combination with RT for 12 weeks resulted in similar increases in biceps brachii thickness [95]. Taken together, results of studies testing plant-derived proteins have shown that ingestion of such protein sources can be used to sustain MPS and support exercise-induced gains in

**Table 1**  
Essential amino acids (EAA) and leucine content in different dietary protein sources.

Protein source	EAA g/100 <sup>a</sup>	EAA g/25 g <sup>a</sup>	Leucine g/100 g <sup>a</sup>	Leucine g/25 g of protein <sup>a</sup>	Protein dose (g) to ingest 3 g of Leucine <sup>a</sup>
<i>Animal-derived proteins</i>					
Egg	16.5	4.1	6.9	1.7	43
Whey	34.1	8.5	10.8	2.7	28
Milk	30.3	7.5	8.7	2.2	34
Caseinate	32.8	8.2	9.0	2.3	33
Casein	24.8	6.2	7.9	2.0	38
<i>Vegetal-derived proteins</i>					
Soy	19.9	4.9	6.8	1.7	44
Wheat	18.0	2.4	6.0	1.5	50
Pea	23.6	5.9	7.1	1.8	42
Potato	29.3	7.3	8.2	2.0	37
Corn	21.0	5.2	13.5	3.4	22
Oat	13.7	3.4	5.7	1.4	52

EAA: essential amino acids.

<sup>a</sup> Values derived from Ref. [92].

muscle mass equivalent to high-quality animal-based protein. Hence, for some plant-based proteins, adjustments of doses focusing on providing enough leucine and other EAA is a reasonable strategy to ensure a maximal MPS response. This is especially important for older subjects, who demonstrate, compared to younger persons, an anabolic resistance at the level of their muscle to an equivalent hyperaminoacidemia [77].

#### 4. Muscle protein breakdown

Although much of the current research centred on the influence of dietary intake on muscle protein turnover has focussed on unravelling the mechanisms of MPS, MPB is also a critical process of skeletal muscle remodelling. Importantly, protein accretion occurs only when an individual is in a state of positive NPBAL, which occurs when MPS exceeds that of MPB achieved through an increased rate of MPS or by the suppression of MPB or a combination of both. The breakdown of skeletal muscle protein is regulated by three inter-connected systems: the ubiquitin-proteasomal pathway (UPP), lysosomal autophagy, and the calpain Ca<sup>2+</sup>-dependent cysteine proteases [96]. The assessment of skeletal MPB is complex, and it can be estimated from static or measured directly by dynamic techniques. Static measurement of muscle protein breakdown is commonly assessed at the gene and protein level predominantly through the determination of molecular, mRNA or protein abundance, responses within each of the three protein breakdown pathways. In addition, the appearance of 3-methylhistidine (3-MH), a post-translationally methylated histidine present in myofibrillar proteins that cannot be metabolized or reutilized within skeletal muscle, within circulation has been used to provide a crude estimate of MPB [97].

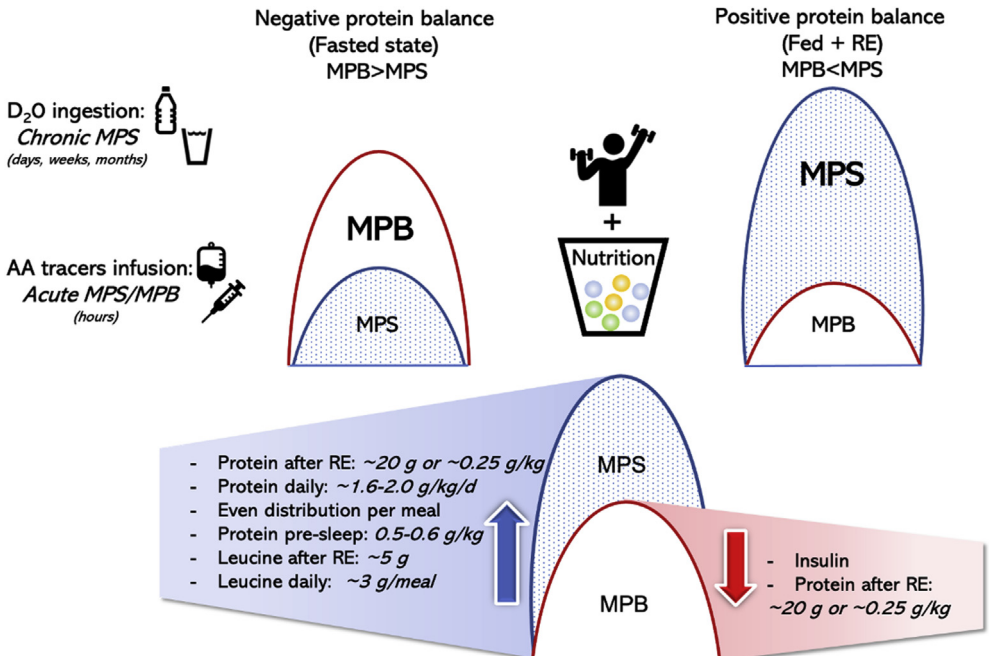
As previously mentioned, the assessment of MPB is complex. Specifically, the use of stable isotopically labelled tracers is an invasive process and requires the infusion of a labelled amino acid combined with multiple arterial and venous blood samples throughout an experiment [96,98]. Briefly, the two-pool arteriovenous (AV) method allows for the determination of release of AA (assumed to be from muscle) into circulation, and by subtraction uptake, of a labelled AA that is not metabolized by the muscle [96]. The three-pool method is very similar to the two-pool method, with the critical distinction that the addition of intramuscular determination of labelled AA enrichment and concentration allows for the calculation of true intramuscular protein breakdown and synthesis and not blood proxies per the two-pool model [96,99]. One fundamental limitation is that the two- and three-pool methods require physiological and isotope steady state, and therefore cannot be conducted following feeding. In addition, in an experimental setting, arterial catheterization is not always possible, therefore the incorporation of the two- and three-pool model may not be feasible. Breakdown can also be determined by the infusion of two different isotopes and the sampling of arterialized venous blood and muscle tissue. Although, this method does not require arterial catheterization, physiological steady state must still be achieved and additional muscle biopsies are required; potentially negating the reduced invasiveness [96,99]. The complexity associated with the assessment of MPB, makes it clear that less information is available that examines the influence of exercise and nutrition on MPB specifically, when compared with the extensive literature that pertains to MPS. Nevertheless, stable isotopes provide a valuable tool in the quest to understand muscle protein turnover, and the associated difficulties of MPB assessments have not hampered the curiosity of researchers to explore the impact of various nutritional interventions of MPB.

##### 4.1. Insulin

The consumption of AA, and ensuing aminoacidemia, stimulates MPS and is especially important in maintaining a positive muscle NPBAL. However, the ingestion of AA also leads to an increase in circulating insulin concentrations (hyperinsulinemia) [34]. Although the systemic rise in insulin has been shown to be permissive for increasing MPS, hyperinsulinemia plays an essential role in the inhibition MPB [100,101]. Using the two-pool method showed a decrease in MPB, as measured by the

rate of appearance (or release) in response to the infusion of AA above postprandial levels and hyperinsulinemia [102]. These results were extended in a series of seminal studies, where Biolo and colleagues, using the three-pool AV method with the infusion of 2 different isotopes, demonstrating that RE increased MPB in the postabsorptive (i.e. fasted) state [103]. However, the rise in MPB was abolished when RE was immediately followed by the infusion of AA [5].

In a hyperlipidemic, hyperglycaemic and hyperinsulinemic state MPB is reduced compared to the post absorptive state [104]. However, Greenhaff and colleagues demonstrated that muscle NPBAL was improved, in a dose-dependent manner (up to a plateau), following the infusion of a range of insulin concentrations (5–167 mU/l) compared to the postabsorptive state [105]. Importantly, increasing the insulin concentration did not augment MPS, highlighting a permissive (not stimulatory) role of insulin in MPS, while MPB was suppressed but not beyond insulin concentration of 30 mU/l [105]. Thus, the consumption of AA is essential to create a positive muscle NPBAL, both through the direct stimulation of MPS and insulin-mediated suppression of MPB. In further support of this, 20 g of EAA combined with high (90 g) or low (30 g) of CHO following RE led to significantly different systemic insulin concentrations, however, MPB, the associated protein signalling and gene expression were not different between conditions [106]. This, and a recent meta-analysis [107], support the notion that the role of insulin to promote a positive muscle NPBAL is driven by the suppression of MPB, up to a point, and not by further stimulation of MPS [100,101]. Thus, the ingestion of a large enough bolus of protein/AA (~0.25 g/kg for younger adults, and 0.4 g/kg for older adults) is sufficient to maximize the acute MPS response, and the ensuing hyperinsulinemia is sufficient to maximally inhibit MPB, optimizing the acute anabolic response to exercise and nutrition; which, when repeated over time leads to protein accretion (this concept is summarised in Fig. 4).



**Fig. 4.** Chronic (free-living) MPS and acute MPS/MPB can be quantified by the infusion of isotope tracers such as D<sub>2</sub>O and labelled AA, respectively. In a fasted state MPB is greater than MPS and thus the muscle is in a catabolic state of negative NPBAL. However, following exercise and an increase in dietary intake, specifically protein, MPS increases, MPB is inhibited and the muscle is in an anabolic state of positive NPBAL. Specifically manipulating dose, timing, distribution and composition of protein intake can maximize the muscle's anabolic response.



#### 4.2. 'Excess' protein consumption

Although it is widely accepted that sufficient AA consumption is essential to generate a positive NPBAL, what happens when an individual consumes protein in excess of what can be used? Several studies have examined the dose–response of protein provision on MPS, the ingestion of 20 g of protein (~0.25 g/kg) maximally stimulates MPS in young individuals [33,34], whereas approximately 10 g of EAA maximally stimulates MPS [108]. When AA are consumed in excess of the amount that can be utilized by protein-requiring processes including skeletal muscle for protein synthesis, and because they are not 'stored' in the traditional sense that fatty acids and carbohydrates are, they are deaminated and some carbon skeletal can then be oxidized. Therefore, the measurement of AA oxidation, of infused and appropriately labelled AA is an indicator of when AA supply exceeds the rates at which AA can be used for protein-requiring processes like protein synthesis [109].

Labelled phenylalanine can be infused intravenously and the oxidation rates of the labelled amino acid can be determined from the conversion of L-[ring-<sup>13</sup>C<sub>6</sub>]phenylalanine to L-[ring-<sup>13</sup>C<sub>6</sub>]tyrosine in plasma enrichment [34]. Oxidation of AA can also be determined via the appearance of an appropriately labelled <sup>13</sup>C-AA and the appearance of this label in expired air (i.e., <sup>13</sup>CO<sub>2</sub>) [33]. In MPS dose–response studies of ingested protein where MPS is maximally stimulated with the ingestion of 20 g of protein following exercise, consumption above 20 g of protein resulted in increased whole-body AA oxidation [33,34]. Additionally, the endogenous urea production rate is an indicator of protein catabolism, as the release of AA nitrogen requires the formation of urea for its safe disposal [110]. The infusion of <sup>15</sup>N<sub>2</sub> urea allows for the determination of urea production rates by determining the ratio of urea enrichment in the plasma and the infusate. Following protein ingestion, similarly to what was observed with AA oxidation, urea production rates were greatest after the consumption of 40 g of whey [34]. These data demonstrate how the use of labelled isotopes can be used to determine whole body AA oxidation following ingestion of various protein doses to confirm that protein consumed in excess is irreversibly loss to oxidation. Important information is gained from studies in which whole-body protein turnover is measured, however it is not specific to the skeletal muscle itself and limitations exist surrounding these measurements [111], therefore researchers must consider practical inferences that can be made based on these results.

### 5. The role of adjunctive nutritional compounds to stimulate MPS

The combination of RE and protein ingestion is well accepted as an effective way to increase muscle mass. Maximizing muscle mass and function is important in various populations such as athletes, older individuals and in several clinical conditions. As such, researchers have focused on other nutritional compounds/supplements which, in addition to protein, may stimulate MPS, the effects of adjunctive nutritional compounds on MPS is summarized in Table 2.

#### 5.1. Omega-3 polyunsaturated fatty acids (n3-PUFA)

Omega-3 polyunsaturated fatty acids (n3-PUFA) are fatty acids that contain two or more double bonds, with the first double bond located three carbons from the terminal methyl group. The most biologically potent n3-PUFA are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), however the *de novo* conversion from its n3-PUFA precursor – alpha linoleic acid (ALA) – to EPA and DHA is fairly limited in humans [112]. With this in mind, EPA and DHA are considered conditionally essential fatty acids, and increasing dietary (i.e. oily fish) and/or supplemental (i.e. fish oil) intake is recommended [113]. N3-PUFA are traditionally known for their potent effects on cardiovascular and neurological function, and recently, n3-PUFA supplementation has garnered considerable attention for its effects on skeletal muscle health [113,114].

Seminal work from Smith and colleagues [115,116] demonstrated that in healthy older adults [115], and young-to-middle-aged adults [116] supplementation with EPA (1.86 g/d) and DHA (1.50 g/d) for 8 weeks, rates of MPS were potentiated during a constant infusion of insulin and AA, compared to a control group consuming corn oil at rest. However, McGlory and colleagues [117] failed to show any benefit of 8 weeks of n3-PUFA supplementation on changes in MPS following either 30 g of whey



**Table 2**  
The effect of various nutritional compounds on MPS.

Supplement	State	MPS measure	Result
Leucine	Fed	acute	increase [70,108,138]
	Fed + RE	acute	increase [70]
	Free-living	integrated	increase [70,76]
n-3 PUFA	Free-living + RE	integrated	increase [70,76]
	Fasted	acute	no change [115,116]
	Fed	acute	increase [115,116]
	Fasted	acute	no change [117]
	Fed	acute	no change [117]
	Fed + RE	acute	no change [117]
Collagen	Free-living (+RE)	integrated	no change [120]
	Free-living (+immobilization)	integrated	increase [114]
	Free-living (+aerobic exercise)	integrated	no change [130]
	Free living (+inactivity and energy restriction)	integrated	no change [124]
	Free living (+RT)	integrated	no change [129]
	Fasted	acute	no change [129]
Creatine	Fed	acute	no change [129]
	Fed + RE	acute	no change [129]
	Fasted	acute	no change [135,136]
	Fed	acute	no change [135]
Arginine	Red + RE	acute	no change [135]
	Fed	acute	no change [139]
Citrulline	Fed + RE	acute	no change [139]
	Fasted	acute	no change [140]
Phosphatidic acid	Fed	acute	no change [140]
	Fed + RE	acute	no change [140]
	Fasted	acute	no change [141]
	RE	acute	reduced [141]

protein ingestion, or when whey protein feeding was combined with RE in young men. It may be that the 30 g dose of whey protein used by McGlory and colleagues [117] saturated the muscle protein synthetic response in younger persons, and that n3-PUFA supplementation conferred no anabolic benefit. In this respect, n3-PUFA supplementation may provide anabolic benefit during time periods where protein consumption is likely to be sub-optimal.

Older adults require a greater relative dose of protein to optimally stimulate rates of MPS [16], however, the recommended dose may be difficult to achieve [118]. With this in mind, n3-PUFA feeding may provide more of an anabolic benefit to older adults. Indeed, compared to younger adults [116], older adults [119] demonstrated greater relative increases in rates of mixed MPS following n3-PUFA feeding. However, Da Boit and colleagues [120] failed to show any measurable effect of n3-PUFA feeding on free-living (integrated) rates of MPS in healthy older adults undergoing RT. It may be that the lack of repeated measures design in the aforementioned study [120] prevented the researchers from finding a discernible effect of n3-PUFA feeding. In addition, due to the lack of monitoring habitual protein intake, we cannot discount the possibility that the older adults recruited by Da Boit and colleagues [120] were habitually consuming adequate dietary protein, effectively masking a discernible effect of n3-PUFA feeding. Nonetheless, future work investigating the effects of n3-PUFA feeding on integrated measures of muscle protein synthesis under conditions of sub-optimal protein intake are warranted.

Periods of physical inactivity (i.e. bed rest, muscle disuse, step reduction) result in decreased MPS in both the post-absorptive and post-prandial state [121]. Reduced rates of MPS during periods of physical inactivity results in a negative NPBAL, leading to a decline in muscle mass and strength over time [122–124]. Strategies such as RT [125] and neuromuscular electrical stimulation [126] are effective in attenuating skeletal muscle disuse atrophy. However, the previously mentioned strategies may not represent the most practical approaches, necessitating alternative strategies to combat disuse-induced skeletal muscle atrophy. Given that n3-PUFA feeding enhances post-prandial increases in MPS [115,116]

it is possible that n3-PUFA may attenuate declines in MPS during periods of skeletal muscle disuse. Recently, we showed that young healthy women supplementing with EPA (2.97 g/d) and DHA (2.03 g/d) had higher integrated rates of MPS during 2 weeks of single-leg immobilization, and upon return to habitual physical activity levels, compared to a control group ingesting sunflower oil [122]. Moreover, EPA and DHA supplementation not only alleviated muscle atrophy during immobilization, but also facilitated full return of skeletal muscle volume after 2 weeks of recovery [127]. It has been proposed that accumulation of short periods of skeletal muscle disuse superimposed onto the natural biological decline in muscle mass with advancing age, may give rise to the development of sarcopenia [114].

5.2. Collagen

Collagen is an extracellular protein that accounts for ~25% of total body protein mass [128]. As a nutritional supplement, collagen peptides are considered a low-quality protein source, due to the complete lack of the EAA, tryptophan, and a low leucine content. As described previously, the EAA content of a protein source, particularly leucine, is pertinent for increasing MPS. Work from our laboratory has demonstrated that, compared to high-quality protein sources, such as whey protein [124,129] and alpha-lactalbumin (a leucine-enriched fraction of whey) [130], collagen peptide ingestion was not effective in mounting comparable increases in MPS, despite all supplements being iso-nitrogenous [124,129,130]. In fact, collagen peptide ingestion did not elevate plasma leucine concentrations, nor did it increase acute and integrated rates of MPS above baseline [129]. Despite this, collagen peptide supplements have garnered considerable attention [128,131,132] for their ability to augment RT-induced increases in skeletal muscle mass that are so large as to position collagen as being as efficacious as testosterone administration [133]. However, in contrast to longer-term trials, our group has consistently demonstrated no benefit of collagen peptide supplementation on indices of skeletal muscle mass during periods of loading (i.e., physical activity [129,130] and unloading (i.e., physical inactivity [124]). Following a single bout of RE [129], or several sessions of aerobic exercise training [130], collagen peptide ingestion was not as effective as whey protein [129] or lactalbumin ingestion [130] to increase MPS in healthy older women [129], or young, endurance trained individuals [130]. In fact, collagen peptide ingestion did not act synergistically with RT to increase integrated rates of MPS above baseline [129]. Furthermore, collagen peptide supplementation did not augment MPS or leg lean mass during convalescence from two weeks of physical inactivity, in healthy older adults; in contrast, whey protein supplementation was able to stimulate recovery of muscle protein synthesis and increase in leg lean mass [124]. Collectively, these findings further emphasize the importance of protein quality as determinant for increasing MPS.

5.3. Creatine

Creatine is essential in ATP re-synthesis and has been used as an ergogenic aid by athletes to improve performance specifically in tasks of repeated high-intensity short duration effort [134]. Supplementation with creatine in combination with RT has been reported to increase lean body mass to a greater extent; when compared with placebo [134]. However, the mechanism by which creatine

**Table 3**

Practical recommendations of protein intake to maximise MPS in young and old adults.

Protein dose	<ul style="list-style-type: none"> <li>• ~1.6 g/kg body mass of protein per day</li> <li>• If total protein intake is not adequate, then consume ~0.4 g/kg body mass of protein at each meal and following RE</li> </ul>
Protein distribution	<ul style="list-style-type: none"> <li>• Evenly space each protein-containing meal</li> </ul>
Protein quality	<ul style="list-style-type: none"> <li>• Consume protein 1–3 h before sleep</li> <li>• Consume high quality (EAA rich) protein</li> <li>• Supplement low dose protein with leucine</li> </ul>
Protein ingestion surrounding RE	<ul style="list-style-type: none"> <li>• Not <u>essential</u> if sufficient AA/EAA are consumed</li> </ul>

MPS, muscle protein synthesis; RE, resistance exercise; EAA, essential amino acids; AA, amino acids.

affects muscle size is largely unknown. To our knowledge only two studies have examined the effects of creatine supplementation on muscle protein turnover. Creatine supplementation for 5 days raised muscle creatine levels [135,136] however basal MPS levels were not affected in either the fasted [135,136], or the fed state [135], nor following RE [137]. Although whole body protein oxidation, assessed via the rate of appearance plasma leucine appearance, was slightly reduced in the fasted state following creatine loading [136], Louis and colleagues observed no changes in MPB following creatine loading in the fasted or fed state [135]. These results support the notion that increased lean mass following creatine supplementation may be due factors other than increased MPS – specifically, creatine serves to increase PCr stores (delaying depletion), facilitate the rapid re-synthesis of PCr and ATP and provide an energy buffer [134] which enables a greater training volume to be completed.

MPS, muscle protein synthesis; RE, resistance exercise; PUFA, polyunsaturated fatty acid.

## 6. Conclusion

Skeletal muscle protein turnover has been, and remains, an important field of study. Muscle mass is determined by the difference between MPS and MPB, creating either a positive (anabolic state) or negative (catabolic state) NPBAL (summarised in Fig. 4). Through the use of labelled stable isotopes researchers have been able to determine the optimal dose, timing, distribution and composition of a protein source to maximally stimulate MPS, inhibit MPB and thus create a positive NPBAL, as summarised in Table 3. An exciting avenue of future research exists in exploring the ability of nutritional supplements, other than protein/AA ingestion, to stimulate MPS, such as the use of omega 3 fatty acids. Furthermore, it is now possible to combine stable isotopes (D<sub>2</sub>O) with proteomic mass-spectrometry to investigate the protein fractional synthesis rates and abundance of hundreds of individual proteins within a given muscle sub-fraction. This can also allow the calculation of the breakdown rate of these individual proteins, which would be extremely valuable information. The integration of stable isotopes and the emerging omics field will enable researchers to further elucidate the ability of nutritional interventions to influence biological networks that regulate muscle protein metabolism.

## Statement of authorship

SJ, JM, CL, EAN, TS, JCM, and SMP wrote the initial draft of the manuscript. All authors edited and approved the final version of the manuscript and agree to be accountable for all aspects of the work.

## Declaration of interests

The authors declare no conflict of interest.

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