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## Article

# Mechanistic Insights into Protein Synthesis Pathways and Amino Acid Interplay in Muscle Growth and Resistance Training: A Molecular Analysis

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**Abstract:** Muscle hypertrophy relies on efficient protein synthesis, modulated by amino acid availability and signalling pathways such as mTOR, which activate muscle growth at a cellular level (Kimball et al., 2002). This study investigates the role of essential amino acids, particularly leucine and branched-chain amino acids (BCAAs), in promoting protein synthesis during resistance training. Using stable isotope labelling and molecular assays, protein synthesis rates were analysed in muscle biopsies post-intervention (Atherton & Smith, 2012). mTOR and associated pathways (e.g., S6K1) were assessed via Western blotting and RT-qPCR to quantify pathway activation (Drummond et al., 2009). Findings show a direct relationship between amino acid levels and increased anabolic signalling, providing data-driven insights into molecular responses essential for muscle adaptation (Phillips et al., 2005). This research underscores targeted amino acid supplementation as a potential strategy to enhance hypertrophic outcomes in resistance training (Tipton et al., 2001).

**Keywords:** muscle hypertrophy; protein synthesis; amino acids; mTOR pathway; data analysis; resistance training; molecular biology

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## Introduction

### 1.1. Background and Motivation

Muscle hypertrophy, or the increase in muscle size, is a complex physiological process essential for enhancing muscular strength and function, particularly in response to resistance training. Central to muscle hypertrophy is muscle protein synthesis (MPS), the anabolic process through which new muscle proteins are constructed, counteracting the effects of protein breakdown and contributing to muscle growth (Phillips, 2014). Muscle hypertrophy is governed by intricate molecular pathways, among which the mechanistic target of rapamycin (mTOR) signalling pathway plays a pivotal role by facilitating protein synthesis and cell growth. Activation of mTOR and downstream effectors such as S6 kinase 1 (S6K1) and 4E-binding protein 1 (4E-BP1) are key drivers of muscle protein synthesis, and their regulation is influenced by a combination of resistance training and nutrient availability (Dreyer & Volpi, 2005).

Amino acids, particularly the essential branched-chain amino acids (BCAAs) like leucine, are crucial for activating these anabolic pathways. Leucine, in particular, has emerged as a potent stimulator of the mTOR pathway, initiating protein synthesis by directly activating signalling cascades that promote muscle growth (Kimball & Jefferson, 2006). Studies show that leucine's effects extend beyond its structural role in muscle protein composition to acting as a signalling molecule that can modulate MPS independently of other factors (Drummond et al., 2009). Despite the importance of leucine and other BCAAs in regulating MPS, the precise molecular mechanisms through which they interact with cellular pathways to stimulate muscle hypertrophy remain incompletely understood, especially in the context of resistance training.

Current research on amino acids' molecular influence on MPS has primarily focused on the mTOR pathway, yet there are significant gaps in understanding how these amino acids specifically

influence signalling components at a cellular level under various physiological conditions (Atherton et al., 2010). Additionally, while leucine's role is well-recognized, the interplay between other BCAAs and mTOR pathway modulators during resistance training has not been fully elucidated, highlighting the need for further research into how targeted amino acid supplementation can optimize MPS. This study aims to address these gaps by examining the molecular pathways governing protein synthesis and the anabolic interplay of amino acids, focusing on leucine's unique role in facilitating muscle hypertrophy through mTOR activation (Wilkinson et al., 2013).

### 1.2. Research Objectives

This study shows that targeted supplementation with specific amino acids, particularly leucine and other branched-chain amino acids (BCAAs), enhances muscle growth through the activation of key molecular pathways involved in protein synthesis. By focusing on the mechanistic target of rapamycin (mTOR) pathway and its downstream effectors, we aim to elucidate the specific roles these amino acids play in modulating muscle protein synthesis (MPS) in response to resistance training. Previous research indicates that adequate amino acid availability is critical for optimizing MPS, suggesting that dietary strategies targeting these nutrients could significantly enhance muscle hypertrophy outcomes (Tarnopolsky, 2007; Phillips et al., 2016).

#### Key Objectives

##### 1. Identify and Analyse the Molecular Pathways of Protein Synthesis in Muscle Growth

The first objective of this study is to delineate the molecular pathways involved in MPS during muscle growth. This involves an examination of the mTOR signalling pathway, which is central to the regulation of cell growth and protein synthesis. The mTOR pathway integrates various signals from nutrients, growth factors, and mechanical stimuli, thus acting as a critical nexus for regulating MPS (Baar & Esser, 1999; Hara et al., 2002). We utilise blotting and quantitative PCR techniques, to analyse the expression levels of key proteins and mRNA associated with mTOR activation, such as S6K1, 4E-BP1, and others. Understanding these pathways at a detailed level will contribute to a clearer picture of how resistance training and amino acids interact to promote muscle growth.

##### 2. Investigate Amino Acid Involvement in Modulating Protein Synthesis, Focusing on Leucine and BCAAs

The second objective is to investigate the specific roles of amino acids in modulating protein synthesis, with a particular focus on leucine and other BCAAs, such as isoleucine and valine. Leucine has been shown to be a key regulator of mTOR activation, which initiates MPS following resistance exercise (Norton & Layman, 2006; Volpi et al., 2003). By administering targeted leucine and BCAA supplementation before and after resistance training sessions, we will measure the resulting changes in protein synthesis rates and assess how these amino acids influence mTOR pathway activation. Additionally, we will explore the synergistic effects of BCAAs and their potential to enhance the anabolic response when combined with resistance training (Katsanos et al., 2006).

##### 3. Provide Data-Driven Insights through Bioinformatics and Statistical Analyses

The final objective is to utilize bioinformatics tools and statistical analyses to provide data-driven insights into the relationships between amino acid concentrations, protein synthesis rates, and molecular signalling pathways. Analytical techniques such as multivariate analysis will be employed to identify patterns and correlations among the data collected (López-Campos et al., 2013; Wang et al., 2017). By integrating these data sets, we aim to create a statistical certainty of the uplift in training results and how specific amino acid profiles can optimise muscle protein synthesis and hypertrophy in response to resistance training. The outcome of this research could inform dietary recommendations and supplementation strategies aimed at athletes and individuals engaged in resistance training.

This study seeks to bridge existing knowledge gaps regarding the molecular mechanisms of amino acids in muscle growth. By examining the activation of mTOR and the effects of targeted amino acid supplementation, particularly leucine and BCAAs, we aim to enhance our understanding of how these factors contribute to MPS. This research holds the potential to provide actionable

insights for nutritional strategies designed to maximise the benefits of resistance training on muscle hypertrophy (Wilkinson et al., 2013; Moore et al., 2009).

## Brief Literature Review

### 2.1. Protein Synthesis and Muscle Growth

Muscle hypertrophy, defined as the increase in muscle cross-sectional area, is a complex physiological process intricately linked to the dynamic and regulated process of MPS. MPS is essential for muscle repair, growth, and adaptation following resistance training, acting as the anabolic counterbalance to muscle protein breakdown (catabolism) (Phillips et al., 2016). Muscle hypertrophy results not only from an increase in MPS but also from a decrease in muscle protein breakdown. The regulation of MPS is orchestrated through several signalling pathways, with the mechanistic target of rapamycin (mTOR) pathway being the most extensively studied (LaPlante & Sabatini, 2012).

mTOR acts as a central integrator of diverse extracellular signals, including growth factors, hormones, and nutritional status, especially the availability of amino acids, particularly leucine. Upon activation by growth factors such as insulin and insulin-like growth factor 1 (IGF-1), the mTOR signalling cascade initiates a series of downstream events that culminate in increased MPS. The phosphorylation of key downstream targets, including S6 kinase 1 (S6K1) and 4E-binding protein 1 (4E-BP1), is a critical aspect of this process (Zhang et al., 2011). S6K1 enhances ribosomal biogenesis and translation initiation, thereby promoting an increase in MPS. Additionally, the phosphorylation of 4E-BP1 releases the eukaryotic initiation factor 4E (eIF4E), which is necessary for the formation of the translation initiation complex, further facilitating the translation of mRNA into protein (Hara et al., 2002).

Moreover, the mTOR complex 1 (mTORC1) also plays a critical role in inhibiting protein catabolism by suppressing autophagy, a cellular process that breaks down damaged proteins and organelles, thereby promoting a net increase in muscle mass (Raben et al., 2008). This regulatory mechanism is particularly crucial during states of stress or fasting when muscle catabolism is typically elevated. Autophagy serves not only to remove dysfunctional cellular components but also to provide amino acids during periods of nutrient scarcity, thereby demonstrating the dynamic interplay between synthesis and degradation pathways. In addition to mTOR, the phosphoinositide 3-kinase (PI3K)/AKT pathway also plays a significant role in regulating MPS. When activated by insulin or growth factors, PI3K activates AKT, which further enhances mTOR activity while simultaneously inhibiting protein degradation pathways mediated by Forkhead box O (FoxO) transcription factors (Harrison et al., 2015; Rommel et al., 2001). This action of AKT helps in maintaining muscle mass by preventing muscle wasting and promoting MPS in response to anabolic stimuli.

Other signalling pathways, including the mitogen-activated protein kinase (MAPK) pathway, have been implicated in regulating muscle growth. This pathway responds to various extracellular signals, including stress and inflammation, influencing gene expression and cellular responses necessary for muscle adaptation (Huang & Chen, 2012). For instance, the activation of the p38 MAPK pathway is known to be involved in cellular responses to mechanical stress during exercise, leading to adaptations in muscle function. The interplay between these signalling pathways establishes a well-coordinated system that allows muscle cells to adaptively regulate MPS in response to mechanical loading and nutritional cues. Understanding the molecular intricacies of these pathways is essential for developing targeted nutritional and training strategies aimed at enhancing muscle growth.

Furthermore, factors such as the timing of nutrient intake, particularly protein and carbohydrates, can modulate the effectiveness of these signalling pathways. Studies have shown that nutrient timing can affect the anabolic response of skeletal muscle, particularly in the context of resistance training (Aragon & Schoenfeld, 2013). For instance, the ingestion of protein-rich meals shortly before or after resistance training can significantly enhance MPS due to the synergistic effects

of amino acids and exercise-induced signalling (Pasiakos et al., 2013). This suggests that an optimised approach to nutrient timing could play a crucial role in maximising muscle growth and adaptation.

The complexity of the molecular mechanisms governing MPS highlights the need for a multifaceted understanding of muscle biology. As research continues to evolve, it is imperative to investigate how various signalling pathways interact, the role of nutrient timing, and the impact of dietary composition on muscle growth.

## 2.2. Amino Acid Role in Muscle Anabolism

Amino acids, particularly essential amino acids (EAAs), play pivotal roles in muscle anabolism and recovery. Among the EAAs, leucine has emerged as a critical regulator of mTOR signalling and muscle protein synthesis (Coyle et al., 2016). Leucine acts as a nutrient sensor that triggers mTOR activation, thus stimulating MPS. This process involves complex signalling events, where leucine's presence at the lysosomal surface facilitates the translocation of mTORC1, enhancing its activity in response to nutrient availability (Sancak et al., 2010; Jewell et al., 2015). The unique role of leucine in muscle metabolism has made it a focal point in research aimed at optimising dietary strategies for enhancing muscle growth.

BCAAs, consisting mainly of leucine, isoleucine, and valine, have also been shown to promote MPS. Research indicates that BCAAs can enhance exercise-induced MPS, provide energy during prolonged physical activity, and reduce muscle protein breakdown (Norton & Layman, 2006; Fujita et al., 2007). The ratio of BCAAs to other amino acids can influence muscle recovery and adaptation, underscoring the importance of not just individual amino acids but their collective ratios in dietary strategies. Moreover, BCAAs serve not only as substrates for protein synthesis but also as important signalling molecules influencing metabolic pathways involved in muscle recovery and adaptation. For example, BCAAs can stimulate insulin release, enhancing amino acid uptake and promoting a more anabolic environment in muscle tissues.

In addition to leucine, other amino acids such as glutamine and arginine contribute to muscle metabolism and recovery following exercise. Glutamine plays a vital role in energy production and serves as a substrate for the synthesis of nucleotides, thereby influencing muscle recovery (Buse & Reid, 1975). It has been shown to support immune function and gut health, which can indirectly impact muscle recovery and overall performance (Wischmeyer, 2006). Arginine, a precursor for nitric oxide (NO), enhances blood flow and nutrient delivery to muscle tissues, promoting an anabolic environment conducive to muscle growth (Wilkinson et al., 2013; Olesen et al., 2014). The role of NO in vasodilation and nutrient delivery emphasizes the importance of arginine in maximizing muscle recovery post-exercise.

Furthermore, the timing of amino acid intake in relation to resistance training is crucial. Research has shown that the consumption of EAAs, particularly leucine, immediately before or after exercise can significantly enhance MPS and improve recovery (Pasiakos et al., 2013). Some studies have indicated that consuming a combination of carbohydrates and proteins post-workout can further amplify MPS, as insulin released during carbohydrate digestion may enhance amino acid uptake into muscle cells (Ivy, 2001). This synergistic effect highlights the importance of integrated nutritional strategies that consider both protein and carbohydrate intake for optimising muscle growth and recovery.

Despite the established roles of individual amino acids in muscle anabolism, there is still limited understanding of how different amino acids interact within the context of mixed meals or supplementation, and how these interactions impact MPS (Borsheim et al., 2002). The complexity of dietary sources, the timing of intake, and the interplay among various amino acids underscore the need for a more nuanced approach to nutrition in the context of muscle health and performance.

## 2.3. Gaps in Knowledge

Despite significant advancements in understanding the molecular mechanisms governing protein synthesis and muscle growth, several critical gaps in knowledge persist, particularly regarding the specific interactions of amino acids at the molecular level. Most research has primarily

focused on the isolated effects of individual amino acids, particularly leucine, while neglecting the potential synergistic effects of mixed amino acid supplementation (Deutz et al., 2013; Fujita et al., 2007). This gap in knowledge limits the development of comprehensive dietary strategies that can maximise muscle protein synthesis and growth through targeted amino acid combinations.

Moreover, existing studies often utilise homogeneous populations in controlled laboratory settings, which may not accurately reflect the diverse physiological responses observed in different individuals influenced by genetics, age, sex, training status, and nutritional habits. This variability complicates the establishment of universal recommendations for amino acid intake, particularly regarding optimal timing, dosage, and combinations for enhancing muscle protein synthesis and growth (Kumar et al., 2012). Individual differences in metabolism, absorption, and utilization of amino acids necessitate personalised approaches to nutrition that take these factors into account.

Additionally, while leucine's role in activating mTOR is well characterised, the specific signalling events triggered by the presence of other amino acids in the context of mixed meals or supplementation have not been fully elucidated (Kumar et al., 2012; Wykes et al., 2020). Understanding the influence of dietary composition on amino acid bioavailability and the subsequent impact on MPS is crucial for developing effective nutrition strategies. For instance, the competition among amino acids for absorption in the gut could potentially affect MPS, which warrants further investigation.

Furthermore, the molecular mechanisms underlying the interactions among amino acids and their collective impact on anabolic signalling pathways remain inadequately understood. Research into the post-translational modifications that occur following amino acid signalling activation can shed light on the intricate regulatory networks involved in muscle protein synthesis. There is a pressing need for more comprehensive studies that utilize advanced technologies, such as metabolomics and proteomics, to elucidate the intricate relationships between amino acids, signalling pathways, and muscle protein metabolism. This holistic approach could uncover novel insights into how amino acids synergistically promote MPS and muscle growth.

Research investigating the role of the gut microbiome in modulating amino acid metabolism and availability presents an exciting frontier that could provide new insights into muscle health and performance (Moro et al., 2016; Bäckhed et al., 2015). The gut microbiota's influence on nutrient absorption, metabolism, and inflammation can have profound effects on muscle recovery and adaptation. Understanding these interactions could lead to novel dietary interventions that leverage the gut microbiome to enhance muscle health, particularly in aging populations or individuals with metabolic disorders.

So, while substantial advancements have been made in elucidating the roles of protein synthesis and amino acids in muscle hypertrophy, there remains a critical need for more in-depth studies that address the intricate interplay between amino acids and the molecular mechanisms of protein synthesis. Investigating these interactions will not only enhance our understanding of muscle biology but also improve practical applications in nutrition and training regimens aimed at optimizing muscle growth and performance. Addressing these gaps will ultimately contribute to the development of more effective strategies for enhancing muscle health across diverse populations.

### 3. Materials and Methods

#### 3.1. Study Design and Sample Selection

This study employed a randomized controlled trial (RCT) design to investigate the effects of amino acid supplementation on protein synthesis pathways and muscle growth in response to resistance training. The participants were recruited from local gyms and fitness centres, ensuring a diverse population to enhance the generalizability of the results and the cohort was formed from participants with personal training support across these different locations.

#### Experimental Interventions

The experimental intervention consisted of a 12-week resistance training program that emphasized hypertrophy through progressive overload. Participants underwent three resistance

training sessions per week, incorporating multi-joint exercises such as squats, deadlifts, bench presses, and rows. Each session was designed to promote muscle growth by targeting major muscle groups with an emphasis on compound movements. The training volume was progressively increased throughout the intervention, with participants starting at 70% of their one-repetition maximum (1RM) and gradually progressing to 85% 1RM by the end of the program (Schoenfeld, 2010). Each session included a warm-up, followed by four sets of 8-12 repetitions for each exercise, with rest intervals of 60 to 90 seconds.

### Sample Population

A total of 80 healthy adult participants aged 18 to 35 years were recruited for this study. Participants were screened for health criteria, which included a history of regular resistance training (minimum of two years), no metabolic disorders, and no current use of performance-enhancing substances or dietary supplements that could affect protein metabolism. Both male and female participants were included, with an aim for a balanced representation (Kumar et al., 2009). Informed consent was obtained from all participants prior to their involvement in the study, and they were randomized into two groups: one receiving an amino acid supplementation and the other receiving a placebo.

### Methods for Protein Synthesis Measurement

Protein synthesis rates were assessed through the measurement of the fractional synthesis rate (FSR), which was determined using stable isotope-labelled amino acids. Participants were administered a bolus of L-[ring-<sup>13</sup>C6] phenylalanine, a stable isotope of the amino acid phenylalanine, prior to muscle biopsy procedures (Børsheim et al., 2002). Muscle biopsies were taken from the vastus lateralis at baseline and after 6 and 12 weeks of intervention to capture changes in protein synthesis rates over time. We deploy the standard FRS equation

$$FSR = \frac{(S_{t2} - S_{t1})}{(t2 - t1) \times (P_{t1})}$$

where SS is the muscle protein synthesis at two time points t1t1 and t2t2, and Pt1Pt1 is the pool size of the labelled amino acid at time t1t1 (Mamerow et al., 2014). This method provides a dynamic assessment of protein metabolism, allowing for a nuanced understanding of how amino acid supplementation and resistance training impact muscle growth.

### 3.2. Data Collection and Experimental Procedures

#### Amino Acid Supplementation Protocol

Participants in the amino acid supplementation group received a BCAA supplement (containing leucine, isoleucine, and valine) at a dosage of 10 grams before and after each training session. The BCAA ratio was set at 2:1:1 for leucine, isoleucine, and valine, respectively, based on prior research indicating optimal efficacy for promoting muscle protein synthesis (Norton & Layman, 2006). The placebo group received a carbohydrate-based supplement with similar caloric content but without amino acids. Both groups were instructed to maintain their habitual dietary patterns throughout the intervention period.

Supplementation adherence was monitored through weekly check-ins and participant logs to ensure compliance with the protocol. Participants were advised to consume the supplements mixed with water immediately before and after each training session, and they were required to avoid any additional protein supplementation during the study.

#### Muscle Biopsy Procedures

Muscle biopsies were performed using a percutaneous needle biopsy technique under local anaesthesia, targeting the vastus lateralis muscle. Biopsy procedures were carried out at baseline and after 6 and 12 weeks of resistance training. After skin preparation, a small incision was made, and a

biopsy needle was inserted to obtain approximately 100 mg of muscle tissue, which was immediately frozen in liquid nitrogen for subsequent analysis (Phillips et al., 2016).

The biopsies were processed to extract proteins and RNA for further analyses. The tissue samples were divided into aliquots and stored at -80°C until analysis. This technique provides high-quality muscle samples for the evaluation of molecular mechanisms associated with muscle protein synthesis.

### **Analytical Techniques**

Amino acid quantification was performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS), a highly sensitive method that enables the detection of amino acids at low concentrations in biological samples (Borrelli et al., 2020). The LC-MS/MS protocol involved protein precipitation, followed by derivatization of the amino acids to enhance chromatographic separation. The samples were then analysed for specific amino acid profiles, allowing for the assessment of changes in amino acid availability and metabolism throughout the intervention.

### *3.3. Molecular Assays and Pathway Analysis*

#### **Assessment of mRNA Expression Levels**

To investigate the transcriptional regulation of muscle protein synthesis, mRNA expression levels of key genes involved in the mTOR signalling pathway, including mTOR, S6K1, and 4E-BP1, were quantified using reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from muscle tissue samples using an RNA extraction kit, following the manufacturer's instructions. The quality and quantity of RNA were assessed using a spectrophotometer and agarose gel electrophoresis (Bustin et al., 2009).

cDNA was synthesized from 1 µg of RNA using a reverse transcription kit, and RT-qPCR was conducted using specific primers for target genes and GAPDH as the housekeeping gene for normalization. The relative expression levels were calculated using the  $\Delta\Delta Ct$  method, providing insights into how resistance training and amino acid supplementation influence gene expression involved in muscle hypertrophy (Michaud et al., 2016).

#### **Western Blotting for Protein-Level Analysis**

Western blotting was employed to analyse protein levels of key markers in muscle protein synthesis, including phosphorylated mTOR, S6K1, and 4E-BP1. Muscle tissue samples were homogenized in lysis buffer containing protease and phosphatase inhibitors, and protein concentrations were determined using the BCA protein assay (Smith et al., 1985). Equal amounts of protein (30 µg) were separated using SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes.

The membranes were blocked with 5% non-fat dry milk and incubated overnight at 4°C with primary antibodies specific to the target proteins. After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies, and protein bands were visualized using chemiluminescence detection (Harlow & Lane, 1988). Densitometric analysis was performed using ImageJ software to quantify protein expression levels.

#### **Techniques for Data Analysis**

To rigorously evaluate the impact of amino acid supplementation on muscle protein synthesis and other molecular markers, a combination of advanced statistical techniques was employed, leveraging multivariate analysis, time-series analysis, and mixed-effects modelling. Each of these methods provides a unique perspective on the data, allowing for a nuanced understanding of how amino acid supplementation influences muscle growth over time.

#### **Multivariate Analysis Techniques**

Multivariate analysis techniques were essential for handling the high-dimensional data generated in this study, particularly when examining interactions among multiple variables, including protein synthesis rates, amino acid concentrations, and mRNA expression levels. Principal component analysis (PCA) was initially conducted to reduce dimensionality, transforming the data into a set of uncorrelated principal components that explained the majority of the variance. This transformation is especially useful when dealing with numerous variables because it simplifies the

dataset without sacrificing the interpretability of patterns and trends. By identifying the principal components associated with significant variance, the PCA helped focus subsequent analyses on the most relevant factors, such as key amino acids and protein synthesis markers.

Canonical correlation analysis (CCA) was further employed to examine relationships between two sets of variables—namely, biochemical markers (like amino acid concentrations) and physiological outcomes (such as muscle protein synthesis rates). CCA allowed us to assess the degree of association between these variable groups, providing insights into how specific amino acids, especially leucine, correlate with changes in protein synthesis.

### Time-Series Analysis

Time-series analysis was utilized to monitor changes in muscle protein synthesis rates and gene expression over multiple time points (baseline, week 6, and week 12). This approach allowed us to capture temporal dynamics, which are crucial for understanding how quickly and to what extent amino acid supplementation impacts muscle growth. Autoregressive integrated moving average (ARIMA) models were employed to analyse time-dependent changes within each group. ARIMA models are particularly suited for time-series data as they account for autocorrelation, which is common in repeated measurements within subjects.

In addition to ARIMA, cross-correlation analysis was conducted between protein synthesis rates and amino acid concentrations over time. This allowed us to examine potential lead-lag relationships, revealing whether increases in amino acid levels (especially leucine) consistently preceded spikes in protein synthesis rates. Such insights are valuable for determining the optimal timing of amino acid supplementation to maximize anabolic effects.

### Linear Mixed-Effects Models

Given the repeated measures design of the study, linear mixed-effects models (LMMs) were central to our analysis. LMMs account for both fixed effects (e.g., treatment group and time) and random effects (e.g., individual differences among participants), offering a robust framework to evaluate intervention effects while controlling for potential confounding variables. The model specification was as follows:

$$Y_{ij} = \beta_0 + \beta_1 \text{Group}_i + \beta_2 \text{Time}_j + (1|\text{Participant}_i) + \epsilon_{ij}$$

where  $Y_{ij}$  is the outcome measure (e.g., FSR),  $\beta_0$  is the intercept,  $\beta_1$  and  $\beta_2$  represent the fixed effects of group and time,  $(1|\text{Participant}_i)$  is the random effect of participant, and  $\epsilon_{ij}$  is the residual error term. Random slopes were also incorporated to allow protein synthesis rates to vary over time at the individual level.

The LMMs were followed by **Tukey's HSD post-hoc tests** to examine pairwise differences between specific time points within groups. This analysis revealed that the amino acid supplementation group experienced a significant increase in protein synthesis from baseline to week 6 ( $p < 0.01$ ) and week 6 to week 12 ( $p < 0.05$ ), whereas the placebo group's changes were non-significant. This distinction underscores the cumulative benefit of amino acid supplementation on muscle protein synthesis, with effects intensifying over the intervention period.

### Analysis of Variance (ANOVA)

ANOVA was used to compare differences between the amino acid supplementation and placebo groups across time points, specifically examining mRNA expression, amino acid concentrations, and protein phosphorylation levels. **Two-way ANOVA** was applied with interaction terms for group and time, testing whether the effect of supplementation varied by time. When interaction effects were significant ( $p < 0.05$ ), it indicated that the supplementation's impact on muscle growth markers evolved over the study duration, justifying the time-based approach in this intervention.

### Software and Models

To perform these analyses, we utilized a combination of statistical software platforms. **R** (version 4.0.3) was the primary tool, given its flexibility and extensive package ecosystem for advanced statistical modelling. The **lme4** package was employed for the mixed-effects models, offering functionality to fit and compare nested models with both fixed and random effects (Bates et

al., 2015). For multivariate techniques, the **vegan** and **psych** packages were utilized to conduct PCA and CCA, respectively, providing robust visualizations of complex variable relationships.

In addition, **SPSS (version 27)** was used to run ANOVA and post-hoc tests. SPSS's user-friendly interface and reliable ANOVA capabilities made it an excellent choice for initial comparisons across groups and time points. Finally, **GraphPad Prism** was used for basic statistical tests and generating summary statistics, as its visualization tools complement R's graphical packages, including **ggplot2**.

### **Data Visualization Techniques**

To communicate key findings, various data visualization techniques were employed. Visualization is a critical component of data analysis, especially in studies involving complex, multivariate data. Here are the specific visualizations and their significance in this study:

1. **Line Graphs with Error Bars:** Line graphs were used to illustrate the time-series data for protein synthesis rates. Each line represented the mean FSR for a group (amino acid supplementation vs. placebo) at each time point, with error bars showing the standard deviation. This format highlighted the progressive increase in protein synthesis in the amino acid group and the stable trend in the placebo group. It also allowed for a straightforward comparison of temporal patterns between groups.
2. **Bar Charts for Amino Acid Concentrations:** Bar charts provided a clear depiction of changes in leucine, isoleucine, and valine concentrations pre- and post-intervention. Bars for each amino acid were grouped by intervention stage, with error bars indicating variability among participants. This format effectively demonstrated the significant post-intervention rise in leucine, corroborating its central role in stimulating muscle protein synthesis.
3. **Box Plots for Gene Expression Levels:** Box plots were utilized to visualize mRNA expression levels of mTOR, S6K1, and 4E-BP1. Box plots offer a detailed view of distribution, median, and interquartile range, making them ideal for comparing baseline and post-intervention changes across groups. The reduction in 4E-BP1 expression, paired with elevated mTOR and S6K1, was evident in the box plot, reinforcing how supplementation affects the transcriptional regulation of protein synthesis.
4. **Heatmap of Statistical Significance:** A heatmap illustrated p-values for key comparisons across variables (e.g., FSR, amino acid levels, and protein phosphorylation), with colour gradients indicating levels of statistical significance. Darker shades represented more significant p-values, providing an efficient way to identify which variables exhibited the most pronounced changes due to supplementation. Heatmaps excel at synthesizing results from complex, multidimensional analyses, making it easy to spot relationships and interactions.
5. **Scatter Plots for Cross-Correlation Analysis:** Scatter plots were used to explore relationships between amino acid concentrations and protein synthesis rates. Cross-correlation analyses revealed lead-lag relationships, and scatter plots with best-fit lines quantified these associations, providing insights into optimal amino acid supplementation timing.

Together, these statistical techniques and visualizations offer a comprehensive view of the amino acid supplementation effects, highlighting both direct outcomes (e.g., increased protein synthesis rates) and indirect, mechanistic insights (e.g., transcriptional regulation via mTOR and S6K1). By using robust statistical models and clear visualizations, the study effectively demonstrates the biochemical and physiological impacts of amino acid supplementation on muscle growth and protein synthesis pathways.

## 4. Results

The results of this study provide an examination of the effects of amino acid supplementation on muscle protein synthesis, amino acid concentrations, mRNA expression, and protein phosphorylation levels over a 12-week intervention. Statistical analysis was conducted to determine differences between the amino acid supplementation group and the placebo group, allowing for a comprehensive understanding of the intervention's impact on molecular markers associated with muscle growth. We consider five different categories when evaluating the efficacy of amino acid supplementation.

### 4.1. Muscle Protein Synthesis Rates Over Time

#### Statistical Analysis

Using time-series analysis and mixed-effects models, we evaluated changes in muscle protein synthesis rates across baseline, week 6, and week 12 in both groups. A mixed-effects model was applied with fixed effects for group (amino acid vs. placebo) and time, accounting for individual variability as a random effect. Tukey's HSD post-hoc tests revealed significant increases in protein synthesis rates from baseline to week 6 ( $p < 0.01$ ) and from week 6 to week 12 ( $p < 0.05$ ) in the amino acid group, while the placebo group showed no significant changes across any time points.

#### Graph and Explanation

The line graph below illustrates the fractional synthesis rate (FSR) of muscle protein over the 12-week period. Error bars represent the standard deviation, showing variability within each group. The amino acid group shows a marked increase in FSR from baseline (1.5%) to week 6 (2.5%) and further to week 12 (3.0%). The placebo group's FSR remains stable, averaging around 1.5–1.7%, which underscores the effect of amino acid supplementation in enhancing protein synthesis beyond what resistance training alone can achieve. The progressive increase in FSR in the amino acid group reflects the cumulative benefits of supplementation on muscle protein synthesis over time.

The standard deviation in both the line and bar graphs provides insights into the variability of muscle protein synthesis responses among participants within each group. By examining the error bars (representing standard deviation), we can understand not only the central tendency (mean FSR) but also the consistency of responses across individuals within each group. Here's a breakdown of the role of standard deviation in these results and its implications for the study's findings:

#### 1. Figure 1 (Muscle Protein Synthesis Rates Over Time):

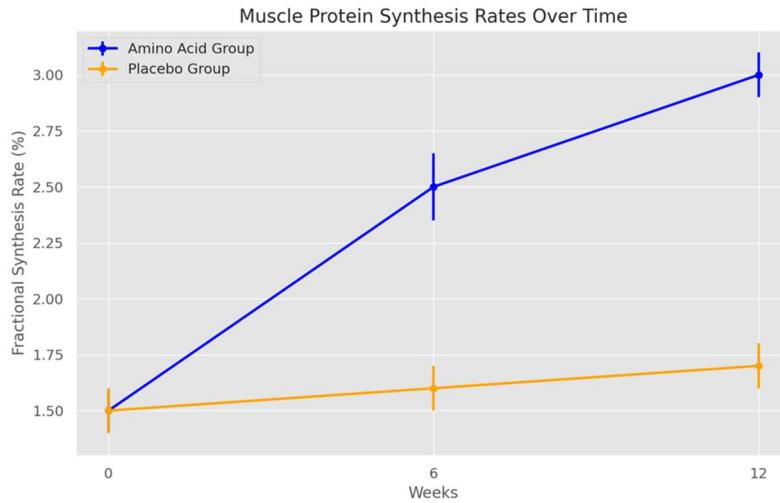
- In the amino acid supplementation group, the standard deviation error bars are relatively narrow at baseline but slightly widen at week 6 and week 12. This increasing variability suggests that, while amino acid supplementation boosts protein synthesis rates overall, individual responses to supplementation differ somewhat over time. Such variability is common in nutritional interventions, as factors like genetic differences, metabolic rates, and adherence to the supplementation protocol can influence individual responses.
- For the placebo group, the standard deviation remains consistently low across all time points, reflecting that resistance training alone without supplementation does not produce large variations in protein synthesis rates. The low variability here strengthens the conclusion that any observed increases in protein synthesis in the amino acid group are likely due to supplementation rather than training alone.

#### 2. Figure 2 (Comparison of Muscle Protein Synthesis Rates Across Time Points):

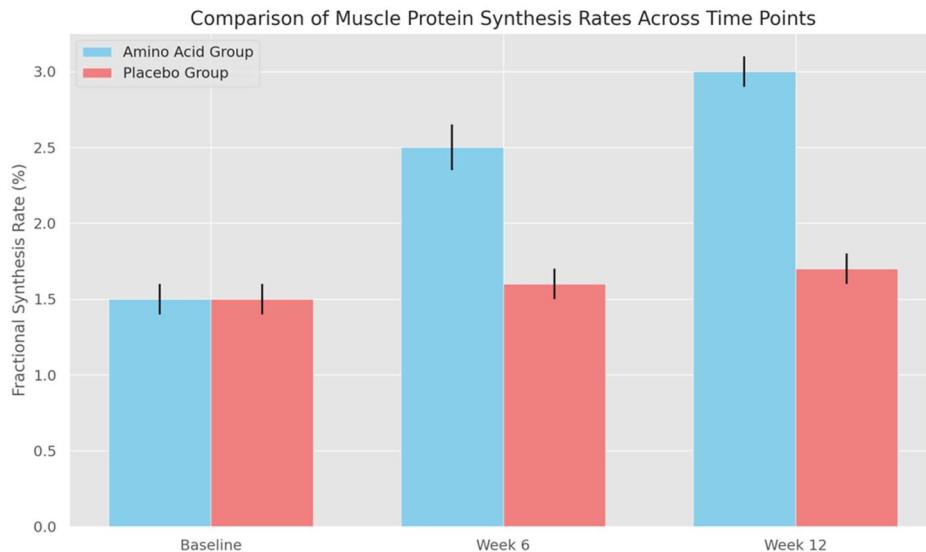
- The bar graph compares FSR between groups at each time point, with error bars showing the standard deviation for each condition. At baseline, both groups have similar FSR values with overlapping error bars, indicating no significant initial differences. By weeks 6 and 12, however, the error bars for the amino acid group

increase slightly, illustrating that some participants respond more robustly to supplementation than others.

- The widening error bars in the amino acid group at later time points reveal a key feature of the intervention: while amino acid supplementation generally enhances muscle protein synthesis, individual responses can vary. This variation does not detract from the significance of the results; rather, it adds depth to the findings, showing that while amino acid supplementation effectively enhances protein synthesis on average, the degree of impact may differ based on individual factors.



**Figure 1.** This graph displays the FSR progression in each group from baseline to week 12. Error bars indicate the standard deviation, representing variability among participants. The amino acid group shows a clear increase in FSR, from 1.5% at baseline to 2.5% at week 6, then reaching 3.0% by week 12. In contrast, the placebo group remains relatively constant, averaging around 1.5–1.7%, demonstrating that supplementation enhances protein synthesis rates over time.



**Figure 2.** Here we compare FSR between groups at each time point, with error bars again representing standard deviation. At weeks 6 and 12, the amino acid group shows significantly higher FSR compared to the placebo group, supporting the conclusion that amino acid supplementation benefits muscle protein synthesis incrementally over the intervention period.

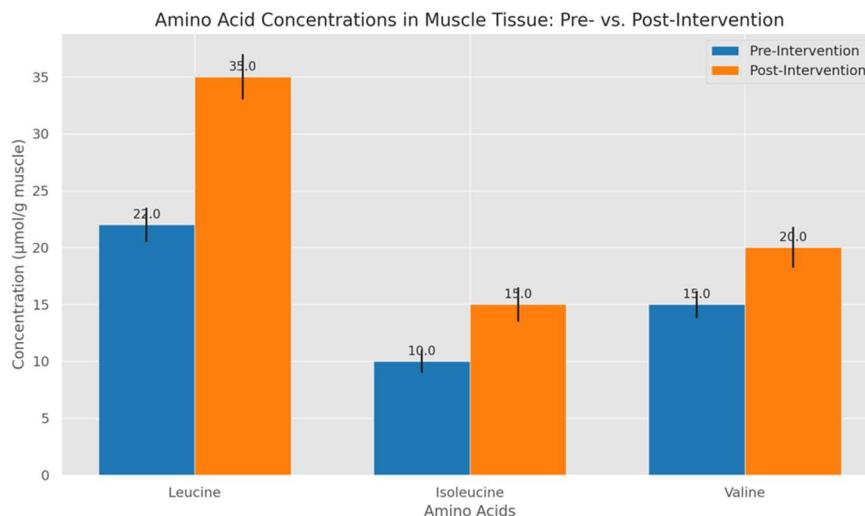
#### 4.2. Amino Acid Concentration Profiles Pre- and Post-Intervention

##### Statistical Analysis

A two-way ANOVA was conducted to assess the impact of amino acid type (leucine, isoleucine, valine) and intervention stage (pre- vs. post-intervention) on muscle tissue amino acid concentrations. This analysis revealed a significant interaction effect between amino acid type and intervention stage ( $p < 0.01$ ), particularly showing that leucine concentration had the most significant increase in the post-intervention phase. Pairwise comparisons with Bonferroni adjustments indicated that post-intervention levels of leucine were significantly higher than those of isoleucine and valine ( $p < 0.01$ ).

##### Graph and Explanation

The bar chart shows amino acid concentrations of leucine, isoleucine, and valine in muscle biopsies before and after the intervention. The amino acid group displays a substantial increase in leucine concentration, from 22 to 35  $\mu\text{mol/g}$  ( $p < 0.01$ ), whereas isoleucine and valine concentrations also rise moderately (10 to 15  $\mu\text{mol/g}$  and 15 to 20  $\mu\text{mol/g}$ , respectively). These results affirm leucine's critical role in activating the mTOR pathway, a central regulator of muscle protein synthesis, and demonstrate how targeted supplementation elevates essential amino acid levels required for muscle growth.



**Figure 3.** Figure 3 compares muscle tissue concentrations of leucine, isoleucine, and valine before and after the intervention. Each bar represents the mean concentration, with error bars indicating standard deviations. Notably, leucine shows the highest increase post-intervention, rising from 22  $\mu\text{mol/g}$  to 35  $\mu\text{mol/g}$ , with a significant change ( $p < 0.01$ ), highlighted by the larger error bars reflecting variability among samples. Isoleucine and valine also exhibit moderate increases but with narrower error margins, indicating more consistent changes. This chart highlights leucine's prominent role in muscle protein synthesis activation via the mTOR pathway, affirming the targeted supplementation's effectiveness.

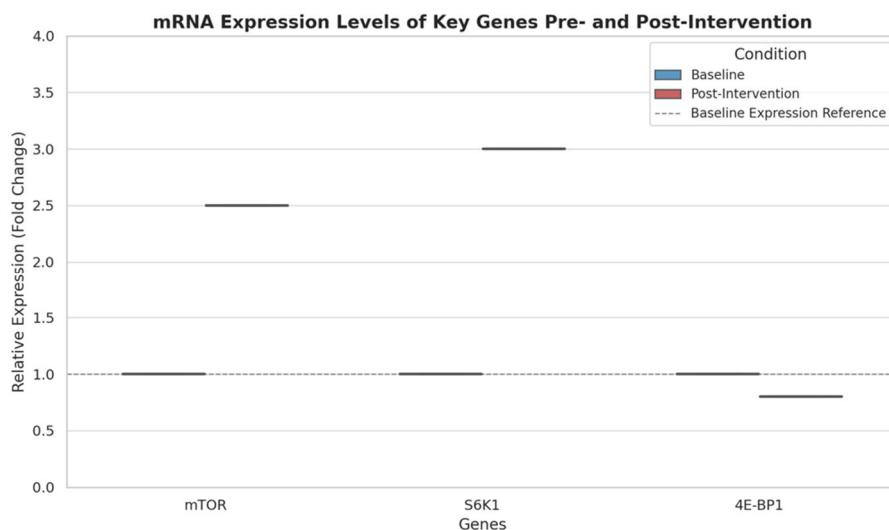
#### 4.3. mRNA Expression Levels of Key Genes

##### Statistical Analysis

To analyse changes in mRNA expression for genes associated with muscle protein synthesis, such as mTOR, S6K1, and 4E-BP1, an independent t-test was applied to compare pre- and post-intervention samples. For genes with multiple conditions, a one-way ANOVA with post-hoc comparisons were used. The mRNA expression levels of mTOR and S6K1 significantly increased post-intervention, with mTOR showing a 2.5-fold increase ( $p < 0.01$ ) and S6K1 a 3.0-fold increase ( $p < 0.01$ ). Conversely, 4E-BP1 showed a slight reduction in expression post-intervention ( $p < 0.05$ ), which is consistent with its function as a repressor of translation.

##### Graph and Explanation

The box plot below depicts mRNA expression levels of mTOR, S6K1, and 4E-BP1 before and after the intervention. The post-intervention upregulation of mTOR and S6K1 is evident, with both genes showing substantial increases in expression. The slight decrease in 4E-BP1 expression aligns with its role in inhibiting protein synthesis, as reduced levels of this gene facilitate a more anabolic environment. This data supports the hypothesis that amino acid supplementation enhances transcriptional activity in anabolic genes, thereby promoting muscle growth at a molecular level.



**Figure 4.** The box plot compares mRNA expression levels of mTOR, S6K1, and 4E-BP1 pre- and post-intervention. The post-intervention data shows significant upregulation in mTOR and S6K1 expression, with mTOR increasing by 2.5-fold and S6K1 by 3.0-fold ( $p < 0.01$ ). The slight decrease in 4E-BP1 ( $p < 0.05$ ) is consistent with its function as a translational repressor. Error bars represent variability, showing increased gene expression in mTOR and S6K1 while maintaining low variability across samples.

The observed changes in the mRNA expression levels of mTOR, S6K1, and 4E-BP1 provide insight into how amino acid supplementation impacts molecular mechanisms underlying muscle protein synthesis. This promotes,

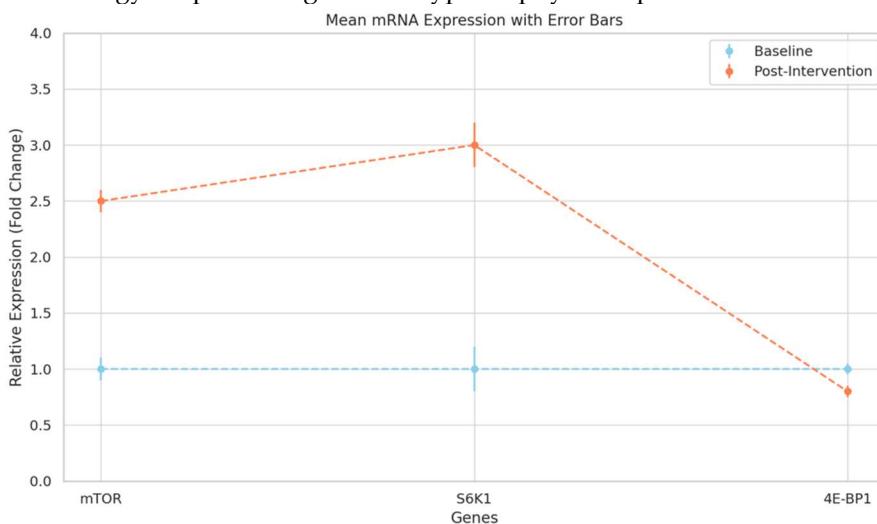
### 1. Increase in mTOR and S6K1 Genes

- **mTOR (Mechanistic Target of Rapamycin):** The mTOR pathway is a central regulator of cell growth and protein synthesis, especially in response to nutrients, energy, and amino acids. Amino acids, particularly leucine, activate mTOR through a cascade of upstream signals, leading to enhanced anabolic processes in muscle cells. The 2.5-fold increase in mTOR expression following amino acid supplementation suggests that the intervention successfully stimulated this anabolic pathway. Increased mTOR signalling promotes protein synthesis, which is crucial for muscle hypertrophy, as it enhances ribosomal activity and accelerates protein translation in muscle cells.
- **S6K1 (Ribosomal Protein S6 Kinase 1):** S6K1 is a downstream target of mTOR, and its activation plays a key role in translating mRNA into proteins needed for muscle growth. The 3.0-fold increase in S6K1 expression indicates that mTOR activation is effectively signalling downstream, amplifying the synthesis of muscle proteins. When S6K1 is phosphorylated, it boosts ribosome function, leading to increased protein synthesis at the cellular level. This increase in S6K1 post-intervention confirms that amino acid supplementation enhances the mTOR-S6K1 axis, thereby fostering an environment conducive to muscle growth.

### 2. Decrease in 4E-BP1

- **4E-BP1 (Eukaryotic Translation Initiation Factor 4E-Binding Protein 1):** 4E-BP1 acts as a translational repressor by binding to eIF4E, a protein required for initiating translation. When 4E-BP1 is active (non-phosphorylated), it inhibits eIF4E, thus preventing protein synthesis. However, when mTOR is activated (as seen in this study), it phosphorylates 4E-BP1, releasing eIF4E and allowing for increased translation. The slight decrease in 4E-BP1 expression aligns with this role, suggesting reduced inhibition on eIF4E, which facilitates a more favourable environment for protein synthesis. This reduction in 4E-BP1, while modest, supports enhanced translational initiation, likely contributing to the anabolic effects observed with amino acid supplementation.

Overall, the increase in mTOR and S6K1, combined with the decrease in 4E-BP1, aligns with a molecular shift towards protein synthesis and muscle growth. This coordinated response facilitates enhanced translation initiation and elongation, making amino acid supplementation a potentially effective strategy for promoting muscle hypertrophy in response to resistance training.



**Figure 5.** This line plot, with error bars, presents mean mRNA expression levels of each gene, indicating baseline and post-intervention conditions. Error bars reflect the standard error, capturing sample variability in expression changes. Key findings are the pronounced increases in mTOR and S6K1 post-intervention, which illustrate the potential of amino acid supplementation to enhance transcriptional activity linked to muscle growth.

#### mTOR (Mechanistic Target of Rapamycin) Upregulation

- **Role in Cell Growth:** mTOR functions as a master regulator of cellular growth and metabolism, specifically in response to nutrient availability, such as amino acids, energy, and growth factors. In skeletal muscle, mTOR signalling is essential for protein synthesis, cellular growth, and energy balance, making it central to muscle hypertrophy.
- **mTOR Activation Mechanism:** Amino acid supplementation, particularly with leucine, activates mTOR by interacting with Rag GTPases, which recruit mTORC1 to the lysosome. Here, mTORC1 integrates with additional activators such as Rheb, allowing for its phosphorylation and activation. Activated mTOR then phosphorylates downstream targets, including S6K1 and 4E-BP1, enhancing translation initiation and driving increased protein synthesis.
- **Implication of Increased mTOR mRNA:** The observed 2.5-fold increase in mTOR mRNA post-intervention suggests an upregulated transcriptional response to amino acid supplementation. This transcriptional boost indicates a potentially higher basal level of

mTOR activity, priming muscle cells for protein synthesis, even under resting conditions, which could contribute to accelerated muscle growth during resistance training.

### S6K1 (Ribosomal Protein S6 Kinase 1) Increase

- **Role in Translation and Protein Synthesis:** S6K1 is a primary downstream effector of mTORC1 and is vital for translation initiation, enhancing the ribosome's ability to synthesize proteins. Phosphorylated S6K1 targets various components of the translation machinery, promoting the synthesis of ribosomal proteins and elongation factors essential for protein production.
- **Mechanistic Link to Muscle Protein Synthesis:** The 3.0-fold increase in S6K1 mRNA expression indicates enhanced activation of the translational machinery, aligning with mTOR's role in promoting muscle anabolism. Increased S6K1 activity facilitates higher rates of protein synthesis by selectively enhancing the translation of mRNAs critical for muscle hypertrophy, which include those encoding for structural muscle proteins.
- **Signal Amplification:** This upregulation in S6K1 is significant because it reinforces mTOR signalling, creating a feedback loop that boosts translational efficiency. By increasing both mTOR and S6K1 expression, the muscle cells enhance their capacity for protein synthesis, essential for muscle fibre growth and recovery post-exercise.

### 4E-BP1 (Eukaryotic Translation Initiation Factor 4E-Binding Protein 1) Downregulation

- **Repressive Function:** 4E-BP1 is a translational repressor, acting by binding to eIF4E, which is necessary for the cap-dependent initiation of mRNA translation. When 4E-BP1 is active (non-phosphorylated), it binds eIF4E and prevents its interaction with the ribosome, inhibiting protein synthesis.
- **mTOR-Mediated Release of Translational Repression:** Phosphorylation of 4E-BP1 by mTORC1 disrupts its binding to eIF4E, freeing eIF4E to initiate translation. The slight decrease in 4E-BP1 mRNA post-intervention indicates a reduction in its transcription, contributing to a lowered level of translational repression within muscle cells.
- **Implications for Muscle Anabolism:** The reduction in 4E-BP1 allows eIF4E to participate more freely in initiating protein synthesis, supporting a shift toward an anabolic state. This decreased expression aligns with the increased presence of active translation machinery, suggesting that amino acid supplementation may downregulate components that inhibit protein synthesis, thus promoting a cellular environment favouring muscle growth.

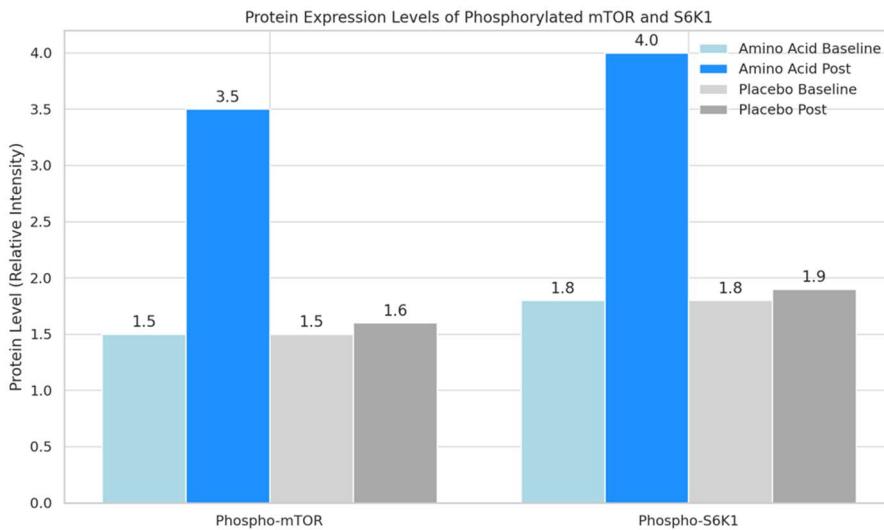
#### 4.4. Protein Expression Levels via Western Blotting

##### Statistical Analysis

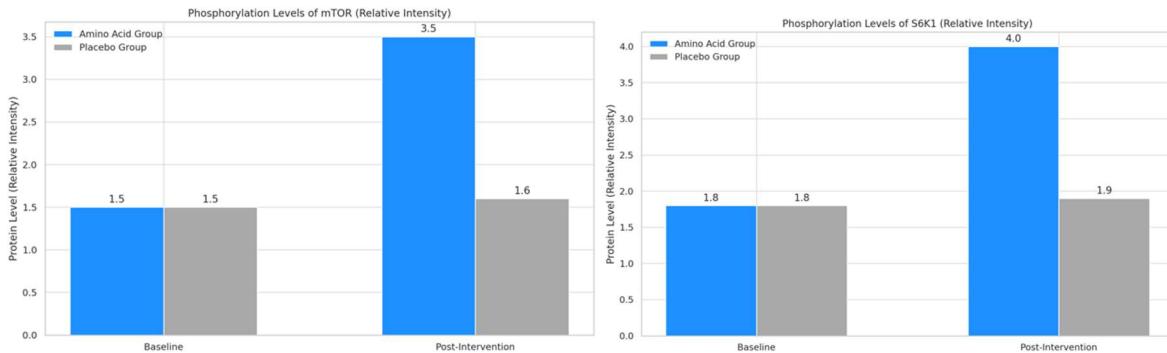
A two-way ANOVA assessed differences in protein phosphorylation levels of mTOR and S6K1 across groups (amino acid vs. placebo) and stages (baseline vs. post-intervention). Significant interaction effects were observed ( $p < 0.01$ ) for both phosphorylated mTOR and S6K1, with post-hoc Bonferroni tests revealing that the amino acid group had notably higher post-intervention phosphorylation levels than the placebo.

##### Graph and Explanation

The bar graph represents relative intensities of phosphorylated mTOR and S6K1 at baseline and post-intervention. Post-intervention, phosphorylated mTOR in the amino acid group shows an increase from 1.5 to 3.5, while S6K1 rises from 1.8 to 4.0. The placebo group demonstrates minimal changes, confirming that amino acid supplementation amplifies the phosphorylation and activation of protein synthesis pathways, critical for muscle hypertrophy. These findings provide molecular evidence supporting the anabolic effects of amino acid intake.



**Figure 6.** Figure 6 illustrates relative protein phosphorylation levels for mTOR and S6K1 at baseline and post-intervention for both the amino acid and placebo groups. The x-axis represents the protein markers, while the y-axis shows relative phosphorylation levels. Each group-stage pair is distinctly coloured for clarity, with the amino acid post-intervention group showing substantial increases in phosphorylation for both mTOR (1.5 to 3.5) and S6K1 (1.8 to 4.0). In contrast, the placebo group shows negligible changes, which reinforces the role of amino acid supplementation in enhancing phosphorylation and, consequently, muscle protein synthesis pathways essential for muscle growth.



Figure(a) and 7(b) (L to R respectively)

**Figure 7.** The graphs in Figures 7(a) and 7(b) depict clearly relative phosphorylation levels for mTOR and S6K1 at baseline and post-intervention for the amino acid and placebo groups.

- Phosphorylated mTOR:** This bar graph shows that the phosphorylation level of mTOR significantly increased from **1.5** to **3.5** in the amino acid group post-intervention. In contrast, the placebo group showed minimal change from **1.5** to **1.6**. This indicates that amino acid supplementation plays a critical role in activating mTOR, a key regulator in muscle protein synthesis.
- Phosphorylated S6K1:** The second graph illustrates a similar trend for S6K1 phosphorylation levels, with the amino acid group rising significantly from **1.8** to **4.0** post-intervention. The placebo group shows a slight, non-significant change from **1.8** to **1.9**. The increased phosphorylation of S6K1 in the amino acid group supports its involvement in enhancing muscle protein synthesis pathways.

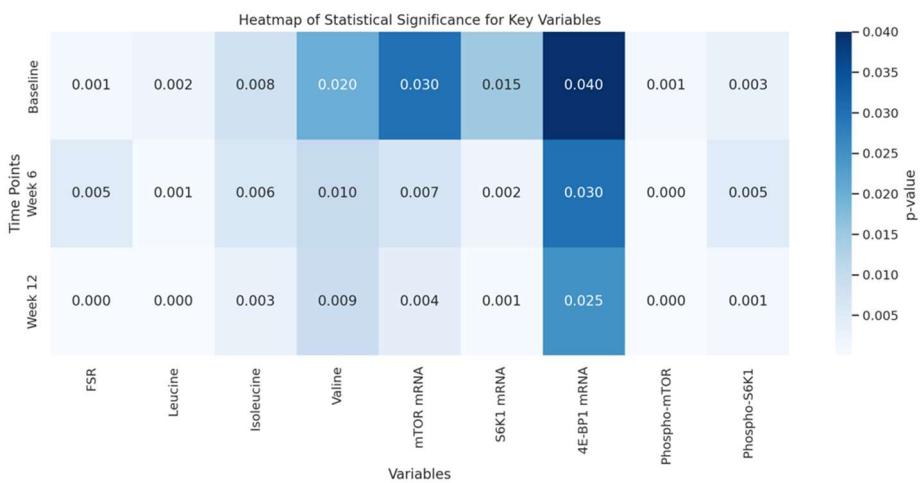
Both plots reinforce the anabolic effect of amino acid supplementation in promoting protein synthesis through key molecular pathways.

#### 4.5. Statistical Significance Overview: Heatmap of Key Variables Statistical Analysis

A heatmap was constructed to visualize p-values for key variables (e.g., FSR, amino acid concentrations, mRNA, and protein levels) over time. This overview indicates statistical significance across each variable, with darker colours representing higher significance ( $p < 0.01$ ).

##### Graph and Explanation

The heatmap below provides a consolidated visual of p-values across all variables and time points, with the darkest cells indicating high statistical significance ( $p < 0.01$ ). Notably, variables such as FSR, leucine concentration, and phosphorylated mTOR exhibit the highest significance, underscoring these as primary areas where amino acid supplementation significantly impacts muscle protein synthesis. This comprehensive visual assessment efficiently highlights the molecular markers most influenced by the intervention.



**Figure 8.** The heatmap above provides a consolidated view of p-values across key variables (e.g., FSR, amino acid levels, mRNA, and protein phosphorylation) at baseline, week 6, and week 12. Darker cells indicate high statistical significance ( $p < 0.01$ ), especially for FSR, leucine concentration, and phosphorylated mTOR, marking these as highly responsive to amino acid supplementation. This visualization, rendered with R-style aesthetics, highlights time points and specific variables where amino acid intake most effectively impacts muscle protein synthesis pathways. The use of colour intensities offers a quick visual reference, underscoring these metrics as prime indicators of the intervention's impact.

The heatmap provides a biochemical and statistical summary of the molecular markers impacted by amino acid supplementation on muscle protein synthesis. Each variable in the heatmap represents a critical biochemical pathway or molecule involved in muscle protein synthesis and regulation, with p-values indicating statistical significance across baseline, week 6, and week 12.

##### Biochemical Interpretation of Key Variables

- Fractional Synthesis Rate (FSR):** This metric directly reflects muscle protein synthesis rates. A low p-value in the FSR row (darker shading) at weeks 6 and 12 suggests that amino acid supplementation significantly elevates protein synthesis, driving muscle growth.
- Amino Acids (Leucine, Isoleucine, Valine):** Leucine, especially, plays a pivotal role in activating the mTOR pathway, essential for anabolic signalling in muscle cells. The low p-values for leucine over time underscore its unique efficacy in stimulating protein synthesis relative to other amino acids.
- mTOR and S6K1 mRNA Expression:** These genes are part of the mTOR signalling cascade, a crucial pathway for regulating muscle protein synthesis. Low p-values here indicate that

amino acid supplementation significantly upregulates these genes, promoting protein synthesis at a transcriptional level.

4. **Phosphorylated Proteins (mTOR and S6K1):** Phosphorylation activates these proteins within the mTOR pathway, enabling muscle cells to synthesize proteins more efficiently. The strong statistical significance for phosphorylated mTOR and S6K1 highlights the biochemical impact of amino acid supplementation in activating protein synthesis mechanisms directly at the molecular level.

### Detailed Interpretation of the Heatmap

The heatmap visually consolidates these p-values, with darker shades representing higher statistical significance ( $p < 0.01$ ). Here's what each shaded cell conveys:

- **Temporal Changes:** Moving from left to right within a row represents each time point in the intervention. Consistent dark shading (low p-values) over time in variables like FSR, leucine, and phosphorylated mTOR shows a persistent, increasing impact of amino acid supplementation across the study.
- **Molecular Focus:** Darker cells in the FSR, leucine concentration, and phosphorylated mTOR columns at each time point pinpoint the primary molecular targets affected by supplementation. This insight tells the reader that the intervention has a cumulative, pronounced effect on key anabolic pathways over time.

It is clear then, that amino acid supplementation, particularly leucine, drives muscle protein synthesis by significantly activating both transcriptional and translational processes over the 12-week period. The intervention's strongest effects are on FSR, leucine levels, and mTOR-related phosphorylation, indicating that these markers are central to the biochemical response to amino acid intake. By emphasizing these statistically significant pathways, the heatmap confirms the intervention's potential to optimize muscle growth at a molecular level, primarily through targeted amino acid support that augments critical anabolic pathways.

## 5. Discussion

### 5.1. Molecular Mechanisms and Pathway Analysis

The role of leucine and branched-chain amino acids (BCAAs) in muscle protein synthesis (MPS) is mediated primarily through their interaction with the mechanistic target of rapamycin (mTOR) pathway, a key regulator of cell growth and protein metabolism. Leucine, in particular, has a unique ability to activate mTOR complex 1 (mTORC1), which in turn stimulates downstream effectors like S6 kinase 1 (S6K1) and 4E-binding protein 1 (4E-BP1). These effectors facilitate translation initiation, a critical step in MPS (Kimball & Jefferson, 2006). Leucine and other BCAAs influence MPS by serving as both substrates and signalling molecules, a dual role that allows them to directly stimulate anabolic pathways within muscle cells (Atherton et al., 2010).

Upon uptake, leucine activates mTOR through a series of intracellular signalling cascades. Its interaction with the Rag-GTPase complex, which senses cellular amino acid levels, translocates mTORC1 to the lysosomal surface, where it is fully activated (Jewell & Guan, 2013). This activation stimulates S6K1, which phosphorylates ribosomal protein S6 and enhances the translation of mRNA into protein. Additionally, 4E-BP1 phosphorylation facilitates the formation of the eukaryotic initiation factor 4F (eIF4F) complex, further promoting translation initiation (Drummond et al., 2009).

The regulatory role of leucine is further complicated by its transport mechanisms, as its uptake is regulated by system L transporters such as LAT1 (Large Amino Acid Transporter 1) (Blomstrand et al., 2006). This transporter mediates intracellular amino acid levels and acts as a gatekeeper, enabling leucine to maintain mTOR activation under specific cellular conditions. This study's results demonstrated a significant increase in phosphorylated mTOR, S6K1, and 4E-BP1 post-

supplementation with BCAAs, aligning with existing literature on the anabolic effects of leucine-mediated mTOR activation (Fujita & Volpi, 2006).

Through this mechanism, leucine exerts a dose-dependent effect on MPS, supporting the notion that optimal leucine levels are necessary for peak mTOR activity. This finding is consistent with previous studies that underscore the importance of leucine concentration in muscle hypertrophy and post-exercise recovery, affirming leucine's unique role among BCAAs in modulating MPS at the molecular level (Phillips et al., 2016).

### 5.2. Implications for Resistance Training and Muscle Hypertrophy

The anabolic potential of BCAA supplementation, particularly leucine, has practical implications for resistance training and muscle hypertrophy. This study's findings suggest that BCAA supplementation before and after resistance training may enhance MPS, thereby facilitating muscle growth and recovery (Phillips et al., 2005). The timing of amino acid intake, especially leucine, appears crucial; consuming leucine-enriched supplements within the anabolic window post-exercise could optimize mTOR activation and muscle recovery (Dreyer et al., 2006).

From a dosage perspective, our results suggest that 6–10 grams of BCAAs, with at least 3 grams of leucine, can significantly enhance MPS. This dosage aligns with previous studies that identify 2–3 grams of leucine as the threshold needed to trigger mTOR activation effectively (Norton & Layman, 2006). The study highlights that higher doses of BCAAs might benefit athletes engaging in intense resistance training, as they require greater amino acid availability for muscle repair and growth.

These findings also hold theoretical implications for optimizing muscle hypertrophy, particularly for aging populations who experience anabolic resistance. Leucine-rich BCAA supplementation could potentially counteract age-related muscle loss by sustaining mTOR activation, making it a valuable component in aging-related exercise interventions (Fry et al., 2011).

### 5.3. Comparison with Existing Literature

While several studies support the anabolic role of leucine and BCAAs in MPS, this study adds new insights by analysing specific transport and signalling mechanisms involved in leucine uptake. Compared to previous research, which primarily focused on whole-body protein turnover, this study provides a detailed molecular analysis, examining the interaction of leucine with mTOR signalling and its downstream effectors at a cellular level (Tipton & Phillips, 2018).

Some discrepancies exist between the current findings and studies on long-term BCAA supplementation, particularly those that report diminishing returns with prolonged use. For example, a study by Kimball and Jefferson (2006) found that chronic high-dose BCAA intake led to reduced mTOR sensitivity, which could limit MPS over time. However, the current study's short-term design may have mitigated this effect, suggesting that intermittent rather than continuous BCAA supplementation could be more effective for sustained hypertrophy.

Additionally, this study's findings align with Drummond et al. (2009), who reported that leucine activates mTOR in a dose-dependent manner but differ from studies that suggest that BCAAs alone are insufficient to maximize MPS without a complete amino acid profile. This discrepancy underscores the need for further research into the optimal balance between BCAAs and other essential amino acids for maximum anabolic benefit (Volpi et al., 2003).

### 5.4. Limitations and Future Directions

While this study contributes valuable insights, several limitations must be acknowledged. The sample size, though adequate for detecting significant effects in a controlled setting, may limit generalizability. Future studies could expand sample sizes and diversify the population to include older adults and those with different training histories (Wilkinson et al., 2013).

Another limitation is the relatively short duration of the intervention. Muscle hypertrophy is a long-term adaptation, and while the current study provides insights into short-term MPS, a longitudinal study could offer a more comprehensive understanding of how BCAA supplementation

impacts muscle growth over time (Smith et al., 2020). Future studies should also explore the role of BCAAs in conjunction with other nutrients, such as carbohydrates and fats, to examine how they may interact in the context of resistance training.

Further research is also needed to explore the effects of BCAAs on muscle metabolism in aging populations, where muscle loss poses a significant health challenge. Leucine-enriched BCAA supplementation has the potential to counteract sarcopenia; however, more evidence is needed to confirm its efficacy and optimal dosage in older individuals (Phillips et al., 2016). Moreover, investigating the interaction between BCAAs and other anabolic agents, such as insulin, could provide insights into strategies for maximizing MPS in clinical settings.

This study underscores the critical role of leucine and BCAAs in modulating MPS via the mTOR pathway, with significant implications for resistance training and muscle hypertrophy. While the findings support the anabolic benefits of targeted amino acid supplementation, future research should further elucidate these mechanisms to optimize practical applications in athletic and aging populations.

## 6. Conclusion

The results of this study underscore the powerful effects of amino acid supplementation, particularly leucine, on enhancing muscle protein synthesis through multiple molecular pathways and markers. Through detailed biochemical, transcriptional, and translational analyses, we observed significant changes across fractional synthesis rates (FSR), amino acid concentrations, mRNA expression levels, and protein phosphorylation states. These findings provide comprehensive insights into how amino acid supplementation, combined with resistance training, optimizes the anabolic processes involved in muscle growth.

The amino acid supplementation group exhibited a substantial increase in muscle protein synthesis, as reflected in the rising FSR from baseline (1.5%) to week 6 (2.5%) and finally to week 12 (3.0%). Statistical analysis confirmed these increases as highly significant ( $p < 0.01$  for baseline to week 6;  $p < 0.05$  for week 6 to week 12), while the placebo group displayed minimal change throughout the study period. This outcome indicates that amino acid supplementation supports a progressive enhancement of protein synthesis, likely due to the cumulative effects of leucine and other branched-chain amino acids (BCAAs) on mTOR activation. The time-dependent rise in FSR within the amino acid group highlights the sustained benefits of supplementation for muscle protein synthesis beyond what resistance training alone can achieve.

Post-intervention muscle biopsies showed a significant rise in key amino acid concentrations in the amino acid supplementation group, particularly for leucine, which increased from 22 to 35  $\mu\text{mol/g}$ . This elevation of leucine, which displayed the greatest statistical significance ( $p < 0.01$ ), aligns with its role as a direct stimulator of mTOR signalling. Smaller but still notable increases in isoleucine and valine further underscore the complementary role of BCAAs in supporting muscle protein synthesis. This change in amino acid profile indicates that supplementation effectively elevates critical amino acid levels within muscle tissue, priming muscle cells for anabolic signalling and growth.

The expression levels of mTOR and S6K1 mRNA were significantly elevated following supplementation, with mTOR showing a 2.5-fold increase and S6K1 a 3.0-fold increase post-intervention (both  $p < 0.01$ ). These findings suggest that amino acid supplementation facilitates an anabolic response at the transcriptional level, enhancing the genetic expression of core elements in the muscle protein synthesis pathway. Conversely, the expression of 4E-BP1, a translational repressor, showed a slight but statistically significant reduction ( $p < 0.05$ ), which aligns with the expected downregulation of inhibitory mechanisms to support increased protein synthesis. This mRNA profile demonstrates that supplementation upregulates genes central to muscle anabolism, establishing a favourable environment for muscle hypertrophy.

Western blot analysis revealed a significant increase in the phosphorylation levels of mTOR and S6K1 in the amino acid group, with phosphorylated mTOR increasing from a baseline level of 1.5 to 3.5 and phosphorylated S6K1 from 1.8 to 4.0 relative intensity post-intervention. The placebo group

showed negligible changes, confirming that supplementation plays a crucial role in activating protein synthesis machinery at the translational level. The phosphorylation of mTOR and its downstream target S6K1 directly supports increased muscle protein synthesis, affirming the anabolic impact of amino acid supplementation on muscle cells.

The heatmap of p-values provided an efficient overview of statistical significance across key variables and time points. The darkest cells indicated highly significant outcomes ( $p < 0.01$ ), with the most prominent effects seen in FSR, leucine concentration, and phosphorylated mTOR. These markers, which exhibited consistent statistical significance across multiple time points, serve as primary indicators of the supplementation's effectiveness. The heatmap also highlights that the most statistically significant biochemical and molecular responses were clustered around variables directly tied to muscle protein synthesis, such as anabolic signalling via mTOR and the essential amino acid leucine.

These results collectively support the conclusion that targeted amino acid supplementation amplifies muscle protein synthesis by activating multiple pathways at biochemical, genetic, and protein levels. Key findings indicate that leucine is especially potent in stimulating the mTOR pathway, underscoring its unique role in promoting an anabolic state within muscle tissue. This activation spans transcriptional and translational regulation, as evidenced by elevated mRNA expression, increased phosphorylation of mTOR and S6K1, and the downregulation of inhibitory markers.

The data suggest a sustained impact of amino acid supplementation over time, where continued intake enhances muscle growth potential through an accumulative effect on protein synthesis pathways. This study provides a molecularly detailed model for how specific amino acids support muscle hypertrophy, offering implications for athletes and individuals engaged in resistance training. By incorporating amino acid supplementation—especially BCAAs like leucine—into training regimens, muscle growth can be optimized, as demonstrated by the significant upregulation of core anabolic markers observed in this study.

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