Skeletal muscle hypertrophy rewires glucose metabolism in mice: an experimental investigation and systematic review

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Running title: Reprogrammed glucose metabolism and changes in muscle size

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ABSTRACT

Objective: Proliferating cancer cells shift their metabolism to anaerobic glycolysis even in the presence of oxygen to generate glycolytic intermediates as substrates for anabolic reactions. We hypothesise that a similar metabolic remodelling also occurs during physiological skeletal muscle hypertrophy.

Methods: We used mass spectrometry in hypertrophying muscles both in C2C12 muscle cells *in vitro* and plantaris mice muscle *in vivo* and assessed metabolomic changes and the incorporation of stable isotope [U-$^{13}C_6$]glucose tracer compared with non-treated controls. We also performed enzyme inhibition for further mechanistic analysis and a systematic review to align any changes in metabolomics during muscle growth with previous findings.

Results: Both our metabolomics analysis and the systematic review reveal altered metabolite concentrations in anabolic pathways such as in the pentose phosphate and serine synthesis pathway to build up biomass, as well as in the hexosamine biosynthetic pathway that serves as a basis for the post-translational so-called *O*-linked glycosylation modification. We further demonstrated that labelled carbon from [U-$^{13}C_6$]glucose is increasingly incorporated into the non-essential amino acids in hypertrophying myotubes. The inhibition of the key enzyme phosphoglycerate dehydrogenase (Phgdh) supresses muscle protein synthesis by 75% highlighting the importance of the serine pathway for maintaining muscle size.

Conclusion: Understanding the mechanisms that regulates skeletal muscle mass will help in developing effective treatments against muscle weakness. Our results provide evidence for metabolic rewiring of glycolytic intermediates into anabolic pathways during muscle growth, such as in the serine synthesis and hexosamine biosynthetic pathways.

INTRODUCTION

Muscle hypertrophy and increased strength are typical phenotypic adaptations to resistance training or chronic muscle overload in mice [1]. Resistance training is an effective low-cost intervention for the prevention and treatment of muscle loss in healthy and diseased individuals [2, 3]. Resistance training not only increases muscle size and strength but also improves glycaemic control, increases glucose uptake as well as insulin sensitivity [3, 4], suggesting metabolic adaptations in healthy as well as diabetic persons. Whilst mechanistic target of rapamycin complex 1 (mTORC1) has been identified as a key mediator of the increased protein synthesis after resistance exercise and during chronic overload [5], there are still many gaps in our understanding of how changes in the muscle metabolism possibly contributes to muscle hypertrophy [1] or atrophy [6].

Research, especially on cancer, shows that proliferating cells reprogram their metabolism and synthesise lactate in the presence of oxygen, which has been termed the Warburg effect. One key function of the Warburg effect is to synthesise glycolytic metabolites and other substrates for anabolic reactions that build biomass [7]. About 20% of the carbon dry mass of cancer cells is delivered from glucose carbon [8], which is accomplished by directing some glycolytic intermediates toward anabolic pathways such as the serine synthesis and pentose phosphate pathway. These two pathways generate critical precursors (e.g., non-essential amino acids and nucleotides) for the synthesis of macromolecules that are required for cancer cell proliferation [9, 10]. Conversely, knockout of the key enzyme phosphoglycerate dehydrogenase (Phgdh), which diverts the glucose-derived 3-phosphoglycerate away from glycolysis into the *de novo* serine synthesis pathway, reduces cancer cell proliferation [11] indicating the importance of this pathway for cellular growth and prolifieration. We recently hypothesised that a Warburg-like metabolic reprogramming also occurs in mature, hypertrophying muscle fibres [12]. Consistent with this, several investigations indicate increased expression of genes that encode enzymes of anabolic pathways [13, 14], and a rewiring of glucose metabolism in hypertrophying muscles [15-18].

A small fraction of glucose from glycolysis can also be shuttled into the hexosamine pathway that generates uridine diphosphate (UDP)-sugar precursors. These activated sugars are required for an important, but often overlooked, post-translational modification, so called *O*-linked glycosylation. Enzymes then catalyse the process to attach these activated sugars onto serine and threonine residues of proteins or lipids [19]. The most abundant metabolites generated via this process in human cells are *O*-N-acetylgalactosamines (*O*-GalNAc), common on membrane glycoproteins, and *O*-N-acetylglucosamines (*O*-GlcNAc). *O*-GlcNAcs are generated via *O*-GlcNAcylation, which coordinates glycolysis and promotes cancer growth [20], and also provides a potential common factor in obesity, diabetes and cancer [21]. A few investigations have shown that *O*-glycosylation is involved in different cellular processes of contracting/exercising skeletal muscle, e.g. in increasing glucose uptake following a resistance exercise bout [22], and that total protein *O*-GlcNAcylation increases by nearly 50% after muscle overload in mice [23] suggesting that the hexosamine pathway may be involved in muscle hypertrophy.

In this study, by using a mass spectrometry-based metabolomics approach in hypertrophy-stimulated muscles both in C2C12 myotubes *in vitro* and in plantaris mice muscle *in vivo*, we identified changes in metabolites linked to the pentose phosphate, serine and hexosamine biosynthetic pathways in hypertrophy-stimulated muscles. A systematic review of previously published metabolomics datasets further indicated a role of anabolic synthesis pathways during muscle growth. stable isotope [U-$^{13}C_6$]glucose tracer revealed that carbon of glucose is increasingly incorporated into non-essential amino acids during muscle growth. Finally, we inhibited the key enzyme of the serine pathway in muscle cells revealing an important role of

this pathway in protein synthesis. Our results further demonstrate that a hypertrophying skeletal muscle reprograms its metabolism to support biomass production.

RESULTS

Muscle growth stimulation with Insulin-like growth factor-1 increases the concentration of most of the glycolytic intermediates in C2C12 myotubes.

To study the metabolic consequences of stimulation and inhibition of muscle protein synthesis in C2C12 muscle cells *in vitro*, we treated myotubes with 100 ng/mL of IGF-1 and/or 10 ng/mL rapamycin for 48 h. As expected, IGF-1 increased myotube diameter whereas rapamycin prevented the IGF-1 induced (ANOVA, all $p<0.001$) (Figure 1A). Treatment with IGF-1 also increased the phosphorylation of the mTORC1 downstream target 70-kDa ribosomal protein S6 kinase (p70S6K) (Figure 1B), which was accompanied later by accretion of protein mass (Figure 1C) implying a positive protein balance, while rapamycin completely abolished basal and IGF-1 stimulated phosphorylation of p70S6k.

Next, we analysed the metabolome of myotubes treated with vehicle control, IGF-1, or rapamycin for 48 h. After rapid quenching and methanol extraction for the profiling of polar metabolites, the samples were analysed with untargeted liquid chromatography-high resolution tandem mass spectrometry (LC-MS/MS). We identified 155 metabolites from myotubes of which 27 showed significant differences between IGF-1 and control, 56 between IGF-1 and rapamycin, and 47 between control and rapamycin group, respectively (false discovery rate [FDR] <0.1). Principal component analysis of the three treatment groups showed a clear separation of the three experimental groups (Figure 1D). IGF-1 treatment changed the concentration of several glycolytic metabolites and amino acids (Supplementary table S4-5).

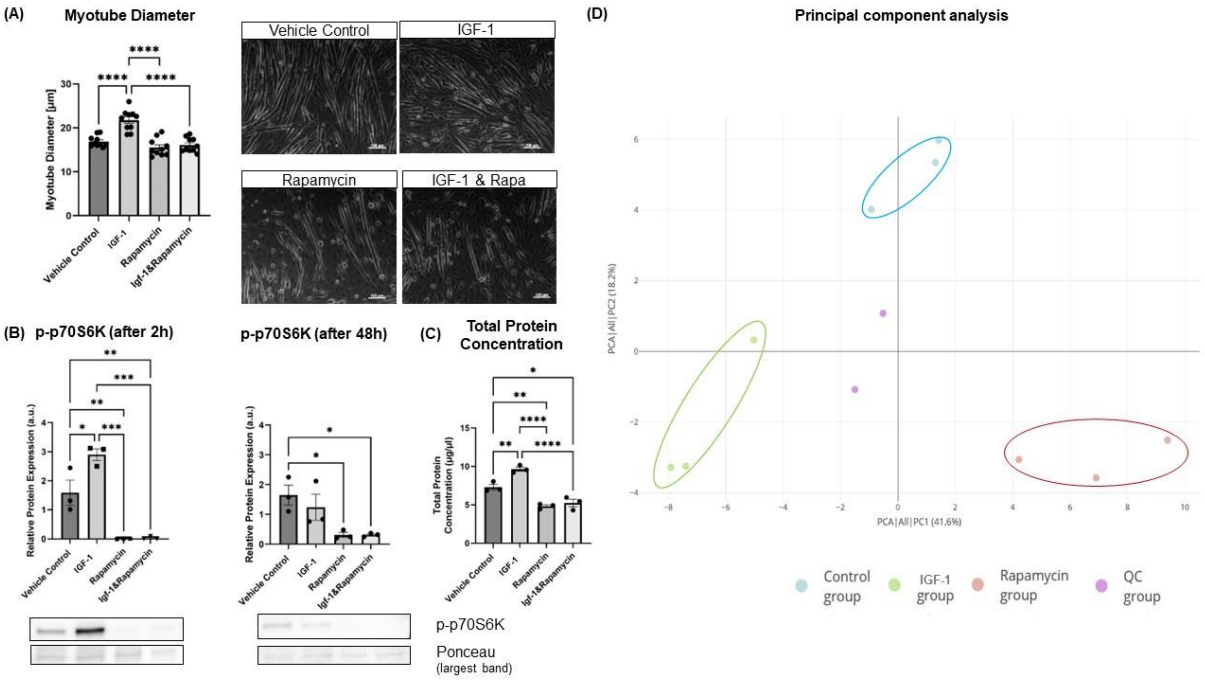

Figure 1 *(A) Myotube diameter measured 48 h after initialisation of treatments, (B) the phosphorylation of 70-kDa ribosomal protein S6 kinase (p70S6K) measured two and 48 h after initialisation of treatments, (C) total protein concentration 48 h after initialisation of treatments, (D) Principal component analysis of metabolites intensities of LC-MS/MS measurements (negative ion mode). *Significant differences between indicated conditions, two-way repeated measures ANOVA, one-way ANOVA with Tukey HSD post-hoc test (p<0.05). Data are expressed as mean ± standard error (SEM).*

We then analysed the metabolic changes within the myotubes and focussed on the glycolytic metabolites that had altered (FDR<0.1) their concentration in response to IGF-1 or rapamycin treatment (Figure 2 and Supplementary table S4-5). IGF-1 treatment significantly increased the concentration of glucose by 513% and lactate by 139% in myotubes (both p<0.0008). With rapamycin treatment, glucose concentration became undetectably low and lactate concentration decreased by 73.3%, when compared to vehicle control (both p<0.002). An elevation of lactate is caused by increased glycolysis that is a phenomenon of the Warburg effect predominantly observed in cancer cells [7]. Concomitant with these changes, the concentrations of phosphorylated sugars were higher by +27.5% (p<0.001) and of ribose-5-phosphate/ribulose-5-phosphate of the pentose phosphate pathway by +40% (p=0.01) in IGF-1 treatment compared to the control group and became undetectably low after rapamycin treatment.

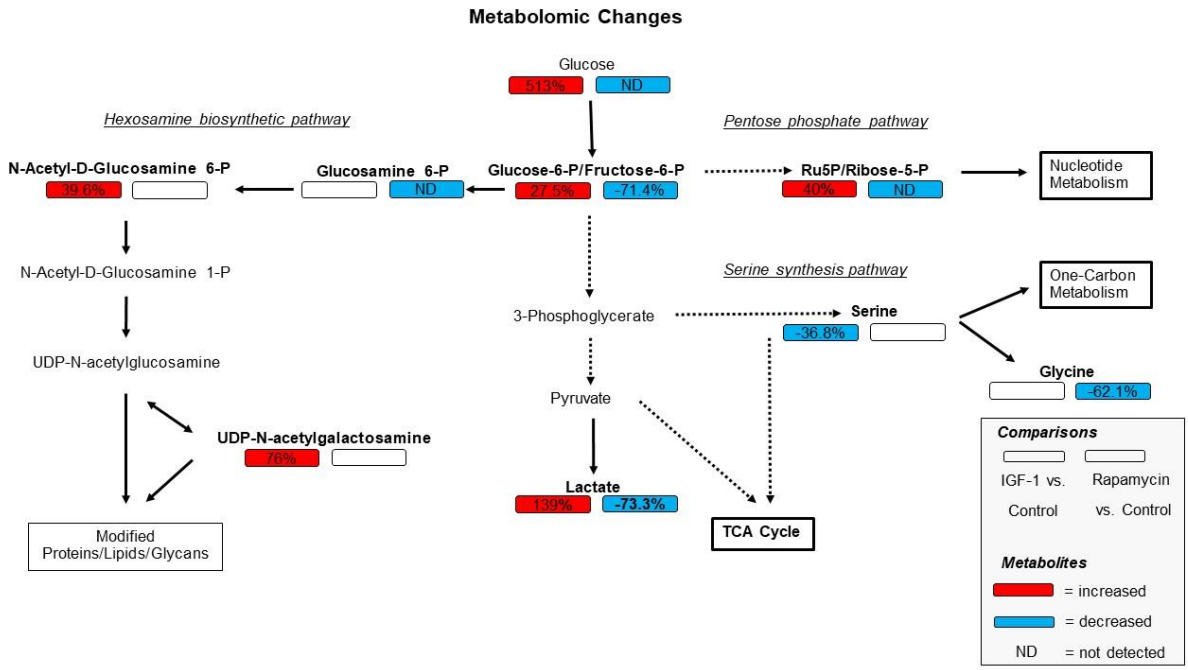

Figure 2 *Metabolomic changes in differentiated myotubes treated with either vehicle control, Insulin-like growth factor-1 (IGF-1; 100 ng/mL) or rapamycin (10 ng/mL) for 48 h (n = 3). Metabolites highlighted in bold were detected by untargeted metabolomics. Left box illustrates IGF-1 vs. control and right box rapamycin vs. control. Boxes highlighted in red and blue show significantly increased and decreased metabolite concentrations, respectively (both FDR<0.1). White box shows no significant changes (FDR≥0.1). P = Phosphate; TCA = Tricarboxylic acid cycle; UDP = Uridine diphosphate.*

Of 12 amino acids detected, IGF-1 treatment elevated the concentration of four (glutamate, leucine/isoleucine, phenylalanine, and valine) amino acids, and rapamycin reduced the concentration of six (alanine, asparagine, glycine, glutamate, proline and threonine) when compared to vehicle control (all p<0.01). Of the detected amino acids, only serine showed a lower concentration in IGF-1 treated myotubes compared to vehicle control (-36.8%; p=0.009). C2C12 myotubes consume rather than release serine into the media [24]. Thus, a decrease in serine concentration in the myotubes can either be based on a decreased activity of metabolite producing enzymes or an elevated activity of metabolite-consuming enzymes [25]. The concentration of glycine (synthesised downstream of serine) did not change with IGF-1 treatment and the concentration decreased by 64% when treated with rapamycin (p=0.001). Thus, we assume that decreased serine concentration after IGF-1 treatment may indicate a high flux through the serine pathway due to an elevated demand of the glycine cleavage

system, which refuels one-carbon metabolism [26]. Further, IGF-1 treatment increased pyrimidine metabolism, i.e., uridine 5'-monophosphate (+82%; p=0.003) and uridine 5'-diphosphate (+130%; p=0.004) compared to control treatment, whereas the former was decreased by 58.7% in rapamycin group, when compared with vehicle control (p=0.016). The pyrimidine biosynthesis pathway is necessary for fundamental cellular functions such as for the DNA and RNA biosynthesis as well as for glucose metabolism.

The metabolomics analysis also revealed changes of metabolites of the hexosamine biosynthetic pathway and to the linked *O*-Glycosylation. Hypertrophy-stimulation by IGF-1 treatment increased the concentration of nucleotide sugars including UDP-N-acetylgalactosamine (UDP-GalNAc) (+76%), and UDP-glucuronic acid (+262%; both p<0.005). By contrast, rapamycin treatment decreased concentration of UDP-glucuronic acid (-73.3%; p=0.007), and glucosamine-6-phosphate (Figure 2) as well as UDP-galactose concentrations to levels under the detection limit. The nucleotide sugars are sugar donors for the synthesis of various cellular glycans and for glycosylation reactions, many of which play important roles in the pathobiology of diseases associated with deranged glucose metabolism, for instance in type 2 diabetes. While UDP-sugars are related to both glycogen synthesis and *O*-Glycosylation, the other sugars (UDP-GalNAc and glucosamine-6-phosphate) are linked with the hexosamine pathway and further indicate that the products of this pathway might play a role during muscle growth.

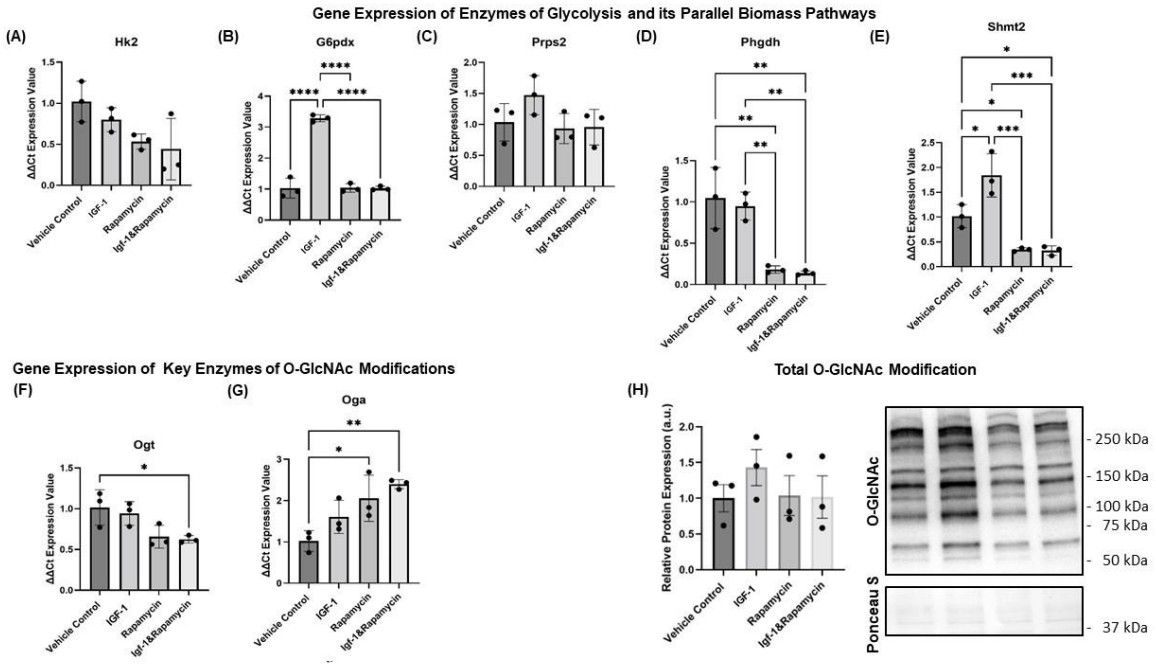

Figure 3 *Gene expressions of (A) hexokinase 2 (Hk2); key enzymes of the pentose phosphate pathway (B) glucose-6-phosphate 1-dehydrogenase X (G6pdx) and (C) phosphoribosyl pyrophosphate synthetase 2 (Prps2); key enzymes of the serine pathway (D) phosphoglycerate dehydrogenase (Phgdh) and (E) serine hydroxymethyltransferase-2 (Shmt2); key enzymes) of the post-translational modification termed O-linked N-acetylglucosaminylation (F) protein O-N-acetylglucosamine (O-GlcNAc) transferase (Ogt) and (G) protein O-GlcNAcase (Oga after 48 h. (H) Protein expression of total muscle O-GlcNAc-modified proteins after 48 h. O-GlcNAc antibody was normalised to the largest band of a Ponceau S stain. *Significant differences between groups, one-way ANOVA with Tukey HSD post-hoc test (p<0.05). Data are expressed as mean ± standard error (SEM).*

Unlike glycolytic intermediates, TCA cycle metabolite concentrations changed little after IGF-1 (Supplementary table S4-5). However, the intermediates fumarate and malate were

decreased by ~70% in myotubes treated with rapamycin (both p<0.0025). Lastly, IGF-1 treatment increased the oxidized form of glutathione 10-fold indicating that IGF-1-induced muscle hypertrophy elevates oxidative stress in myotubes.

To investigate whether the changes of metabolite concentrations were accompanied by changes in the expression of enzymes, we measured the expression of genes that encode metabolic enzymes. Elevated gene expression of key enzymes of the glycolysis and its parallel biomass pathways after treatment with IGF-I further supported the metabolic rewiring during skeletal muscle hypertrophy (Figure 3A-E). More specifically, glucose-6-phosphate dehydrogenase X (*G6pdx*), which encodes the rate-limiting enzyme of the oxidative pentose phosphate pathway, increased 3-fold in response to IGF-1 treatment. The serine-pathway-related enzymes *Phgdh* and serine hydroxymethyltransferase-2 (*Shmt2)* revealed also changes between the intervention groups, and especially *Shmt2* increased ~2-fold in abundance in hypertrophying muscle cells. The upregulation of these enzymes further suggests that the lower concentration of the serine metabolite as mentioned above was indeed caused by an increased flux through the serine pathway. Lastly, the gene expression of *O*-GlcNAc transferase (*Ogt*) and *O*-GlcNAcase (*Oga*), both enzymes responsible for attaching and removing GlcNAc from substrates, also decreased and increased, respectively in myotubes inhibited by rapamycin (Figure 3F-G). Thus, we further assessed the abundance of total muscle *O*-GlcNAc-modified proteins and observed no significant differences between the groups, indicating that *O*-GlcNAcylation is not unidirectionally changed on a global level *in vitro* (Figure 3H).

To summarise, the above data show that IGF-1 treatment reprograms the metabolism of myotubes towards enhanced utilization of glycolytic intermediates into anabolic pathways and that rapamycin has an opposite effect.

The hexosamine pathway is also increased in hypertrophying murine muscle.

To study the muscle metabolome response to a more physiological hypertrophic stimulus *in vivo*, we used an established muscle overload model [27]. Here, we induced compensatory hypertrophy of the right plantaris muscle in eight-month-old mice (n=6; body weight=31.3±2.6 g) by denervation of gastrocnemius and soleus muscles. The advantage of this model is that the plantaris muscle exhibits significant physiological hypertrophy (i.e. about 32% in 6 weeks ([28], and yet is associated with less invasive surgery and inflammatory response than during overload induced by ablation of synergists. We chose to examine the muscle metabolome at 14 days after denervation at a time when there was yet only a small 13.5 ± 3.51% (p=0.007) increase in skeletal muscle mass, when compared to intact contralateral plantaris muscle, to capture a timepoint of a dynamic and physiological increase in muscle size.

After rapid quenching of the muscles, we determined metabolite concentrations using an untargeted LC-MS/MS method. Two metabolites were altered in both hypertrophied muscles in mice *in vivo* as well as in hypertrophying myotubes *in vitro.* Similar to the hypertrophy-stimulation with IGF-1 in skeletal muscle cells *in vitro*, the concentration of the dipeptide L-carnosine (known for its antioxidant activity and antiglycation effects) was decreased by 25.9% (p<0.014) and of UDP-GalNAc was increased by 252% (p<0.042) during skeletal muscle growth *in vivo (*Supplementary table S6-7*)*. When comparing the results of the *in vivo* muscle overload model with metabolite concentration changes between hypertrophy stimulating IGF-1 group and the absence of muscle growth represented by the rapamycin group *in vitro*, the concentrations of pyroglutamic acid (*in vivo*: +40.2%), threonine (*in vivo* +24.9%), glutamate (+64.9%), and aminoisobutyric acid (*in vivo:* +63.2%) were also increased in both experimental models of muscle hypertrophy (p<0.05). We also tested the total muscle *O*-GlcNAc-modified

proteins in the *in vivo* overloaded plantaris muscles and could not find any differences compared to the control muscles ($p>0.05$; data not shown).

Systematic review further demonstrates a metabolic rewiring of glycolytic intermediates in growing muscles

Currently, no single metabolomics methodology currently covers the complete collection of metabolites in tissue [29]. To address this issue, we compared our findings to previously published metabolomics datasets of *in vitro* murine muscle cells and *in vivo* murine experiments to gain a comprehensive insight in metabolomic changes during and after skeletal muscle hypertrophy. For this, we performed a systematic review (PROSPERO access number: CRD42022318998) following PRISMA guidelines [30] and searched the literature in three literature databases (Pubmed, Scopus, Metabolight) using the PICO framework [31]. To assess the quality and risk of bias of the *in vivo* studies, the 'Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies' (CAMARADES) was used [32] (Table S3). The results display metabolites that showed significantly changed concentrations in at least two experiments (Figure S6).

Table 1 *Design and characteristics of the individual studies (n=8) included in the systematic review.*

Author (year)	**Model Intervention (n)**	**Model Control (n)**	***Sex***	**Body Weight**	**Tissue analysed**	**Muscle weight**	**Age [weeks]**	**Intervention**	**Detection method**
Baumert et al. (2022)	C2C12 muscle cells	C2C12 muscle cells	NA	NA	NA	NA	5d after different.	IGF-1 incubation	LC-MS/MS
Baumert et al. (2022)	C57BL/6 mouse (6)	C57BL/6 mouse (6)	Male	31.3g	Plan	C: 21mg I: 24mg	32	Synergist ablation	LC-MS/MS
Cheng et al. (2014)	Akt1 DTG mouse (6)	C57BL/6 mouse (6)	Male	C: 38g I: 38g	GA	C: 180mg I: 220mg	12	Akt1 induction	H NMR
Collins-Hooper et al. (2015)	$Mstn^{-/-}$ mouse (Nk)	C57BL/6 mouse (Nk)	Both	C: 26g I: 22g	GA	C: 100mg I: 180mg	16	Myostatin KO	H NMR
Hoshino et al. (2020)	C2C12 muscle cells	C2C12 muscle cells	NA	NA	NA	NA	6d after different.	EPS	CE-TOF-MS
Jiang et al. (2021)	C57BL/6 mouse (16)	C57BL/6 mouse (16)	Both	C: 26g I: 26g	GA	C: 0.42%* I: 0.50%*	8	Body Vibration	LC-MS/MS
Weyrauch et al. (2020)	C57BL/6 mouse (7-9)	C57BL/6 mouse (7-9)	Both	27g	Plan	C: 21mg I: 30mg	6	Synergist ablation	UPLC-MRM/MS
Wu et al. (2017)	Akt1 DTG mouse (Nk)	C57BL/6 mouse (Nk)	Both	C: Nk I: Nk	GA	C: 0.5%* I: 0.7%*	16	Akt1 induction	LC-MS/MS

Different. – Differentiation; C – Control; EPS – electro pulse stimulation; IGF-1 – Insulin-like growth factor-1; GA – Gastrocnemius muscle; I – Intervention; KO – gene knockout; NA – not applicable; Nk – not known; Plan – Plantaris; * Gastrocnemius:body weight ratio.

After removing duplicates, we screened 269 articles. Next to both present hypertrophying C2C12 muscle cells *in vitro* and plantaris muscle overload *in vivo* studies, we finally included another six publications that matched our eligibility criteria (see method section), which investigated changes in the metabolome of skeletal muscle tissue *in vivo* or myotubes *in vitro* during skeletal muscle hypertrophy [15, 16, 33-36] (Table 1). We found 20 metabolites that were identified in at least two experiments (Figure 4A). About 65% of the observed metabolites that demonstrated changes in at least two experiments were detected in both study designs either analysed hypertrophying mice muscles *in vivo* or in growing differentiated muscle cells *in vitro* (Figure 4B). This confirms that *in vitro* studies mimic the metabolic response to muscle hypertrophy stimulation. These results align to gene expression outcomes, which also revealed a great overlap between bioengineered muscles compared to acute resistance exercise in humans [37].

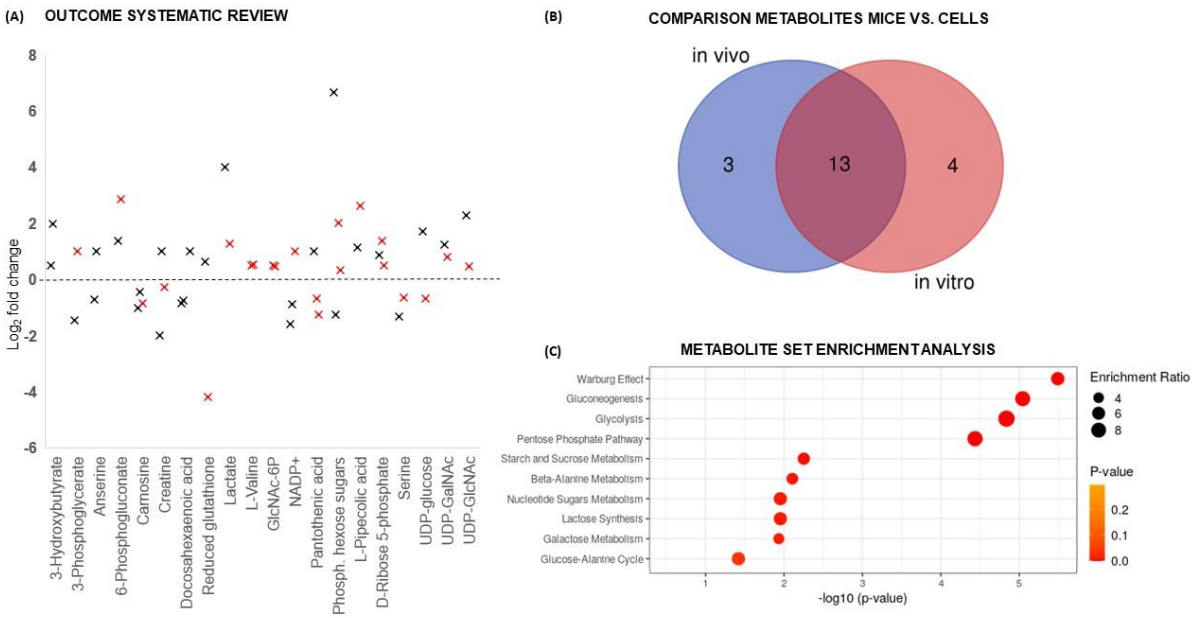

Figure 4 *(A) Metabolic changes in response to resistance exercise (log2 fold change versus rest), black cross – in vivo studies; red cross – in vitro studies. (B) Venn diagram showing overlap and unique metabolites associated with muscle growth between studies performed in mice in vivo (Animals) or in vitro (Cell Culture). (C) Enrichment analysis with MetaboAnalyst 5.0. GlcNAc-6P= N-acetylglucosamine-6-phosphate; NADP+ = oxidized nicotinamide adenine dinucleotide phosphate; UDP = Uridine diphosphate; UDP-GalNAc = UDP-N-acetylgalactosamine.*

Twelve of this 20 metabolites were directly related to glycolysis and to its linked anabolic pathways. Phosphorylated sugars were the most frequently identified metabolites and their concentration was elevated in three of four experiments. Ribose-5-phosphate/ribulose-5-phosphate was also increased 1.5-2.5 fold. Both lactate and valine concentrations were increased up to 16-fold and 1.5-fold during muscle hypertrophy, respectively. Supporting the *in vitro* investigation presented here, serine concentration was decreased also in [16]. Four sugar metabolites associated with *O*-glycosylation were mostly elevated during muscle hypertrophy (Figure 4A).

Metabolic set enrichment analyses of different metabolite concentrations following muscle hypertrophy-inducing treatments revealed a significant increase in the pentose phosphate pathway, glycolysis and gluconeogenesis [in muscle this refers to partial gluconeogenesis in which gluconeogenesis intermediates are produced [38]], all characteristic of the Warburg effect (Figure 4C). These results, similar to the observed changes in our muscle cell *in vitro*

and mice study *in vivo*, indicate that skeletal muscle hypertrophy, like in cancer, causes a metabolic reprogramming to generate biomass.

[U-$^{13}C_6$]Glucose tracer confirms increased amino acid carbon incorporation in IGF-1 stimulated C2C12 myotubes

An observation that we made in our initial studies was that the concentration of glycolytic intermediates changes during muscle hypertrophy. This observation was supported by the metabolic set enrichment analysis of the systematic review that revealed a strong association with the Warburg effect. We next investigated the fate of glucose during skeletal muscle growth. For this, we used stable isotope [U-$^{13}C_6$]glucose tracer to identify, whether stimulation with IGF-1 changes glucose carbon incorporation into the protein biomass of C2C12 myotubes over 24 h. Intermediary metabolism of U-$^{13}C_6$ labelled glucose results in ^{13}C labelled carbon being incorporated into non-essential amino acids. The enrichment of ^{13}C-glucose within the conditioned media, measured in atom percent excess (~23.5 APE), did not differ between the groups at 24 h post-tracer treatment (p=0.95; Figure 5A). However, the IGF-1 treated cells showed significant additional ^{13}C-enrichment in the media lactate and alanine compared to control, suggesting that lactate and alanine was increasingly synthesised from glucose and then released into the media (Figure 5B-D). Glycine (p=0.068; Figure 5D) and proline [multiply labelled proline compounds were observed, reflecting the different routes of carbon into *de novo* proline synthesis; control: 1.40 vs. IGF-1: 3.66 APE regarding M+1 (p=0.016); control: 5.07 vs IGF-1: 6.47 APE regarding M+2 (p=0.002); control: 2.02 vs IGF.1: 2.39 APE regarding M+3 (p=0.108)] were also enriched with ^{13}C in the IGF-1 group within the conditioned media, respectively, reflecting the flux of glucose carbon into the non-essential amino acid pools. The enrichments were slightly higher in the IGF-1 group, possibly reflecting fluxes through de novo synthesis pathways but also inhibition of breakdown. We then estimated the fractional rate of protein synthesis (FSR) with the ^{13}C-enriched alanine that is incorporated into muscle protein, and FSR was elevated by ~13% with IGF-1 treatment (p=0.001; Figure 5E). This indicates that ^{13}C labelled carbon of glucose is increasingly converted to pyruvate, which is then transaminated to form alanine, which is then incorporated into protein.

Inhibition of the key serine synthesis pathway enzyme PHGDH reveal a key regulatory or allowing role of the serine pathway in maintaining muscle size

Our metabolomics *in vitro* study as well as the systematic review revealed a decreased serine concentration in growing muscles and an upregulation of enzymes that are linked to the serine pathway. With stable isotope [U-$^{13}C_6$]Glucose tracer, we also confirmed increased incorporation of glucose-derived carbon into glycine suggesting that there may be increased flux through this pathway during muscle growth. Further, we recently reported that Phgdh knock down is linked to a smaller myotube size [39]. Thus, we next inhibited Phgdh to investigate its role in muscle protein synthesis during muscle cell differentiation. Chemical inhibition of Phgdh with NCT-503 (50 µM) led to delayed decreased protein synthesis by 74.9% measured by puromycin labelling (Figure 5F), which resulted in 23.9% lower total muscle cell protein concentration (Figure 5G). Extracellular lactate dehydrogenase (LDH) activity was not changed thus indicating that the Phgdh inhibition did not cause major cytotoxic effects (Figure 5H).

Collectively, these data suggest that the serine synthesis, pentose phosphate and hexosamine pathways are reprogrammed during skeletal muscle hypertrophy to support biomass production while inhibiting biomass pathways decreases protein synthesis. Skeletal muscle

converts carbon from glucose into non-essential amino acids and the rate of incorporation increases during muscle hypertrophy.

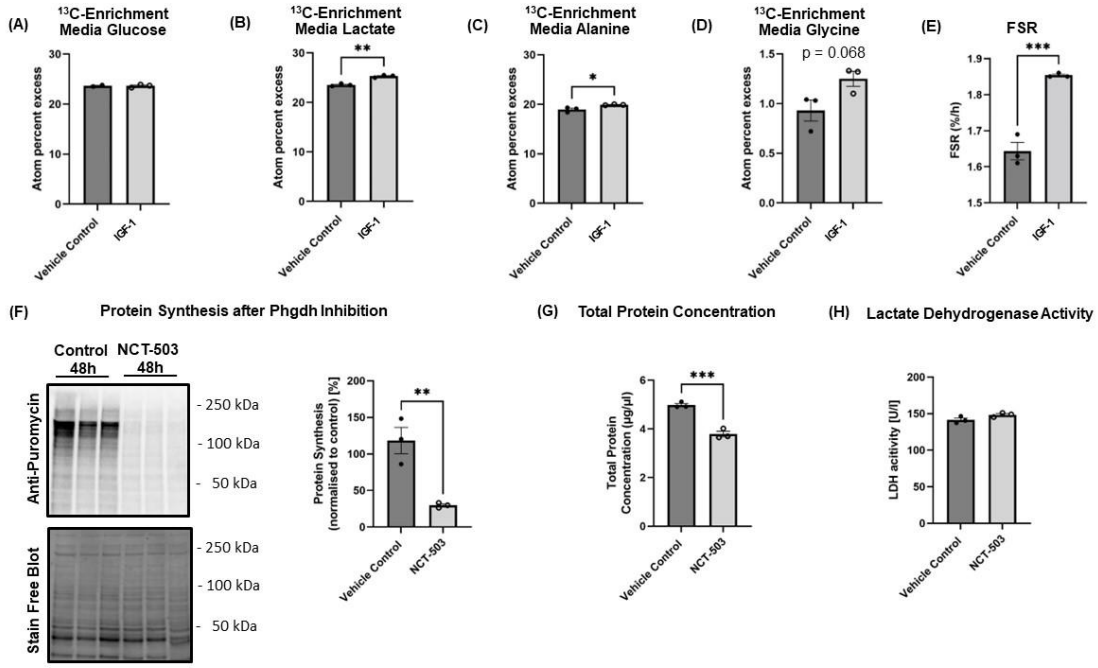

Figure 5 *13C-Enrichment of (A) glucose, (B) lactate, (C) alanine and (D) glycine between IGF-1 and vehicle control in conditioned media after 24 h. (E) Fractional synthesis rate (FSR) based upon ^{13}C-alanine incorporation. (F) Protein synthesis determined using puromycin, (G) total protein concentration of C2C12 myotubes, and (H) lactate dehydrogenase (LDH) activity in conditioned media measured including NCT-503 inhibitor or vehicle control after 48 h. *Significant differences between groups, unpaired two-tailed Student's t-test (p<0.05). Data are expressed as mean ± standard error (SEM).*

DISCUSSION

The major finding of this study is that a hypertrophying skeletal muscle remodels its metabolism so that carbon from glucose increasingly diverts into anabolic pathways for building biomass. This is indicated by altered intermediate metabolite concentrations of anabolic pathways. This was further supported by stable isotope [U-$^{13}C_6$]glucose tracer experiments that demonstrated that labelled carbon from glucose is incorporated into the non-essential amino acid alanine in normal growing myotubes and more labelled alanine is incorporated in hypertrophying myotubes. Further mechanistic analysis revealed that the inhibition of Phgdh supresses muscle protein synthesis, highlighting the importance of the serine synthesis pathway for muscle maintenance and potentially also in the prevention of muscle loss.

Metabolic reprogramming

Of the detected amino acids, only the concentration of serine was lower in hypertrophy-simulated muscle cells. We also found that hypertrophy-stimulated muscles did not show any changes of Phgdh gene expression, the rate-limiting enzyme of the serine synthesis pathway, but had an increased expression of the *Shmt2* gene, an enzyme downstream of Phgdh. The gene encodes the serine hydroxymethyltransferase 2 enzyme that catalyses the reversible reaction of serine to glycine, thus promoting the production of one-carbon units that are indispensable for cell growth [26]. Other studies also showed a reduction of muscle serine during muscle growth over time [16]] and that C2C12 myotubes take a lot of serine from the media and secretes glycine in the media [40]. Therefore, the lower serine concentration in hypertrophying muscles might be due to an increased activity of the serine consuming enzyme serine hydroxymethyltransferase 2 for providing precursors for macromolecules. In support of this, we found -74.9% lower protein synthesis in Phgdh-inhibited myotubes, demonstrating that serine biosynthesis limits protein synthesis.

We estimated with stable-isotope labelled [U-$^{13}C_6$]glucose tracers the fractional synthesis rate of ^{13}C-enriched alanine that is incorporated into muscle protein and the results revealed an increase of 13% in the IGF-1 group compared to control. We also saw a small additional enrichment of carbon from glucose in the detected non-essential amino acids glycine, proline, alanine and especially lactate within the conditioned media, when myotubes were stimulated with IGF-1. This suggests that the rate of incorporation of amino acids into skeletal muscle is greater during muscle growth and that the increased carbon demand for the *de novo* biosynthesis is met by an elevated glycolysis combined with a redirection of glucose intermediates toward anabolic pathways.

Hexosamine biosynthetic pathway

O-glycosylation is a post-translational modification resulting from sugar and it is among the most complex post-translational modifications. Here, we detected via untargeted metabolomics an increased concentration of different activated sugar metabolites in both present *in vivo* and *in vitro* muscle hypertrophy experiments, as well as in the systematic review, suggesting that the formation of UDP-sugars may be demanded for posttranslational protein modification. This increased *O*-glycosylation is linked to the hexosamine biosynthetic pathway and to the most frequent subtypes of *O*-glycosylation (*O*-GalNAcylation and *O*-GlcNAcylation). Previous investigations showed that a specific genetic variations of N-acetylgalactosaminyltransferase-like-6 (GALNTL6) [41] and GALNT13 [42] genes, which encodes enzymes that transfers a GalNAc residue to a serine or threonine residue e.g. on a protein, is associated with power and strength athletes. We specifically found elevated concentrations of UDP-GalNAc in both hypertrophy-stimulated skeletal muscle cells *in vitro* and plantaris muscles *in vivo* showing that O-GalNAcylation is linked to muscle growth. Presumably, the preferential genotypes of these genetic variations elevate the enzyme activity

of the corresponding gene, which facilitates muscle hypertrophy and the development of the extracellular matrix over time. The involvement of *O*-GlcNAcylation was also evident on the gene level by a more subtle but significant increased OGT and decreased OGA expression. In contrast to [23], however, we did not find any significant increase of total muscle *O*-GlcNAc-modified proteins in C2C12 muscle cells *in vitro* and in the *in vivo* overloaded plantaris muscles. The cause underlying this difference is not clear and could indicate that *O*-GlcNAcylation is not unidirectionally changed on a global level during muscle hypertrophy [22], and further mass spectrometry-based approaches are needed to uncover the fine details of cellular glycosylation in future.

Our investigation shed further light on the metabolism during muscle growth. Hypertrophying muscle rewires the metabolism accompanied with metabolic changes in pathways associated with the Warburg effect. The results of our investigation might help to develop effective nutritional and exercise therapies against muscle wasting during ageing and metabolic diseases. Future studies should investigate whether inhibition of other key glycolytic enzymes linked with the Warburg effect will further shed light on the metabolic response to muscle hypertrophy.

Limitations

We demonstrated a regulatory function of the serine pathway in muscle size regulation by inhibiting Phgdh with NCT-503. Further studies should repeat this experiment with several inhibition and activation strategies for other key enzymes of the serine and pentose phosphate pathway. Additionally, our results of the increased Shmt2 gene expression and the relative lower metabolite concentration of serine suggest an increased metabolite flux through the serine pathway. To validate our findings, ^{13}C-glucose metabolic flux analysis could directly assess the metabolic flux in the serine and other pathways. The ^{13}C-enrichment in muscle protein alanine was increased. However, we have only measured FSR, and the enrichment could also reflect inhibition of muscle breakdown.

CONCLUSION

Taken together, our results provide further evidence for metabolic remodelling for *de novo* syntheses of non-essential amino acids like alanine, and also for a role of *O*-glycosylation during muscle hypertrophy. Understanding of the mechanisms regulating skeletal muscle mass can assist in developing treatments for muscle weakness.

MATERIAL AND METHODS

Animals and *in vivo* study design

All procedures involving mice were approved by the Lithuanian State Food and Veterinary Service (Animal ethics number: 2018-06-14 Nr. G2-90). Male mice were used in all experiments. Mice were housed in standard cages, one to three mice per cage at a temperature of 22–24°C and 40–60% humidity as in our previous studies (Ratkevicius et al., 2010; Kilikevicius et al., 2012). Animals were fed standard chow diet and received tap water ad libitum.

Six mice were subjected to muscle overload of the plantaris muscle for 14 days starting at the age of 32 weeks, as described previously [43]. After 14 days, mice were fasted overnight, and they were sacrificed by the exposure to CO_2. Immediately afterwards, plantaris muscle was dissected and in isopentane pre-cooled with liquid nitrogen and stored at − 80 °C until further metabolomics analysis. For a detailed description, see supporting information.

Cell culture and *in vitro* study design

Mouse C2C12 myoblasts cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum. To induce differentiation, the myoblasts were cultured until confluence and then the medium was switched to DMEM with 0.2% FBS. After 72 h of differentiation, C2C12 Myotubes were serum starved (DMEM only) for 4 h and then treated with DMEM including 0.2% dialysed FBS and vehicle control (10 mM HCL, 0.1% bovine serum albumin; BSA), IGF-1 (100 ng/ml; recombinant Human LONG R^3 IGF-1, Sigma-Aldrich, Cat# I1271, MO, USA), rapamycin (10 ng/ml; Calbiochem, Cat#553210, Watford, Hertfordshire, UK) and IGF-1 + rapamycin, for 48 h. For Phgdh inhibition experiments, we treated C2C12 cells for 48 h with chemical PHGDH inhibitor NCT-503 (50 µM; MCE, HY-101966; MedChemExpress) five days after the initiation of differentiation. To measure carbon incorporation in IGF-1 stimulated C2C12 myotubes, 4.5 g/L glucose consisting of ~20% [U-$^{13}C_6$]glucose and of 80% non-labelled glucose was added into glucose-free DMEM supplemented with 2 % horse serum (Sigma-Aldrich, H1138, NY, USA). Cells were incubated for 24 h with this enriched media on day 3 post differentiation after which the media was collected, and cells were harvested in 1 M perchloric acid (PCA) for stable isotope analysis. Please see the Supporting Information for detailed experimental procedures.

RNA isolation, reverse transcription, and quantitative real-time PCR

C2C12 myotubes were washed twice with PBS at room temperature and then lysed in lysis T buffer (Peqlab Biotechnology GmbH, reference number 12-6634-01). Total RNA was then extracted with the peqGOLD Total RNA Kit C-Line (Peqlab Biotechnology GmbH, reference number: 12-6634-01) according to the manufacturer's instructions. For mRNA analysis, we synthesised cDNA using 2 µg of total RNA and cDNA was amplified with a PerfeCTa qPCR fluorescent SYBR Green SuperMix (Quantabio) using real-time quantitative PCR (Rotor-Gene RG 6000— QIAGEN). The primer sequences used for the gene expression are shown in Table S2. Please see the Supporting Information for detailed experimental procedures.

Protein determination and western blot analysis

Cells were lysed on ice in 100 µl of radioimmunoprecipitation assay (RIPA) including 0.1% SDS,0.5 M sodium orthovanadate, 0.5% sodium deoxycholate, 50 mM NaF, 1 mM EDTA,150 mM NaCl, 1%Triton-X 100 and with protease and phosphatase inhibitor cocktail (Peqlab Biotechnology GmbH, Germany). For the Phgdh experiment. protein synthesis in C2C12 myotubes was measured using SUnSET protein synthesis measurement as earlier [44]. Protein quantification by western blot analysis was performed as recently described [45]. Please see the Supporting Information for detailed experimental procedures.

Metabolomics and GC-MS analysis

For untargeted metabolomics, muscle cells and plantaris muscle tissue were prepared based on [46], and a Nexera UHPLC system (Shimadzu, Nakagyo-ki, Kyoto, Japan) coupled with a Q-TOF mass spectrometer (TripleTOF 6600, AB Sciex, Framingham, MA, USA) was used. Separation of the samples was performed using a HILIC UPLC BEH Amide 2.1 × 100, 1.7 µm analytic column (Waters Corp., Milford, MA, USA), and a reversed-phase Kinetex XB-C18, 2.1 × 100, 1.7-µm analytical column (Phenomenex, Torrance, CA, USA), respectively. The ^{13}C labelling of the protein-bound alanine (product) was determined using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) following derivatization as its MCME derivative. The media alanine (precursor) enrichment is determined by gas chromatography mass spectrometry. Please see the Supporting Information for detailed experimental procedures.

Statistical Analysis

Data are expressed as mean ± SEM for the indicated number of observations. Data were assessed for statistical significance by one-way or two-way ANOVA tests, followed by Tukey HSD, or by using a two-tailed unpaired Student's t-test, as appropriate. A p-value of <0.05 was considered statistically significant. The resultant p values for all metabolites in the cell culture study were subsequently adjusted to account for multiple hypotheses testing. The specific statistical tests that were used are indicated in the figure legends. Please see the Supporting Information for detailed experimental procedures.

Systematic Review

This systematic review is registered on PROSPERO (access number: CRD42022318998; (https://www.crd.york.ac.uk/PROSPERO/). One researcher searched in PubMed, Scopus and metabolite using the PICO framework [31] leading to the following search strategy: ((mice or mouse) and (metabolomics or metabolome or metabolomes) and (skeletal muscle)) and ((increase or increased or increases or increasing or increasingly) or (mass gain) or (hypertrophy)) until May 11, 2022. Two researchers then screened the search results. Two researchers individually assessed the quality of each individual study using the 10-item checklist of CAMARADES (Collaborative Approach to Meta Analysis and Review of Animal Experimental Studies) (Table S3). A metabolite set enrichment analysis was performed using the MetaboAnalyst 5.0 web platform [47]. Please see the Supporting Information for detailed experimental procedures.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

ETHICS STATEMENT

All procedures involving mice were approved by the Lithuanian State Food and Veterinary Service (Animal ethics number: 2018-06-14 Nr. G2-90) under consideration of the well-being of the animals and the 3R (replace, reduce, and refine) principle. The manuscript does not contain clinical studies or patient data.

AUTHOR CONTRIBUTION

P.B., H.W., M.S., T.V., H.S., A.R., H.D., and J.H. conceived and designed the research; P.B., S.M., A.S., P.M., and M.D. performed the experiments; P.B., M.S., S.M., K.G., C.M., and M.G. analysed the data; P.B., H.W., M.S., H.D., A.R. and J.H. interpreted the results of the experiments; P.B. prepared the figures; P.B. drafted the manuscript; P.B., H.W., M.S,, S.M., A.S., P.M., M.D., T.V., H.S., H.D., A.R. and J.H. edited and approved the final version of the manuscript.

DATA SHARING

The datasets that support the findings of this study are available as Supplementary Information files.

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