Dose-dependency of a combined EPA:DHA mixture on incorporation, washout, and protein synthesis in C2C12 myotubes

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PII: S0952-3278(24)00045-0
DOI: https://doi.org/10.1016/j.plefa.2024.102651
Reference: YPLEF 102651

To appear in: *Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA)*

Received date: 21 May 2024
Revised date: 12 September 2024
Accepted date: 20 September 2024

Please cite this article as: M. Banic , M. van Dijk , F.J. Dijk , M.J.W. Furber , O.C. Witard , N. Donker , M.J.A. Becker , S.D. Galloway , N. Rodriguez-Sanchez , Dose-dependency of a combined EPA:DHA mixture on incorporation, washout, and protein synthesis in C2C12 myotubes, *Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA)* (2024), doi: https://doi.org/10.1016/j.plefa.2024.102651

Highlights:

- Low and high dose EPA:DHA result in unique incorporation and washout patterns
- High dose of EPA:DHA blunts the formation of DPA
- Only low dose EPA:DHA results in upregulation of MPS above insulin/leucine trigger
- This upregulation of MPS does not increase phosphorylation of mTORC1 proteins

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FUNDING

This study was part funded by Danone Global Research & Innovation Center B.V., Utrecht, the Netherlands.

DATA AVAILIBILITY

Data presented in this study are available on request from the corresponding author upon reasonable request.

DISCLOSURES

The authors have no relevant conflicts of interest to disclose. F.J.D., M.v.D., M.J.W.F, and M.J.A.B., are employees of Danone Global Research & Innovation Center B.V., Utrecht, the Netherlands.

SUMMARY

We demonstrate divergent incorporation and washout patterns for EPA and DHA following high and low-dose EPA+DHA incubation in C2C12 myotubes, with higher concentrations favoring *n*-3 PUFA incorporation. Lower *n*-3 PUFA concentrations increased MPS without further upregulating the mTORC1 signaling pathway. Our study provides novel insights into the temporal incorporation and washout dynamics of EPA and DHA and, specifically, their combined effect on MPS, thereby advancing knowledge regarding dietary *n*-3 PUFA prescription to promote skeletal muscle health in humans.

KEYWORDS

Dose; fish oil; mTORC1 signaling; muscle; n-3 PUFA; time-course

1. INTRODUCTION

A key mechanism that underpins the pathophysiology of muscle atrophy in older adults is anabolic resistance which describes the age associated impaired stimulation of muscle protein synthesis (MPS) in response to anabolic stimuli, namely protein feeding and muscle loading [1]. Emerging evidence exists to suggest a therapeutic role of long chain omega-3 polyunsaturated fatty acids (*n*-3 PUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), in modulating skeletal muscle protein turnover [2]–[5]. For instance, seminal *in vivo* studies have reported chronic (8 weeks) fish oil-derived *n*-3 PUFA supplementation to potentiate rates of MPS and molecular readouts of anabolic cell signaling in response to a hyperinsulinemic / hyperaminoacidemic clamp in young and older adults [4], [5]. The biological actions of *n*-3 PUFA in skeletal muscle are primarily mediated by the incorporation of EPA and DHA into the cellular membranes of skeletal muscle cells, thus altering cell membrane phospholipid composition [6], [7]. Additionally, the ingestion of *n*-3 PUFA leads to the secretion of specialized pro-resolving mediators (SPM), which compete with pro-inflammatory eicosanoids derived from *n*-6 PUFA arachidonic acid [8]. These SPM help mitigate the upregulation of catabolic signaling pathways by resolving inflammation, specifically in critical care settings [9]. However, the optimal dosing strategy for *n*-3 PUFA ingestion to maximize EPA and DHA membrane phospholipid composition and upregulate the stimulation of MPS and associated signaling proteins remains unclear.

The dynamics of EPA and DHA incorporation into blood, adipose tissue and skeletal muscle is dose and time-course dependent, as evidenced by multiple human studies [10]–[12]. Accordingly, high dose (4.4g EPA + DHA) *n*-3 PUFA ingestion over a 4-week period induced a similar increase in muscle EPA and DHA incorporation compared to low dose (3 – 3.36g EPA + DHA) *n*-3 PUFA ingestion over extended time periods (8-12 weeks) in humans [5], [11], [13]. In contrast, only 6 days of high dose (6% of total energy) *n*-3 PUFA ingestion led

to an increased composition of *n*-3 PUFA in the phospholipid membrane composition of muscle tissue [14]. Collectively, these data suggest that higher doses of ingested *n*-3 PUFA lead to a more rapid and pronounced incorporation of EPA and DHA into skeletal muscle tissue. In addition, several *in vitro* studies have assessed changes in *n*-3 PUFA membrane composition in response to EPA or DHA incubation. For example, incubation with 20 µM EPA or DHA modified membrane lipid composition during 2-4 days of differentiation in L6 skeletal muscle cells [15]. Similarly, incubating C2C12 myotubes for 72 h with 50 µM EPA or 50 µM DHA markedly altered membrane *n*-3 PUFA composition, while incubation with EPA also increased docosapentaenoic acid (DPA) content and resulted in an increased protein accretion [16]. However, the time-course of changes in *n*-3 PUFA composition in an *in vitro* model in response to different doses of combined EPA/DHA remains unclear.

The dose and duration of *n*-3 PUFA ingestion may impact the washout of EPA and DHA from the skeletal muscle phospholipid membrane. The washout time course of EPA and DHA after cessation of n-3 PUFA rich fish oil supplementation has been characterized in several blood fractions, including erythrocytes [10], [17], [18], whole blood [18], and plasma [17]–[20] in human studies. However, the washout dynamics of EPA and DHA following cessation of *n*-3 PUFA supplementation has yet to be elucidated in any skeletal muscle model. Given that distinct differences exist in the incorporation rates of EPA and DHA into erythrocytes and skeletal muscle tissue [11], it is plausible that the washout profile of EPA and DHA measured in blood does not translate to skeletal muscle tissue. Hence, it is crucial to investigate washout profiles of EPA and DHA from skeletal muscle.

The incorporation of EPA and DHA into the phospholipid membrane of skeletal muscle cells results in the modulation of muscle protein turnover, as evidenced by findings from *in vivo* human studies [3]–[5] and *in vitro* cell models [16], [21], [22]. While some studies report increased rates of MPS following *n*-3 PUFA supplementation is accompanied by the increased

phosphorylation of associated signaling proteins [4], [5], this finding is not universal [23]. Preliminary evidence in human muscle suggests that changes in skeletal muscle phospholipid membrane composition resulting from the incorporation of EPA and DHA may enhance signaling efficiency [23].

Moreover, there is evidence that EPA and DHA exhibit differential effects on muscle protein turnover. For instance, incubation of C2C12 myotubes with EPA resulted in a 25% greater stimulation of MPS, with no effect of DHA incubation on MPS rates [21]. Nevertheless, both EPA and DHA incubation increased the phosphorylation status of p70S6K1$^{[Thr389]}$. However, Wang et al. [24] observed a more pronounced reduction in protein breakdown with DHA compared to EPA incubation in C2C12 myotubes, as mediated by an upregulation of signaling proteins involved in the PPARγ/NFκB pathway. Hence, these studies indicate distinct roles for EPA and DHA in regulating muscle protein turnover when administered independently in cellular models. However, EPA and DHA are commonly co-administered in commercially available products, which emphasizes the need to understand the dynamics of EPA and DHA uptake into the phospholipid membrane and subsequent effects on muscle protein metabolism when EPA and DHA are administered in combination at different doses. To this end, no *in vitro* experiment has incubated cells with high and low doses of combined EPA and DHA.

Despite accumulating evidence regarding the effects of *n*-3 PUFA ingestion in modulating skeletal muscle protein turnover [2]–[5], the dose and time-course dependent rates of EPA and DHA incorporation and washout in relation to skeletal muscle remain poorly understood. Therefore, the aim of this study was to determine temporal changes in phospholipid membrane incorporation and washout of EPA and DHA, in concert with measured MPS rates, in response to incubation with combined EPA + DHA at high or low concentration in C2C12 myotubes. In addition, we assessed the impact of high and low concentrations of combined

EPA and DHA incubation and washout on the phosphorylation status of associated mTORC1 pathway signaling proteins after a 16 h incorporation period and a 24 h washout period.

2. MATERIAL AND METHODS

2.1 Chemicals and reagents

Fetal bovine serum (FBS; Cat.No: 10270-106) was purchased from ThermoFisher Scientific. Horse serum (HS-hi; Cat.No: VX16050122), phosphate buffered saline (PBS; Cat.No: VX14190169) and Trypsin-EDTA (trypsin; Cat.No: VX25300096) were purchased from Gibco. Penicillin-streptomycin (pen-strep; Cat.No: VX15140130) and Hanks' Balanced Salt Solution (HBSS; Cat.No: VX14175) were purchased from Invitrogen. EPA (≥99%, cis-5,8,11,14,17-Eicosapentaenoic acid, Cat.No: E2011-50MG) and DHA (≥98%, cis-4,7,10,13,16,19-Docohexaenoic acid, Cat.No: D2534-100MG) were purchased from Sigma-Aldrich. Reagents for culture medium (DMEM powder; Cat.No: D5648, $NaHCO_3$), Insulin from bovine pancreas (Cat.No: I6634-100MG), L-Leucine BioUltra (Cat.No: 61819), were purchased from Sigma-Aldrich. Puromycin (Cat.No: 540411-100) was purchased from Merck and anti-puromycin (Cat.No: EQ0001) was purchased from Kerafast. Anti-mouse Detection Module (Cat.No: DM-002), Anti-rabbit Detection module (Cat.No: DM-001), and 12-230 kDa Wes Separation Module (Cat.No: SM-W004) were purchased from Bio-Techne. Anti-4EBP1 (Cat.No: CST9452), anti-phospho-4EBP1 (Thr37/46) (Cat.No: CST2855), anti-p70S6K1 (Cat.No: CST34475), anti-phospho-p70S6K1 (Thr421/Ser424) (Cat.No: CST9204), Akt (pan) (40D4) Mouse mAb (Cat.No: CST2920240), and Phospho-Akt (Ser473) (D9E) XP(R) Rabbit mAb (Cat.No: CST4060P) were purchased from Cell Signaling Technology.

2.2 Cell lines, cell culture and treatment

For all experiments, 1.25×10^5 cells/mL C2C12 myoblasts (Maastricht University) were seeded in 6-well plates in 3 mL DMEM containing 10% FBS and 1% pen-strep and incubated overnight at 37°C and 5% CO_2. Differentiation was induced by changing medium to DMEM with 2% HS-Hi and 1% pen-strep and refreshed every other day. Experimental treatments were

initiated on day 7 of differentiation. Solutions of 100 mM EPA and DHA were prepared by dissolving EPA (≥99%) and DHA (≥98%) in 100% ethanol, and further diluted in PBS + 2.5 % fatty acid free BSA to a final concentration of 10-20 mM. Exact fatty acid concentration was measured and aliquots were stored at -80°C until further use. Cells were treated with EPA:DHA in a 3:2 ratio complexed with 2.5% albumin dissolved in PBS in the following concentrations: high concentration (HC): 50 µM EPA + 33.3 µM DHA or low concentration (LC): 12.5 µM EPA + 8.33 µM DHA. This ratio was chosen based on commercially available products, and previous studies using concentrations of 50 µM EPA or DHA to study rates of muscle protein synthesis [16], [21]. Differentiation medium was used as washout medium, and differentiation medium containing 100% ethanol diluted to the same concentration as HC (1.28%) with PBS + 2.5% albumin was used as a vehicle control (VC). Treatments were freshly prepared on the morning of the experiment by adding stock EPA, DHA, or ethanol-PBS-albumin to the differentiation medium and heated at 37°C.

2.3 Experimental design

C2C12 myotubes were treated with 3 mL of HC, LC, or VC. The incorporation and washout rate of EPA, DHA and DPA were determined in two separate experiments. A third experiment was conducted to assess rates of MPS and the phosphorylation status of associated mTORC1 proteins. To assess the incorporation of EPA, DHA and DPA (**Figure 1A**), myotubes were incubated for 1, 2, 4, 8, 16 or 24 h before phospholipid membrane fractions were collected (as described below). To determine the washout rate (**Figure 1B**), C2C12 myotubes were treated with 3 mL of HC, LC, or VC and incubated 16 h (n=3) or 24 h (n=1). Treatments were then switched to a washout medium, and cells were collected at 0, 2, 4, 8, 16 or 24 h. To assess rates of MPS and the phosphorylation status of mTORC1 associated cell signaling proteins (**Figure 1C**), C2C12 myotubes were incubated for 16 h with HC, LC, or VC, with the addition of an insulin/leucine (I/L) trigger. Treatments were then switched to a washout medium for 24 h.

Measurement of MPS and anabolic cell signaling were determined after the 16 h incorporation period and after a 24 h washout period. A schematic overview of the experimental design is displayed in **Figure 1)**.

2.4 Cell collection and fatty acid analysis

Myotubes were washed twice with cold PBS and then lysed with 200 µL PBS + 1 % Triton X-100 (VWR). Cells were detached and resuspended following 5 min of incubation at 4°C. In total, 150 µL from suspended cell lysates were collected in glass tubes and analyzed by gas chromatography with flame ionization detection (GC-FID). In brief, a known amount of 1,2-dinonadecanoyl-sn-glycero-3-phosphocholine was added as an internal standard to 150 µl for phospholipid analysis. The lipids were extracted according to a modified procedure of Bligh and Dyer (2 mL dichloromethane, 2 mL methanol and 2 mL 1% EDTA solution) [25]. After vortexing and centrifuging at 3000 RPM, the dichloromethane layer containing the lipids was collected in a new glass tube. The phospholipid fraction was separated from the other lipid classes by SPE (Solid Phase Extraction). To convert the fatty acids into fatty acid methyl esters (FAME), 80 µl of concentrated sulphuric acid was added to the extracted phospholipids in 4 mL methanol to yield a 2% concentrated sulphuric acid in methanol solution and heated at 100 °C for 60 minutes [26]. Once cooled, the fatty acid methyl esters were extracted with 2 mL hexane and 0.5 mL 2.5 mol/L sodium hydroxide solution. After vortexing, the upper layer, hexane with FAME, was collected and dried using a SpeedVac®. Dried samples were subsequently dissolved in 80 µl iso-octane and analyzed with an in-house validated analysis method using GC-FID (Shimadzu Corporation, Kyoto, Japan) with a CP-SIL88 for FAME column (60 m × 0.25 mm id. 0.20 µm film thickness; Agilent Technologies, Inc., Santa Clara, CA, USA). Fatty acids were identified based on retention time using reference standard GLC-569B (Nu-Chek Prep,Inc., Elysian, MN, USA). The relative concentration of the identified FAME in the samples was calculated via the peak area and total concentration of fatty acids in the sample was calculated via the internal standard.

2.5 Muscle protein synthesis treatment

Cells were washed with 1 mL pre-warmed HBSS and incubated in DMEM without insulin/leucine for 4 h at 37°C and 5% CO_2. Thereafter, DMEM + 1 mM leucine and 100 nM insulin or control was added and incubated at room temperature for 30 min. DMEM without I/L trigger was employed as a basal control to confirm the trigger worked. Next, 10 µL puromycin for a total concentration of 300 µM was added and incubated for another 30 min. Cells were washed with PBS and homogenized with 250µL cell lysis buffer. Cell lysates were collected and centrifuged at 2000 RPM for 5 min at 4°C. The supernatant was collected for analysis on the Wes™ system.

2.6 Protein Simple Western™ Analysis

Incorporation of puromycin into proteins was measured using Simple Western™ analysis on the Wes™ system (ProteinSimple, a Bio-Techne brand, San Jose, USA). Samples were diluted in 0.1× sample buffer to 0.5 g/L cell lysate and combined with Fluorescent Master Mix in a ratio of 4:1. Samples were vortexed, heated for 5 min at 100 °C and subsequently centrifuged at room temperature for 5 min at 10,000 RPM. Next, 4 µL of sample was loaded onto the WES plate and centrifuged for 5 min at 2500 RPM. The assay was run using the 12-230 kDa Separation Module (ProteinSimple). Anti-puromycin was diluted 5x and loaded on the WES plate and Anti-mouse Detection Module for Wes™ (ProteinSimple) was used as secondary antibody and detection. The run was performed using 30 min separation time, 375 V separation voltage, 30 min antibody diluent time, 30 min primary antibody time and 30 min secondary antibody time. Total area under the curve of puromycin was calculated by the Compass for SW software (ProteinSimple), with ratios to the basal control group calculated as a measure of puromycin incorporation.

2.7 Measurement of mTORC1 pathway proteins 4EBP1, p70S6k, Akt

The phosphorylation status of mTORC1 pathway proteins 4EBP1, p70S6k, and Akt was determined with the Wes™ using the same settings as for MPS (see above). Anti-4EBP1 (10×

diluted) and anti-phospho-4EBP1 (Thr37/46) (10× diluted) antibodies were used for 4EBP1; anti-p70S6k (10× diluted) and anti-phospho-p70S6k (Thr421/Ser424) (10× diluted) antibodies were used for p70S6K1; Akt (pan) (40D4) Mouse mAb and Phospho-Akt (Ser473) (D9E) XP(R) Rabbit mAb were used for Akt. A concentration of 0.313 g/L protein for $4EBP1^{[Thr37/46]}$ and 0.625 g/L protein for $Akt^{[Ser473]}$ and $p70S6K1^{[Thr421/Ser424]}$ were loaded onto the Wes™. The total peak area was calculated by the Compass for SW software (ProteinSimple), with ratios to the basal control group calculated for each antibody. Signaling data were expressed as the ratio of phosphorylated proteins to unphosphorylated proteins. The 12–230 kDa Separation Module (ProteinSimple) was used for all signaling pathway proteins. The Anti-rabbit Detection Module or Anti-mouse Detection Module (ProteinSimple) was used for detection. The total peak area was calculated by the Compass for SW Software.

2.8 Statistical analysis

All statistical analyses were conducted in R (version 4.3.1). An independent samples Student's t-test was used to analyze differences in EPA, DHA and DPA uptake at each timepoint between concentrations. Temporal changes in EPA, DHA and DPA incorporation into C2C12 myotubes, MPS rates and the phosphorylation status of mTORC1 pathway proteins were analyzed using linear mixed-effect models followed by Tukey's post-hoc analysis. Statistical significance was set at $P < 0.05$.

3. RESULTS

3.1 Dose-response of EPA and DHA incorporation into the phospholipid membrane

The proportion of EPA incorporated into the phospholipid membrane was higher in HC compared to LC ($P < 0.05$) at all time points except for 16 h ($P = 0.06$). No changes in EPA incorporation were detected from 8 h onwards after incubation with HC and LC ($P > 0.05$; **Figure 2A**). EPA incorporation increased from 10.3 ± 0.2% of total FA profile at 1 h to 18.1 ± 1.9% at 24 h after incubation with HC, while after incubation with LC, EPA incorporation

increased from 8.3 ± 0.8% of total FA profile at 1 h to 13.0 ± 2.0% at 24 h. The proportion of DHA incorporated into the phospholipid membrane was similar between HC and LC at all timepoints ($P > 0.05$, **Figure 2B**). In contrast to EPA, the incorporation of DHA increased moderately during the 24 h period after incubation with HC and LC and did not reach a plateau. DHA incorporation increased from 3.4 ± 0.6% of total FA profile at 1 h to 14.8 ± 2.8% at 24 h after incubation with HC, and from 2.8 ± 0.6% of total FA profile at 1 h to 10.7 ± 2.6% at 24 h after incubation with LC.

3.2 Dose-response of DPA incorporation into the phospholipid membrane

We assessed temporal changes in DPA expressed as %DPA of total fatty acid profile (**Figure 3**), given that previous research indicated the formation of DPA due to EPA supplementation [16]. We observed no detectable incorporation of DPA after incubation with HC. However, we detected a significant incorporation of DPA into the phospholipid membrane after 24 h incubation with LC ($P = 0.01$) whereby DPA levels increased from 2.5 ± 0.5% of total FA profile at 1 h to 4.3 ± 0.4% at 24 h.

3.3 Washout of EPA and DHA from the phospholipid membrane

After the overnight incubation period, incorporation of EPA was 16.0 ± 0.3% of total FA profile in HC and 11.8 ± 0.3% of total FA profile in LC at the onset of the washout period (t0). Similarly, DHA incorporation was 13.6 ± 0.9% of total FA profile in HC and 8.5 ± 0.6% of total FA profile in LC at the onset of the washout period following overnight incubation. Incubation with HC and LC led to sustained EPA and DHA levels above the vehicle control throughout the 24 h washout period ($P < 0.05$; **Figure 4**). Additionally, HC resulted in consistently higher EPA and DHA incorporation into the phospholipid membrane compared to LC during the 24 h washout period ($P < 0.05$). After 24 h of washout, EPA levels in the phospholipid membrane were 19.0 ± 0.4% greater with HC than LC (**Figure 4A**).Following overnight incubation with HC, EPA levels in the phospholipid membrane decreased after 2 h

when switching to the washout medium ($P = 0.02$) and continued to decrease at 16 h ($P < 0.001$). Likewise, EPA levels after overnight incubation with LC decreased 2 h after switching to washout medium ($P = 0.01$), but with no further decline thereafter ($P > 0.05$). No decline in EPA level was observed between 0-8 h in either condition. The overall decline in EPA over 24 h was 2.8 ± 0.6% after treatment with HC and 0.6 ± 0.3% after treatment with LC. DHA incorporation into the phospholipid membrane was 136.9 ± 0.5% greater with HC than LC after a 24 h washout (**Figure 4B**). Conversely, an increase in DHA incorporation was observed after 8 h when switching to washout medium after overnight incubation with HC ($P < 0.001$), and further increased between 8–16 h ($P < 0.001$). After incubation with LC, no differences in DHA incorporation in the phospholipid membrane were detected from the start of the washout period ($P > 0.05$).

3.4 Formation of DPA during washout in response to EPA:DHA supplementation

We observed an increase in DPA levels between 1–8 h of washout ($P < 0.001$; **Figure 5**) in both concentrations, and an increase in DPA in the vehicle control from 16 h onwards compared to start of washout ($P < 0.001$). DPA incorporation differed at all timepoints between HC and LC ($P < 0.05$). Compared to VC, DPA levels were lower following treatment with HC after 0, 2 and 4 h after washout ($P < 0.05$), with no differences after 8 h ($P > 0.05$). No additional DPA formation compared to VC was observed at any timepoint after incubation with LC ($P > 0.05$).

3.5 Protein synthesis rates in response to an insulin/leucine stimulus

The insulin/leucine stimulated condition significantly upregulated protein synthesis rates and the phosphorylation status of mTORC1 pathway proteins compared to the basal control after the 16 h incorporation and 24 h washout period ($P < 0.05$), with the exception of $4EBP1^{[Thr37/46]}$ phosphorylation after the 24 h washout period, whereby no difference was detected between conditions (**Supplemental Figure 1**). We report no further increase in MPS after 16 h of incubation with HC or LC compared to VC ($P > 0.05$; **Figure 6A**). Moreover, there were no

differences in the phosphorylation status of 4EBP1$^{[Thr37/46]}$, p70S6K1$^{[Thr421/Ser424]}$, or Akt$^{[Ser473]}$ after 16 h of incubation with HC or LC (**Figure 6C, E, G**). The LC condition exhibited a 19.7 ± 1.2% higher MPS rate compared to VC after the 24 h washout period (P = 0.01; **Figure 6B**). However, there were no differences in the phosphorylation status of 4EBP1$^{[Thr37/46]}$, p70S6K1$^{[Thr421/Ser424]}$, or Akt$^{[Ser473]}$ after 24 h washout (**Figure 6D, 6F, 6H**).

4. DISCUSSION

This *in vitro* study examined the incorporation and washout profiles of EPA and DHA in the phospholipid membrane of C2C12 myotubes during incubation with a low or high concentration EPA:DHA mixture, in combination with measurements of stimulated rates of MPS and mTORC1 signaling. The study revealed four main findings. First, the higher concentration EPA:DHA mixture resulted in a greater incorporation of EPA into the phospholipid membrane over the 24 h incubation period in C2C12 myotubes, with no differences in DHA incorporation between HC and LC treatments. Second, EPA and DHA exhibited a distinct temporal pattern of uptake and washout, whereby a plateau in EPA uptake was observed after 8 h of incubation and exhibited a partial washout, whereas DHA uptake increased over the entire 24 h incubation period in both conditions, and during washout in HC. Third, DPA formation was observed after 24 h of incubation in LC, whereas pre-incubation with HC inhibited DPA formation during the washout phase. Finally, incubation with LC followed by a 24 h washout resulted in increased insulin/leucine stimulated MPS rates above VC, albeit independently of changes in the phosphorylation status of 4EBP1$^{[Thr37/46]}$, p70S6K1$^{[Thr421/Ser424]}$, and Akt$^{[Ser473]}$. Taken together, these *in vitro* data indicate a finite effect of EPA:DHA incorporation into the phospholipid membrane in upregulating the stimulation of MPS in C2C12 myotubes.

Our cell culture model revealed a dose-dependent, *n*-3 PUFA-specific, pattern of *n*-3 PUFA uptake into the phospholipid membrane of C2C12 myotubes and indicate that provision

of a high dose EPA:DHA mixture elicits a greater uptake of EPA into the muscle phospholipid membrane, with no apparent dose effect of *n*-3 PUFA provision on DHA uptake into the phospholipid membrane fraction. The differential incorporation and washout profile of *n*-3 PUFA with EPA + DHA incubated was likely mediated, at least in part, by the distinct lipid structures of EPA and DHA and variable distributions within the phospholipid membrane. Consistent with this notion, EPA is preferentially incorporated into phosphatidylcholine species of erythrocytes that are primarily located on the outer segment of the membrane [27]. In contrast, DHA is preferentially incorporated into phosphatidylethanolamine species located on the inner segment of the phospholipid membrane and requires the transport of DHA across the cellular membrane for incorporation [28], [29]. These divergent physical properties of *n*-3 PUFA species may explain the slower incorporation of DHA into the cellular membranes of skeletal muscle cells [29]. Moreover, it is plausible that the distinct structural characteristics of EPA and DHA also play a role in modulating the washout profile of *n*-3 PUFA. Accordingly, multiple human studies have investigated the washout profile of *n*-3 PUFA in erythrocytes [10], [17], [18], whole blood [18], and plasma [17]–[20] and revealed a more rapid washout of EPA compared to DHA. In humans, the skeletal muscle washout profile of EPA and DHA in response to different doses of *n*-3 PUFA are yet to be characterized. However, in the present *in vitro* study we observed a similar washout pattern for EPA between dose conditions. Despite a more pronounced decline in absolute EPA levels, the incorporation of EPA into the phospholipid membrane remained higher in HC compared to LC, indicating that a higher *n*-3 PUFA concentration is required to preserve the EPA content of the skeletal muscle phospholipid membrane.

In contrast to EPA, no decline in DHA was observed during the washout period in the present experiment. In fact, we observed an increase in the muscle phospholipid membrane content of DHA during the washout period in HC. One plausible explanation for this increase

in DHA content during the washout period relates to the observation that EPA undergoes metabolic conversion to DHA [30]. While several studies in humans have reported a limited conversion rate of EPA to DHA in plasma and erythrocyte phospholipids following EPA supplementation [31], [32], a recent tracer study demonstrated an increase in plasma DHA level that was primarily attributed to exogenous EPA supplementation [33]. Moreover, an *in vitro* study demonstrated that incubation of C2C12 myotubes with 50 μM EPA for 72 h failed to elevate DHA levels in the phospholipid membrane [16]. However, it is plausible that the EPA:DHA mixture administered in the present study facilitated an increased DHA content during the washout period by converting a proportion of EPA to DHA. Interestingly, the amount of DHA formed during the washout is not linear to the loss of EPA from the phospholipid membrane observed during the washout period. Hence, it is plausible that the EPA loss from other cellular fractions, rather than the phospholipid membrane, are elongated, desaturated and β-oxidized to DHA, leading to increased DHA being observed in the phospholipid membrane during the washout phase in our study. However, since we did not use compound specific isotope analysis in our experiment, we cannot confirm this hypothesis. Future tracer studies in muscle tissue and C2C12 myotubes are warranted to confirm this notion. Nevertheless, our data indicate that the metabolic interplay between EPA and DHA modulates their incorporation and washout profiles, and this process appears to be influenced by the administered dose of *n*-3 PUFA.

The formation of DPA following EPA administration has previously been demonstrated in C2C12 myotubes [16] and the skeletal muscle tissue of tumor-bearing mice [34]. Consistent with these observations, we observed an increase in DPA formation after 24 h of incubation with LC whereas DPA formation was negligible in HC. Although speculative, we suggest that competition between EPA and DHA in HC could explain this observation, suggesting that the accumulation of DHA impairs the conversion of EPA to DPA [29]. Accordingly, findings from

tracer studies in rodents [35] and humans [33] suggest that DHA accumulation mediates a reduced conversion of EPA to DPA. Moreover, a recent comprehensive study from the same group confirmed that increasing DHA levels impaired elongation of EPA to DPA, as mediated by a negative feedback system via the elongation enzyme ELOVL2 [36]. Taken together, these findings suggest that the higher *n*-3 PUFA concentration provided in our study may have led to competition between EPA and DHA, slowing down the conversion of EPA to DPA and DHA. Moreover, in the present study DPA accumulation was blunted during the initial 4 h period in HC which further supports the hypothesis that higher DHA levels impair the conversion of EPA to DPA.

The greater incorporation of EPA and DHA into the muscle phospholipid membrane with HC did not translate to a further increase in stimulation of MPS beyond VC. Instead, we observed an increased stimulation of MPS after 16 h of incorporation followed by a 24 h washout in the LC condition only. A potential explanation for this observation relates to the observation that the total concentration of EPA + DHA in HC induced cellular stress, as evidenced by visual inspection of protein content. Consequently, this increased stress response may have negated any increased stimulation of MPS. In the present study, we administered a combination of EPA + DHA to simulate the *n*-3 PUFA content of common fish oil supplements administered in human studies [37]. Conversely, previous *in vitro* studies reported an increase in MPS and suppression of muscle protein breakdown [21] or increased protein accretion [16] after incubation with 50 µM EPA, whereas DHA had no effect. Hence, we postulate that the higher dose of DHA in addition to EPA in HC in our study attenuated the independent effect of EPA on muscle protein metabolism and that there may be interplay and/or competition between EPA and DHA in terms of regulating muscle protein metabolism. Moreover, we observed an increased stimulation of MPS after the 24 h washout phase, albeit not in response to the 16 h incorporation phase. Further analysis of our incorporation data revealed that the

compositional profile of EPA + DHA in LC after the 24 h washout period was similar to the 16 h incorporation period, but with a marked rise in DPA levels. Although EPA is commonly regarded as the most bioactive *n*-3 PUFA species in terms of stimulating MPS [37], our data suggest that the presence of DPA in the skeletal muscle membrane in addition to EPA could modulate the stimulation of MPS. Taken together, the greater MPS response compared with VC following the 24 h washout phase with LC incubation may be attributed to factors such as the cumulative quantity of *n*-3 PUFA administered, the potential interaction between EPA and DHA that are likely less pronounced in LC, or the increased formation of DPA, which was markedly higher after LC compared to HC.

The mTORC1 pathway consists of cell signaling proteins known to regulate the stimulation of MPS [38]. Despite observing an increased stimulation of MPS after the 24 h washout period in LC, we failed to detect any changes in the phosphorylation status of 4EBP1$^{[Thr37/46]}$, p70S6K1$^{[Thr421/Ser424]}$, or Akt$^{[Ser473]}$. This disassociation between molecular signals that regulate MPS has also been observed in previous *in vitro* studies [16], [21]. In the present study, a possible explanation for the increased stimulation of MPS in the absence of an upregulation of mTORC1 signaling relates to the notion that incorporation of EPA and DHA after incubation with LC may have accelerated or optimized signaling. Accordingly, we previously demonstrated that a lower and more efficient kinase activity was required to maximize MPS following an 8-week period of fish oil supplementation [23]. These findings indicate that fish oil supplementation results in a shift in kinase signaling and MPS, suggesting that a reduced kinase activity is required to maximize MPS. This observation could explain, at least partially, the disconnect between the phosphorylation status of signaling proteins and MPS rates observed in our C2C12 myotubes, however this hypothesis warrants further investigation.

We acknowledge that static measurements of kinase activity at a single time-point likely resulted in missing some peak readouts of the kinase activity. We measured MPS and associated kinase phosphorylation 30 minutes after exposure to an insulin/leucine trigger, following a 16 h incorporation + 24 h washout period with EPA and DHA. While the time-course of elevated MPS post anabolic trigger has been investigated in humans [39], our reliance on a single time point within our *vitro* model may have limited our potential to capture the optimal kinase signaling window in response to EPA and DHA.

It is important to acknowledge that the observed incorporation and washout profile of EPA and DHA, and subsequent stimulation of MPS and molecular signaling, are confined to a mouse muscle cell model, and may not directly translate to human skeletal muscle. The concentrations of EPA and DHA utilized in *in vitro* studies range between 1-750 μM [40], and the shift in relative fatty acid profile observed in our C2C12 myotubes exceeded those observed in a human population [37]. Moreover, given the current recommendation to consume at least one portion of oily *n*-3 PUFA rich fish per week [41], it is noteworthy that intermittent eating patterns deviate significantly from the practice of constant daily consumption [42], emphasizing the importance of studying the washout profile of *n*-3 PUFA species in human skeletal muscle. Furthermore, the focus of the present study was on MPS rather than muscle protein breakdown. Previous cell-based investigations in C2C12 myotubes have revealed a metabolic action of EPA [16], [21], [24] and DHA [24] in attenuating muscle protein breakdown rates. Taken together in combination with findings from other animal [43], [44] and cell [45] studies, this observation emphasizes a potential role for *n*-3 PUFA in regulating muscle protein breakdown. Future investigations are warranted to investigate the time-dependent uptake and washout dynamics of EPA, DHA, DPA, and related lipid classes in human skeletal muscle. Additionally, it is important to examine the influence of different EPA

+ DHA concentrations on subsequent measurements of MPS and anabolic cell signaling pathways.

In conclusion, our data demonstrate that treatment with higher concentrations of EPA and DHA resulted in a greater incorporation of *n*-3 PUFA into the muscle phospholipid membrane and led to a greater preservation of *n*-3 PUFA following washout, with divergent patterns for EPA and DHA. However, only treatment with the low concentration of EPA and DHA stimulated increased rates of MPS without modulating the phosphorylation status of mTORC1 signaling proteins. The distinct dynamics of EPA and DHA in altering muscle cell lipid composition has the potential to impact physiological outcomes related to muscle protein turnover, with possible clinical implications for the prescription of omega-3 polyunsaturated fatty acid supplementation protocols to promote human health.

ACKNOWLEDGEMENTS

We thank Martin Balvers for analyzing fatty acid composition.

AUTHOR CONTRIBUTIONS

M. Banic: Conceptualization, Validation, Formal analysis, Investigation, Writing – Original Draft, Visualization, Project administration. **M. van Dijk**: Conceptualization, Resources, Writing – Review & Editing, Supervision, Project administration. **F. J. Dijk**: Methodology, Validation, Investigation, Writing – Review & Editing. **M. J. W. Furber**: Conceptualization, Resources, Writing – Review & Editing, Funding acquisition. **O. C. Witard**: Conceptualization, Writing – Review & Editing, Supervision, Funding acquisition. **N. Donker**: Validation, Investigation, Writing – Review & Editing. **M. J. A. Becker**: Validation, Investigation, Writing – Review & Editing. **S. D. Galloway**: Conceptualization, Writing – Review & Editing, Supervision, Funding acquisition. **N. Rodriguez-Sanchez**: Conceptualization, Writing – Review & Editing, Supervision, Funding acquisition.

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FIGURE LEGENDS

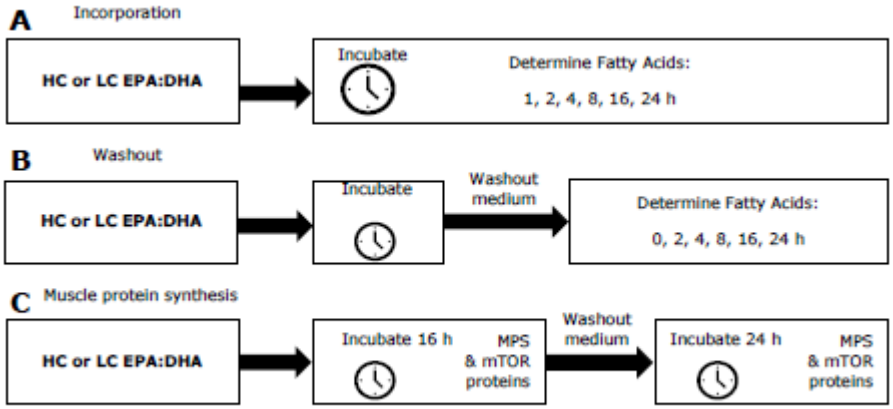

Figure 1). Schematic overview of incorporation (A), washout (B), and muscle protein synthesis experiments (C). HC = high concentration (50 µM EPA + 33.3 µM DHA), LC = low concentration (12.5 µM EPA + 8.33 µM DHA). EPA = Eicosapentaenoic acid, DHA = Docosahexaenoic acid. MPS = muscle protein synthesis. mTORC1 = mammalian target of rapamycin complex 1.

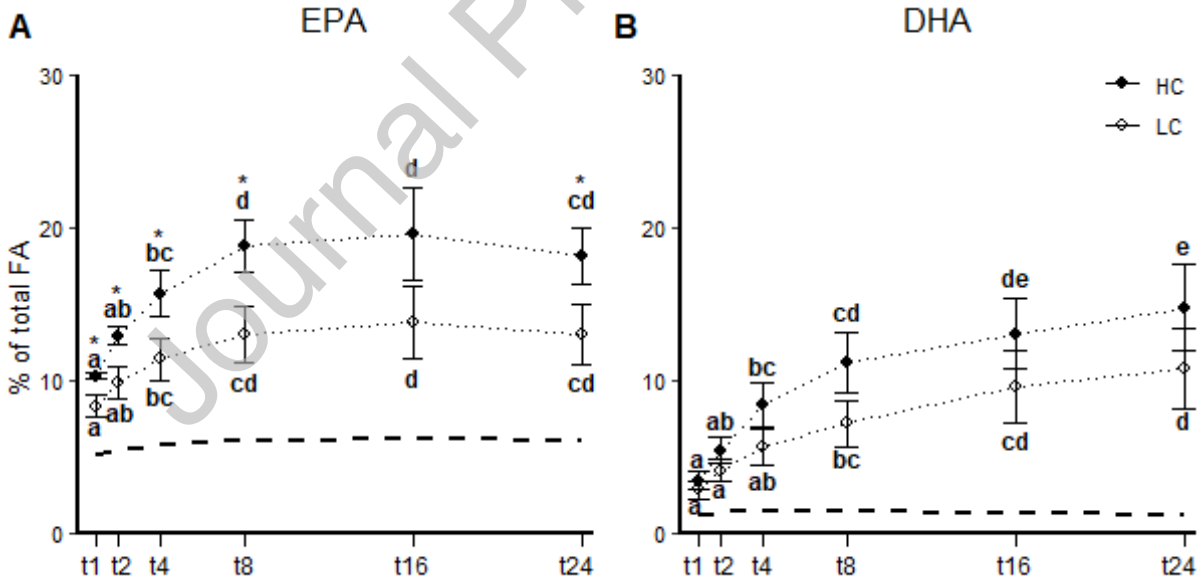

Figure 2). Temporal (24 h) changes in incorporation of EPA (A) and DHA (B) into the phospholipid membrane of C2C12 myotubes following incubation with HC (50 µM EPA + 33.3 µM DHA) or LC (12.5 µM EPA + 8.33 µM DHA) treatments. Values are means ± SD (*n*=3). Dashed line represents vehicle control (VC) over time. Mean values that do not share a

common letter within HC or LC are statistically different from one another (P < 0.05). Significant differences between HC and LC at each timepoint are denoted by an asterisk (*; P < 0.05). EPA = Eicosapentaenoic acid, DHA = Docosahexaenoic acid, HC = high concentration, LC = low concentration.

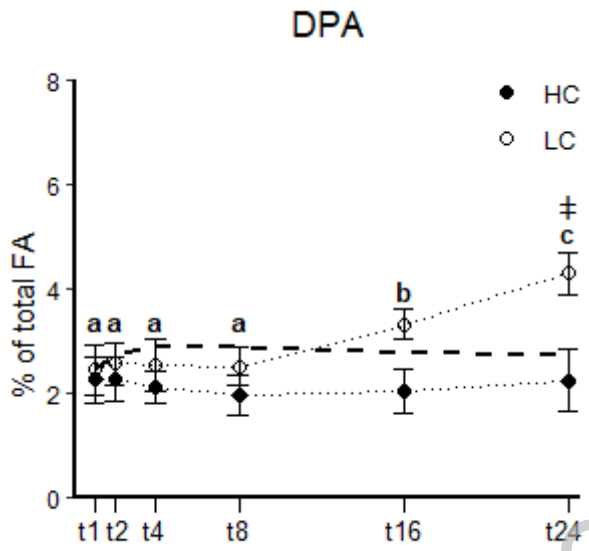

Figure 3). Temporal (24 h) changes in incorporation of DPA into the phospholipid membrane of C2C12 myotubes following incubation with HC (50 µM EPA + 33.33 µM DHA) or LC (12.5 µM EPA + 8.33 µM DHA) treatments. Values are means ± SD (*n*=3). Differences from vehicle control (VC; represented by a dashed line) are denoted by '‡' (P < 0.05). Mean values that do not share a common letter within HC or LC are statistically different from one another (P < 0.05). Levels of DPA did not differ between HC and LC at any timepoint (P > 0.05). DPA = docosapentaenoic acid, HC = high concentration, LC = low concentration, FA = fatty acids.

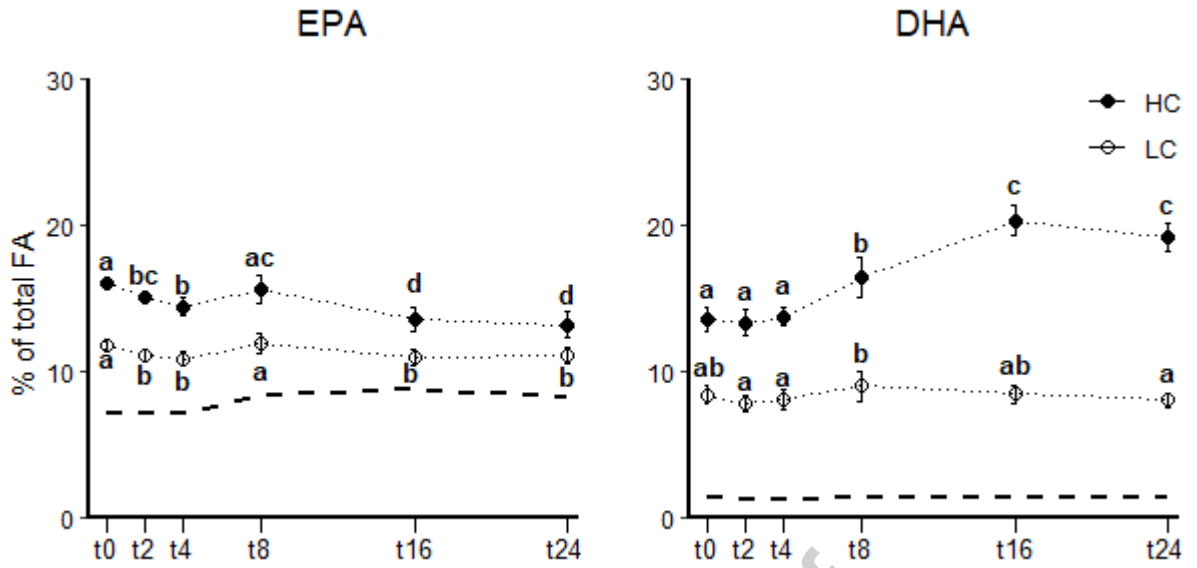

Figure 4). Temporal (24 h) changes in washout of EPA (A) and DHA (B) from the phospholipid membrane of C2C12 myotubes following overnight incubation with HC (50 µM EPA + 33.33 µM DHA) or LC (12.5 µM EPA + 8.33 µM DHA). Values are means ± SD (*n*=4, except t16 HC, where *n*=3). Dashed line represents vehicle control (VC) over time. Mean values that do not share a common letter within HC or LC are statistically different from one another ($P < 0.05$). EPA and DHA levels differed significantly between HC and LC treatments and from VC at all timepoints ($P < 0.05$). EPA = Eicosapentaenoic acid, DHA = Docosahexaenoic acid, HC = high concentration, LC = low concentration, FA = fatty acids.

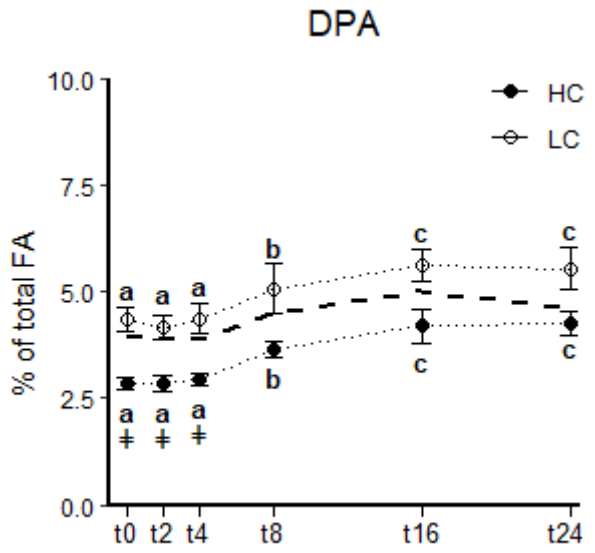

Figure 5). Temporal (24 h) changes in incorporation of DPA during the washout period in the phospholipid membrane of C2C12 myotubes following overnight incubation with HC (50 µM EPA + 33.33 µM DHA) or LC (12.5 µM EPA + 8.33 µM DHA). Values are means ± SD (*n*=4, except t16 HC, where *n*=3). Differences from vehicle control (VC; represented by a dashed line) are denoted by '‡' ($P < 0.05$). Mean values that do not share a common letter within HC or LC are statistically different from one another ($P < 0.05$). Levels of DPA differed at all timepoints between HC and LC ($P < 0.05$). DPA = docosapentaenoic acid, HC = high concentration, LC = low concentration, FA = fatty acids.

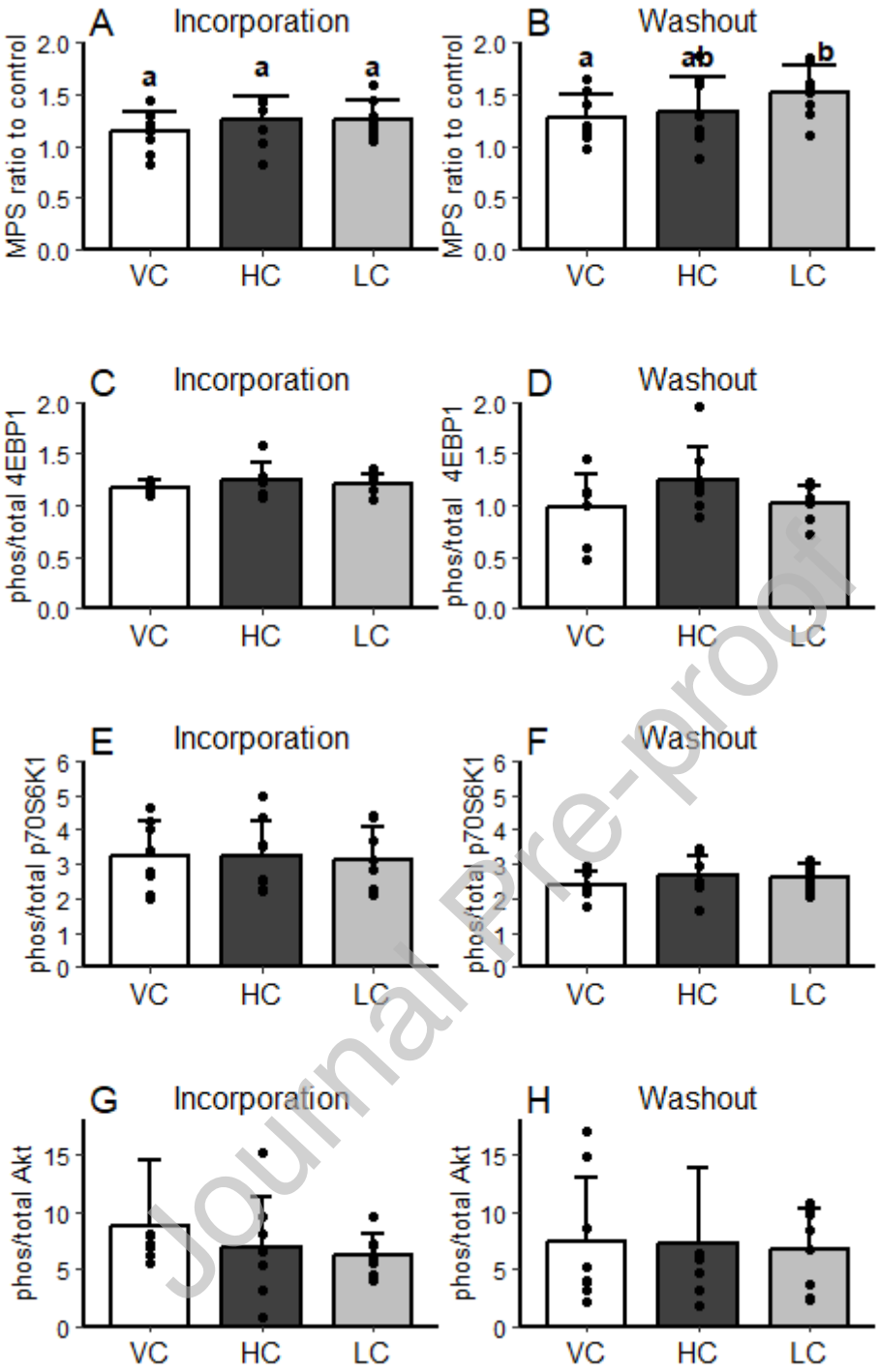

Figure 6). Protein synthesis following 16 h incorporation (A) or 24 h washout (B) and phosphorylation status of signaling pathway proteins following 16 h incorporation (C, E, G) or 24 h washout (D, F, H) in C2C12 myotubes after incubation with VC (vehicle control), HC (50 µM EPA + 33.33 µM DHA) or LC (12.5 µM EPA + 8.33 µM DHA). Data represents protein

synthesis relative to basal control (A, B) and phosphorylated/total 4EBP1$^{[Thr37/46]}$, p70S6K1$^{[Thr421/Ser424]}$, and Akt$^{[Ser473]}$ (C, D, E, F, G, H). Values are means + SD (*n*=4 in duplicate). Mean values that do not share a common letter are statistically different from one another ($P < 0.05$). MPS = Muscle Protein Synthesis. EPA = Eicosapentaenoic acid, DHA = Docosahexaenoic acid, HC = high concentration, LC = low concentration. 4EBP1 = eukaryotic translation initiation factor 4E-binding protein 1. Akt = Protein Kinase B. p70S6K1 = ribosomal protein S6 kinase beta-1.

Supplementary figure 1). Protein synthesis following 16 h incorporation (A) or 24 h washout (B) and phosphorylation status of signaling pathway proteins following 16 h incorporation (C, E, G) or 24 h washout (D, F, H) in C2C12 myotubes after incubation with CON (basal control), IL (1 mM leucine + 100 nM insulin trigger) or VC (vehicle control; IL + 100% ethanol diluted to 1.28% with PBS + 2.5% albumin). Data represents protein synthesis relative to basal control (A, B) and phosphorylated/total 4EBP1$^{[Thr37/46]}$, p70S6K1$^{[Thr421/Ser424]}$, and Akt$^{[Ser473]}$ (C, D, E, F, G, H). Values are means + SD (*n*=4 in duplicate). Mean values that do not share a common letter are statistically different from one another ($P < 0.05$). MPS = Muscle Protein Synthesis. 4EBP1 = eukaryotic translation initiation factor 4E-binding protein 1. Akt = Protein Kinase B. p70S6K1 = ribosomal protein S6 kinase beta-1.

CRediT author statement

- **M. Banic**: Conceptualization, Validation, Formal analysis, Investigation, Writing – Original Draft, Visualization, Project administration.
- **M. van Dijk**: Conceptualization, Resources, Writing – Review & Editing, Supervision, Project administration.
- **F. J. Dijk**: Methodology, Validation, Investigation, Writing – Review & Editing.
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- **N. Donker**: Validation, Investigation, Writing – Review & Editing.
- **M. J. A. Becker**: Validation, Investigation, Writing – Review & Editing.
- **S. D. Galloway**: Conceptualization, Writing – Review & Editing, Supervision, Funding acquisition.
- **N. Rodriguez-Sanchez**: Conceptualization, Writing – Review & Editing, Supervision, Funding acquisition.