OMEGA-3 FATTY ACIDS AND VITAMIN D IN IMMOBILISATION: PART A- MODULATION OF APPENDICULAR MASS CONTENT, COMPOSITION AND STRUCTURE

E.L. BOSTOCK, C.I. MORSE, K. WINWOOD, I.M. MCEWAN, G.L. ONAMBÉLÉ

Health, Exercise and Active Living Research Centre, Department of Exercise & Sport Science, Manchester Metropolitan University, Crewe Green Road, Crewe, CW1 5DU, United Kingdom. Corresponding author: Gladys Onambele-Pearson, Health, Exercise and Active Living Research Centre, Department of Exercise & Sport Science, Manchester Metropolitan University, Crewe Green Road, Crewe, CW1 5DU, United Kingdom. Tel: +44 (0) 161 247 5594; Email: g.pearson@mmu.ac.uk

Abstract: *Objectives:* Muscle size decreases in response to short-term limb immobilisation. This study set out to determine whether two potential protein-sparing modulators (eicosapentaenoic acid and vitamin D) would attenuate immobilisation-induced changes in muscle characteristics. *Design:* The study used a randomised, double-blind, placebo-controlled design. *Setting:* The study took part in a laboratory setting. *Participants:* Twenty-four male and female healthy participants, aged 23.0±5.8 years. *Intervention:* The non-dominant arm was immobilised in a sling for a period of nine waking hours a day over two continuous weeks. Participants were randomly assigned to one of three groups: placebo (n=8, Lecithin, 2400 mg daily), omega-3 (ω-3) fatty acids (n=8, eicosapentaenoic acid (EPA); 1770 mg, and docosahexaenoic acid (DHA); 390 mg, daily) or vitamin D (n=8, 1,000 IU daily). *Measurements:* Muscle and sub-cutaneous adipose thickness (B-mode ultrasonography), body composition (DXA) and arm girth (anthropometry) were measured before immobilisation, immediately on removal of the sling and two weeks after re-mobilisation. *Results:* Muscle thickness (-5.4±4.3%), upper and lower arm girth (-1.3±0.4 and -0.8±0.8%, respectively), lean mass (-3.6±3.7%) and bone mineral content (BMC) (-2.3±1.5%) decreased significantly with limb immobilisation in the placebo group (P<0.05). Despite no significant effect of group, ω-3 and vitamin D supplementation showed trends (p>0.05) towards attenuating the decreases in muscle thickness, upper/lower arm girths and BMC observed in the placebo group. The ω-3 supplementation group demonstrated a non-significant attenuation of the decrease in DXA quantified lean mass observed in the placebo group. Sub-cutaneous adipose thickness increased in the placebo group (P<0.05). ω-3 and vitamin D both blunted this response, with ω-3 having a greater effect (P<0.05). All parameters had returned to baseline values at the re-mobilisation phase of the study. *Conclusion:* Overall, at the current doses, ω-3 and vitamin D supplementation only attenuated one of the changes associated with non-injurious limb immobilisation. These findings would necessitate further research into either a) supplementation linked to injury-induced immobilisation, or b) larger doses of these supplements to confirm/refute the physiological reserve potential of the two supplements.

Key words: Docosahexaenoic acid, eicosapentaenoic acid, immobilisation, lecithin, omega 3, vitamin D.

Introduction

Skeletal muscle undergoes structural adaptations in response to changes in functional demand. Prolonged periods of reduced muscle activity and mechanical loading, e.g. immobilisation, limb-suspension or bed-rest, result in changes in skeletal muscle structure and function, bone mineral density (BMD) and intermuscular adipose content (1-6). Musculoskeletal trauma and sports injuries are often treated with orthopaedic surgery/ limb immobilisation, which inevitably leads to periods of inactivity and disuse. The prognosis of orthopaedic surgery patients is poor especially in the older population with an ~6 to 17% mortality rate within three years of hip and knee joint replacement (7). Exercise could be beneficial in these circumstances, but is not always practical. In addition, there is a notable need to identify non-pharmacological interventions since polypharmacy in itself is conducive to skeletal tissue loss (8).

Eicosapentaenoic acid (EPA) is an n-3 polyunsaturated fatty acid with anti-inflammatory properties, which is synthesised from ingested alpha-linolenic acid or consumed in fish, or in fish oil, such as sardines and cod liver oil. Despite there being no established Dietary Reference Intake for n-3 fatty acids, adequate intake (AI) is set at 1.6 and 1.1 g/day for men and women, respectively (9). There is evidence to suggest that EPA may reduce the pro-inflammatory cytokines associated with muscle damage-induced inflammation (10). Magee et al. (2008) demonstrated in vitro that EPA inhibits the effects of TNF-α by reducing its apoptotic effects and enabling myogenesis (10). It is unclear whether this supplement would have a beneficial effect during immobilisation, where it is generally accepted that there is muscle atrophy (11), which is associated with decreased protein synthesis (12) but scant evidence for increased protein breakdown (13).

Another non-pharmacological agent that may potentially be used against the asthenia and sarcopenia induced through immobilisation is vitamin D. Vitamin D is required to absorb calcium and phosphorus and plays a crucial role in maintaining bone, muscle function, modulation of cell growth, neuromuscular and immune functions, and reduction of

inflammation. The main source of vitamin D is sunlight, with smaller amounts found in certain foods. The Recommended Dietary Allowance for vitamin D is 600 IU/day (14). Vitamin D supplementation is another way of making sure the recommended allowance is achieved. Vitamin D has been shown to have direct effects on muscle (15); however, the exact mechanisms remain unknown. To date, research has indicated an association between genetic variation in the vitamin D receptor gene and muscle strength, fat mass and body mass in premenopausal women (16). Moreover, vitamin D has been reported to impact on both the trans-membranous flows of calcium and phosphate in skeletal muscle, and the synthesis rate of contractile properties (17). Vitamin D supplementation reduced falls by 49% and improved musculoskeletal function in frail elderly women with vitamin D deficiency (18). It remains to be seen whether vitamin D supplementation in healthy persons with no known vitamin D deficiency, would lead to any enhancement in muscle structural and contractile properties in the presence of immobilisation.

In the present study, an arm immobilisation model was chosen as it is relatively less restrictive on daily life and causes fewer burdens on participants. The aim was to determine the role that the two potential protein-sparing modulators (EPA or vitamin D supplementation) may play in attenuating atrophy induced through a model that would emulate relatively short-term decreased local mobility/activity in humans. The differential effect of immobilisation on in vivo muscle (thickness), limb composition (lean mass, bone parameters and fat mass), and anthropometry (arm girth) was systematically monitored. Participants received either omega 3 (ω-3, a fish oil of a complex of EPA and docosahexaenoic acid (DHA)), vitamin D or placebo (Lecithin), hereafter simply referred to as EPA, vitamin D or placebo supplementation. It was hypothesised that muscle thickness, lean mass and arm girth will decrease with limb immobilisation. It was also hypothesised that EPA will be the most effective supplement at minimising these changes, since it is understood to act on the protein synthesis pathways.

Method

Participants

Twenty-four healthy volunteers were recruited from the local university campus. All participants provided written informed consent before taking part in this study, which was approved by the local Ethics Committee of Manchester Metropolitan University. Exclusion criteria were any conditions requiring the use of medication likely to affect muscle function or musculoskeletal health (e.g. statins and oral steroids), and any current or history of kidney/liver disease, as those suffering with such conditions are more susceptible to the side effects of nutritional supplementation. An in-house designed physical activity and general health questionnaire, generally used in our research laboratories for teaching and research purposes, was completed by each participant, to ascertain health, habitual physical exercise levels and supplement history prior to the study. This questionnaire confirmed all participants were recreationally active (defined here as undertaking three hours or less of low-to-moderate intensity exercise per week.), free from recent (last 6 months) upper limb injury, were not currently taking any supplements and had no history of ω-3 or vitamin D supplementation within at least the last year. Participants were randomly assigned to one of three groups: placebo [PLA: n = 8]; EPA [EPA: n = 8]; or vitamin D [Vit-D: n = 8]. For anthropometric measurements (height, weight and BMI) in our three populations, please see the results section.

Study design

The study used a randomised, double-blind, placebo-controlled design. All participants attended a familiarisation session at least one week prior to the first testing session. All testing sessions were completed after an overnight fast. After baseline testing, the non-dominant arm was immobilised in a sling, with the correct sling wearing procedure demonstrated to each participant (Figure 1). Participants were required to wear the sling for nine waking hours a day for two continuous weeks, removal of the sling was permitted only when necessary (e.g. taking a bath/shower, driving, sleeping etc.), minimising any movement medio-laterally at the elbow and shoulder, whilst requiring participants to not contract the upper musculature (including the hands) during the hours of immobilisation. Measures of upper arm muscle and subcutaneous fat thickness, body composition (lean mass, bone parameters and fat mass), and upper and lower arm girth were taken immediately before immobilisation (Pre), on removal of the sling (Post), and two weeks after re-mobilisation (Post2).

The placebo group consumed two 1200 mg capsules of Soya Lecithin (Holland & Barrett, UK) a day, each daily dose typically providing 1464 mg of Phosphatides. The EPA group consumed three softgel of High EPA Formula (MorEPA, Minami Nutrition, UK), with the daily dose providing 1770 mg EPA and 390 mg docosahexenoic acid (DHA). The Vit-D group consumed one softgel of Vitamin D3 (Now Foods, Bloomingdale, U.S.A.), each dose providing 1,000 IU of Vitamin D3. The participants consumed the nutritional supplements during the two weeks of limb immobilisation. Participants were asked to maintain their habitual diet and not to perform any unaccustomed strenuous exercise during the 2 weeks of immobilisation and remobilisation. To monitor this, during the immobilisation period participants completed a 3-day food diary, a daily activity log (including sling-wear hours) and wore a pedometer (Omron Walking style III step counter, Omron Healthcare Co., Ltd, Kyoto, Japan) to record the number of steps taken each day. The food diaries were analysed for macronutrient and micronutrient average intake using Microdiet Plus 1.2 (Microdiet, Downlee Systems Ltd, UK). All participants recorded their daily sling wear hours. In addition, since they were university-based local recruits, they

were in full view of at least one member of the research team and/or other university staff on campus who had been made aware of the study requirement, during their hours on campus. Last but not least, participants were questioned during their final testing session, to confirm their compliance with the protocol.

Muscle and sub-cutaneous adipose thickness measures

All images were recorded after approximately 20 minutes seated rest to avoid fluid shifts that might induce interstitial and/or intracellular changes (19). Images of the muscle and sub-cutaneous adipose tissue of the upper arm were obtained using B-mode ultrasonography (AU5, Esaote, Genoa, Italy). A 7.5-MHz linear phased-array probe (image depth: 37.1-92.8mm) was applied in the sagittal plane with minimal pressure to the tissue area of interest to avoid image distortion. This method has previously been shown to be highly reliable for determining muscle and adipose thickness (20-22). Images were recorded using Adobe Premiere 6.0 (Adobe Systems, USA) and stored for later analysis.

Images were obtained with the participant in an upright, seated position with their arm abducted square to the body (elbow at 180˚) and resting on the ultrasound machine. In the upper arm, the proximal and distal insertions of the biceps and triceps brachii were identified by sonography and marked on the skin. The midpoint (L50) and a third of the distance (L33) from the distal end of the biceps and triceps brachii were identified and marked onto the skin. Upper arm ultrasonography images were collected in the sagittal plane, at both sites on both the biceps and triceps brachii. Muscle thickness was measured as the distance from the top of the superficial muscle aponeurosis to the bone at both sites along the biceps and triceps brachii. Ultrasound assessment of muscle tissue content has previously been validated (23-26). Sub-cutaneous adipose thickness was measured as the distance from the bottom of the epidermis to the top of the superficial muscle aponeurosis in the biceps and triceps at both sites. Each of these distances were measured at three standardised points along the width of the probe to obtain average muscle and sub-cutaneous adipose thicknesses using ImageJ analysis software (ImageJ 1.37, Maryland, USA).

Body composition analysis

Body composition was determined using dual-energy X-ray absorptiometry (DXA) scanner (Hologic Discovery; Vertec Scientific, Reading, Berkshire, UK). Whole body scans were performed lasting approximately seven minutes with a dose-area product (DAP) of 21 cGy*cm^2. The appendicular mass was isolated from the trunk and head using DXA regional computer-generated default lines, with manual adjustment, on the anterior view planogram. Measures of BMD, bone mineral content (BMC), lean mass, fat mass and fat percentage are reported for the immobilised arm only.

Arm girths

Participants assumed a relaxed standing position with arms hanging by the sides and palms facing the hips. A measuring tape was used to measure upper arm girth at the mid-acromial-radial and lower arm girth at a fixed point a third of the way (from the proximal end) along the length of the radiale-stylion. Measurements were repeated three times at each point and average girths were calculated.

Measurement reliability

All protocols were assessed for intra as well as inter-day reliability. This utilised five participants and entailed carrying out measurements three times on day 1, and repeating these on day 2, approximately a week later. Within-day coefficient of variation (CV) of 0.1 %, 0.1 %, 0.4 %, 0.2 %, 0.3 % and 0.3 %, and between-day CVs of 0.4 %, 0.2 %, 0.6 %, 0.2 %, 0.2 % and 0.4 % were yielded for upper arm girth, lower arm girth, biceps muscle thickness, triceps muscle thickness, biceps subcutaneous adipose thickness and triceps subcutaneous adipose thickness, respectively. DXA reliability is reported with a CV of 1.0 %.

Statistical analyses

Data were analysed using IBM SPSS v21 (IBM Inc, USA). The Shapiro-Wilk test revealed some of the data to be non-parametric (upper arm muscle and adipose thickness, upper and lower arm girths). The effect of immobilisation was examined by assessing the changes seen in the PLA group by either repeated measures ANOVA (parametric data) or a Friedman test (non-parametric data). Parametric change relative to baseline values (Pre-to-Post: (Post-Pre)/Pre; and Pre-to-Post2: (Post2-Pre)/Pre) were analysed using a repeated measures ANOVA, with post-hoc Bonferonni corrected 2-tailed t-tests to determine group difference. Non-parametric between group effects on change data were analysed using the Kruskal Wallis test, with post-hoc Mann-Whitney U tests. All data are presented as mean ± standard deviation (SD). Statistical significance was set with alpha at ≤ 0.05.

Results

The non-immobilised limb was also monitored throughout the immobilisation and supplementation phase. When the immobilised limb data was normalised for the non-immobilised limb data there was no effect on the outcome measures. Data for the non-immobilised limb is therefore not reported in this paper.

Homogeneity of sample

There were no significant differences in baseline characteristics between the groups (Table. 1). The groups similarly did not differ in baseline muscle size (e.g. bicep muscle thickness (L50) - PLA: 28.7 ± 7.1 mm; EPA: 31.1 ± 5.4 mm; Vit-D: 32.1 ± 6.5 mm), arm girth (PLA: 28.8 ± 3.0 cm;

EPA: 28.1 ± 4.0 cm; Vit-D: 29.4 ± 3.2 cm), body composition (e.g. lean mass – PLA: 1874.5 ± 595.8 g; EPA: 2361.0 ± 1116.8 g; Vit-D: 2531.1 ± 832.6 g).

Table 1
Baseline characteristics of all participants

	PLA	EPA	Vit-D
Age (years)	26 ± 6.7	19 ± 1.6	23 ± 5.9
Gender			
Males	n = 2	n = 4	n = 3
Females	n = 6	n = 4	n = 5
Height (cm)	168.7 ± 11.1	169.5 ± 12.0	172.2 ± 8.0
Weight (kg)	69.1 ± 14.2	69.6 ± 23.1	75.2 ± 14.5
BMI	24.1 ± 3.7	24.1 ± 5.1	24.4 ± 5.0

Mean values ± SD for baseline characteristics.

Figure 1
Image demonstrating correct sling wear

Front, side and back view demonstrating sling wear. Sling wear was initially demonstrated by the experimenter and on subsequent applications, sling application was self-administered by each participant. The sling was worn for a minimum of nine waking hours a day for 14 days. The elbow joint was positioned at approximately a 90° angle.

Daily physical activity and nutritional intake

No significant differences were observed in calorific intake (PLA: males 3134 ± 247 kcal/day and females 1352 ± 506 kcal/day; EPA: males 1826 ± 780 kcal/day and females 1468 ± 121 kcal/day; Vit-D: males 2658 ± 679 kcal/day and females 1541 ± 431 kcal/day (p>0.05)) or in habitual physical activity (PLA: males 8216 ± 34 steps/day and females 6361 ± 1681 steps/day; EPA: males 9648 ± 2934 steps/day and females 5410 ± 2231 steps/day; Vit-D: males 7795 ± 1050 steps/day and females 5156 ± 1940 steps/day (p>0.05)) between the groups during the course of the immobilisation. This effect was true for the EPA, Vit-D and PLA groups. Further diet composition analyses revealed no group differences in protein (PLA: 1.1 ± 0.3 g·kg-1·bw/day; EPA: 1.0 ± 0.3 g·kg-1·bw/day; Vit-D: 1.0 ± 0.6 g·kg-1·bw/day), carbohydrate (PLA: 3.1 ± 1.5 g·kg-1·bw/day; EPA: 3.1 ± 1.1 g·kg-1·bw/day; Vit-D: 3.0 ± 1.0 g·kg-1·bw/day), fat (PLA: 1.0 ± 0.5 g·kg-1·bw/day; EPA: 1.0 ± 0.5 g·kg-1·bw/day; Vit-D: 1.0 ± 0.3 g·kg-1·bw/day), vitamin D (PLA: 1.7 ± 0.7 ug/day; EPA: 1.6 ± 0.9 ug/day; Vit-D: 2.0 ± 1.6 ug/day) or ω-3 (PLA: 0.37 ± 0.30 g/day; EPA: 0.36 ± 0.19 g/day; Vit-D: 0.36 ± 0.20 g/day) intake over the immobilisation period between the three groups.

Results of the questionnaire revealed that participants walked to and from work, university and/or shopping a minimum of 30 minutes per day. Examination of the activity diaries completed by the participants themselves during the immobilisation period, confirmed that the majority of participants spent at least half an hour outside each day.

Figure 2
Muscle thickness changes in response to immobilisation and supplementation
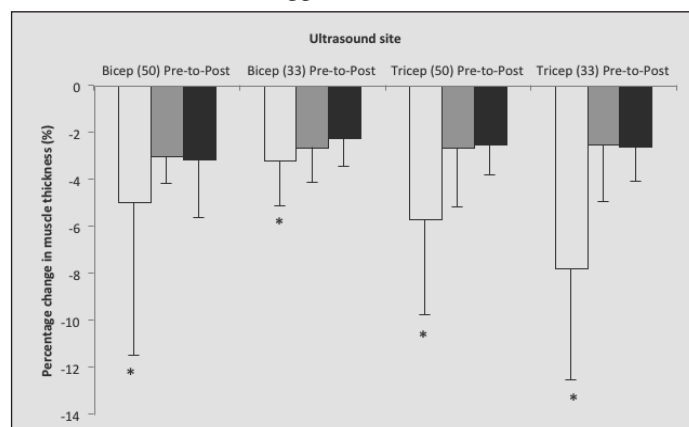
Percentage change (Mean difference (%) ± SD) in muscle thickness from Pre-to-Post for PLA (white bars), EPA (grey bars) and Vit-D (black bars) at the midpoint (L50) and a third of the distance (L33) along the length of the biceps and triceps brachii. * Significant difference between Pre and Post immobilisation in the PLA group.

Muscle and sub-cutaneous adipose thickness measures

Muscle thickness decreased from Pre to Post at both sites (L50 and L33) on the biceps and triceps brachii (p<0.05) in the placebo group. The percentage decrease in muscle thickness was significantly greater in the triceps brachii (L50 & L33) than the biceps brachii (L33) (p<0.05) The percentage change in muscle thickness at both sites on the biceps and triceps brachii did not significantly differ between the groups (Figure. 2), though both EPA and Vit-D groups showed a non-significant trend towards differing from the PLA group. Sub-cutaneous adipose thickness increased significantly post immobilisation at L50 of the biceps brachii (p<0.05), with no significant change at L33 of the biceps brachii or either site on the triceps brachii. The percentage change in adipose thickness was not significantly different between groups at both sites on the triceps brachii (Figure 3). Percentage change in adipose thickness was significantly greater in the PLA than the EPA group (p<0.05) and in the Vit-D than the EPA group (p<0.05) at L50 and L33 along the biceps brachii.

Body composition

There was a significant decrease in lean mass Post immobilisation (p<0.05) but no significant effect of supplement group on the percentage change in lean mass (Figure 4). Percentage changes in BMC, BMD, fat mass and fat percentage

are displayed in Table 2. No significant change was observed in BMD, fat mass or fat percentage. There was a significant decrease in BMC from Pre to Post immobilisation in the PLA group (p<0.05). There was no significant difference in percentage change in BMC, BMD, fat mass or fat percentage between the groups Post or Post2 (p>0.05).

Figure 3
Sub-cutaneous adipose thickness changes in response to immobilisation and supplementation
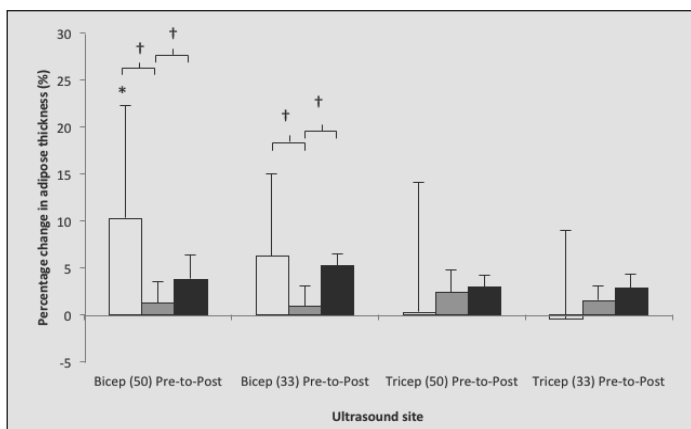
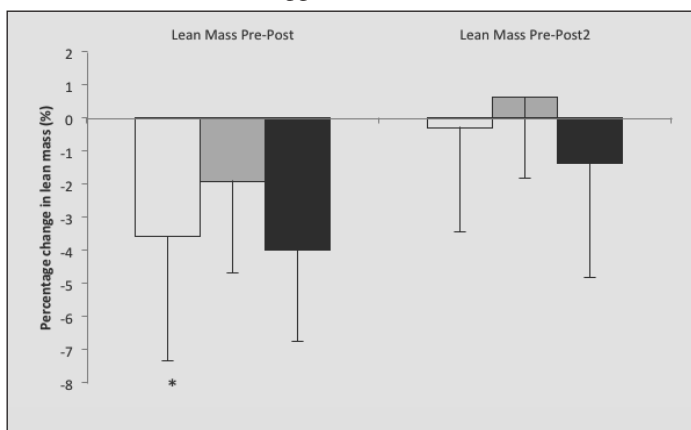

Percentage change (Mean difference (%) ± SD) in adipose thickness from Pre-to-Post for PLA (white bars), EPA (grey bars) and Vit-D (black bars) at the midpoint (L50) and a third of the distance (L33) along the length of the biceps and triceps brachii. * Significant difference between Pre and Post immobilisation in the PLA group. † Significant difference in % change between groups.

Arm girths

Upper and lower arm girths significantly decreased from Pre to Post immobilisation (p<0.05) in the PLA group. There was no significant difference in the percentage change in upper or lower arm girth between groups (Figure 5).
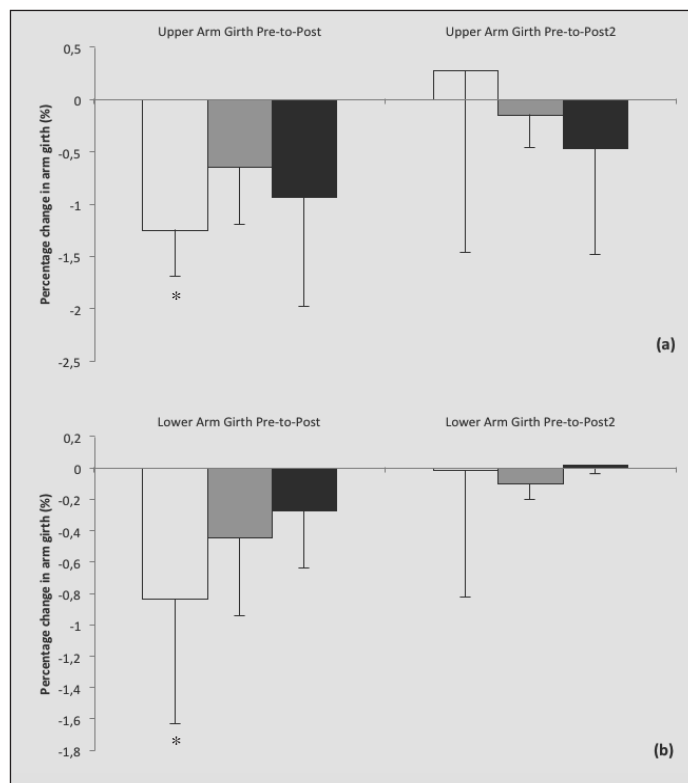
Figure 4
Lean mass changes in response to immobilisation and supplementation

Percentage change (% ± SD) in lean mass from Pre-to-Post and Pre-to-Post2 for PLA (white bars), EPA (grey bars) and Vit-D (black bars). * Significant difference between Pre and Post immobilisation in the PLA group.

Figure 5
Arm girth changes in response to immobilisation and supplementation

Percentage change (% ± SD) in upper (a) and lower (b) arm girth from Pre-to-Post and Pre-to-Post2 for PLA (white bars), EPA (grey bars) and Vit-D (black bars). * Significant difference between Pre and Post immobilisation in the PLA group.

Discussion

The purpose of this study was to determine the role that two potential protein-sparing modulators (EPA or vitamin D supplementation) may play in attenuating the deleterious physiological changes induced through 2 weeks of 9-waking-hours-per-day combined arm and shoulder immobilisation. We hypothesised that muscle thickness, lean mass and arm girth would decrease with limb immobilisation. We found evidence to support this, with significant decreases in muscle thickness (PLA: -3.2 to -7.8% dependent on anatomical site), arm girth (PLA: -0.8 to -1.3% dependent on anatomical site) and lean mass (PLA: -3.6% for the immobilised limb). In addition, we found a significant increase in sub-cutaneous adipose thickness (PLA: 10.3%) and a significant decrease in BMC (PLA: -2.3%). It was also hypothesised that EPA would be the most effective supplement at minimising the effects of immobilisation. In fact, our data show that neither EPA nor vitamin D had any significant effect on the responses to non-injurious immobilisation, other than on the associated accumulation of sub-cutaneous fatty tissue. Nonetheless, a few trends towards attenuations in deleterious physiological events were observed, in the EPA and Vit-D treated groups.

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Table 2
Changes in immobilised limb composition in response to immobilisation and supplementation

	PLA		EPA		Vit-D	
	Pre-to-Post	**Pre-to-Post2**	**Pre-to-Post**	**Pre-to-Post2**	**Pre-to-Post**	**Pre-to-Post2**
BMC	-2.3 ± 1.5 *	-1.5 ± 1.1	-0.3 ± 1.0	0.6 ± 1.9	-0.7 ± 1.9	-0.6 ± 0.7
BMD	-1.6 ± 2.6	0.4 ± 2.2	-0.5 ± 3.2	-0.3 ± 1.1	0.0 ± 1.5	0.5 ± 2.1
Fat mass	2.0 ± 2.4	-1.4 ± 6.7	3.2 ± 3.2	0.2 ± 1.7	2.0 ± 3.5	1.9 ± 1.8
Fat %	2.1 ± 5.9	0.0 ± 5.2	2.6 ± 5.3	-1.2 ± 4.3	3.4 ± 2.6	0.4 ± 3.6

Percentage change (% ± SD) in immobilised limb composition parameters from Pre-to-Post and Pre-to-Post2 for PLA, EPA and Vit-D. * Significant difference between Pre and Post immobilisation in the PLA group.

We discuss the observed trends for the attenuation of some parameters.

A significant change in upper limb muscle thickness is evidenced in our ultrasound data, with a significant decrease in biceps and triceps brachii muscle thickness with immobilisation. The ultrasound data demonstrate a greater decrease in triceps brachii muscle (L50 and L33) thickness than the biceps brachii (L33) in the PLA group. We had, in fact, expected the muscle held in the shortened position (i.e. the biceps) to be impacted on more than the muscle held in the lengthened position (i.e. the triceps); previous research suggests that sarcomeres are added in series when the muscle is immobilised in the lengthened position, and sarcomeres are lost when the muscle is immobilised in the shortened position (27). In the present study, the elbow was immobilised at a 90° angle and, as such, would not have exerted maximal lengthening or shortening on the triceps and biceps brachii. The EPA and Vit-D supplementation groups demonstrated non-significant trends towards smaller decreases in muscle thickness in response to immobilisation, suggesting that EPA and vitamin D may have some role to play in attenuating muscle atrophy associated with disuse. A recent study in an animal model demonstrated distinct effects of EPA and DHA on protein metabolism (protein synthesis and breakdown) with EPA showing a greater ability to result in skeletal muscle protein accretion. Further work in humans is required to further investigate this and the effects EPA may have in human disuse models. Indeed, it is likely that the dose, duration and/or whole body measurement of muscle mass that was adopted in these animal studies diminished the comparability of animal models from our immobilisation model.

Upper and lower arm girths were shown to decrease significantly post immobilisation in the PLA group. We used girth as a gross marker of skeletal muscle atrophy, as previously used by Matsumura et al (28). A decrease in upper arm girth (in the PLA group) suggests a decrease in muscle CSA in the upper arm and this supports our ultrasound data along with previous findings of a decrease in elbow flexor muscle CSA and volume with arm immobilisation (29). The decrease in lower arm girth suggests a decrease in forearm muscle CSA, and this is in line with finding from Miles et

al. who reported a decrease in forearm muscle CSA with 9 days arm casting (30). The significant decrease in limb CSA is reflected in our DXA data, with a significant decrease in lean mass with immobilisation. The decrease in upper arm girth appeared to be attenuated by EPA and vitamin D supplementation, with EPA having the greater effect. Similarly, in the lower arm, supplementation showed a trend towards attenuating the losses in arm girth, this time with vitamin D having a slightly greater effect. The EPA group appear to show a smaller decrease in lean mass than both other groups.

The ultrasound data revealed no significant change in sub-cutaneous adipose thickness in the triceps brachii over the immobilisation period. Biceps sub-cutaneous adipose thickness, however, did significantly increase at the midpoint site in the placebo group. Manini et al. (6) reported a significant increase in intermuscular adipose tissue and no significant change in subcutaneous adipose tissue in response to 4-weeks unilateral lower limb suspension. The difference between the response of sub-cutaneous adipose tissue to disuse in the current study in comparison to Manini et al. may be due to the different techniques used to assess the parameter (i.e. ultrasound vs. magnetic resonance imaging) and/or the differing modes of immobilisation (i.e. arm immobilisation vs. unilateral lower limb suspension). It is possible that the more stringent immobilisation in their study (throughout daily mobile activities, for four weeks), as well as the adiposity site (intermuscular), may account for the fact that they observed significant increases in adiposity. The present study partially agrees with their findings as our data showed a significant increase at one region, subcutaneously, along the upper arm (i.e. 50% of upper limb length). The EPA group demonstrated a significantly smaller increase in biceps sub-cutaneous adipose thickness following immobilisation than both the PLA and Vit-D groups. This effect may, indeed, be meaningful given that diet was monitored and the dietary records demonstrated neither time nor between group differences in total calories, macronutrients or vitamin D intakes, throughout the study. EPA supplementation potential for attenuating increases in sub-cutaneous adipose thickness in response to disuse. The Vit-D group also demonstrated a trend towards an attenuation in the increase of sub-cutaneous adipose thickness in comparison to

the PLA group.

DXA analysis revealed no significant changes in fat mass or fat percentage with limb immobilisation. The difference in the response of adiposity to disuse in the current study in comparison to Manini et al. (6), who reported a significant increase in intermuscular adipose tissue, may be due to the different techniques used to assess the parameter (i.e. DXA vs. magnetic resonance imaging) or the different mode and/ or duration of disuse (i.e. 2 weeks arm immobilisation vs. 4 weeks unilateral lower limb suspension). Interestingly, the increases we reported in sub-cutaneous adipose thickness were not matched by our DXA analysis of fat mass or percentage. Possible explanations for this include: a) the DXA takes the average of the whole limb rather than regional data and hence would have averaged-out the regional changes observed with the ultrasound; b) it is possible that the tissue density changes with immobilisation mean that the DXA may have miss-qualified muscle and fat after immobilisation (31).

We reported no significant change in BMD (-0.8%/week), but a significant decrease in BMC (-1.2%/week) following immobilisation. Decreases in bone are often reported in more severe and longer periods of disuse. For example, Rittweger et al. reported significant decreases in BMC of the tibia (-0.1 to -0.5/week) and radius (-0.03 to -0.05%/week) in response to 90 days bed rest (5). Marchetti et al. found significant decreases in BMD (-1.0 to 2.3%/week) in response to 6 weeks arm immobilisation, however, the participants had also undergone surgery, which could contribute to greater decreases in BMD (32). Values for BMC and BMD changes with immobilisation showed a trend towards attenuation of changes with EPA and vitamin D supplementation. Previous research indicates that BMD is affected by changes in body weight and composition (33), therefore our observed changes in muscle and sub-cutaneous adipose thickness may have influenced our BMD values. The value of BMD can also be limited by the inherent limitations of using a two-dimensional x-ray projection to estimate bone area and geometrical changes. The use of peripheral quantitative tomography (pQCT) may have been more advantageous to examine these specific changes.

A recent study in our own laboratory, found that a lower dose of EPA and DHA than that used in the current study, was not sufficient to decrease inflammation (34). We therefore chose to increase the EPA and DHA of our ω-3 supplement in the current study, in line with previous levels used in the literature (35-37). The vitamin D dose of 1000 IU is equal to 25 ug. Public Health England suggest that adults at risk of vitamin D deficiency should take a daily supplement containing 10 ug of vitamin D (38). Our selected dose is much higher than this and as such should be sufficient in the healthy population used in the current study. The formula of each supplement was chosen so that the dose provided would be able to be bought over the counter and be taken without prescription. The lack of the effectiveness of vitamin D and EPA in the current study may be due to the dosage of the supplements used and therefore this needs to be altered, potentially increased, in future studies.

The NHS consensus on vitamin D states that if people achieve a sufficient supply of vitamin D in the summer, most should keep levels greater than the deficiency threshold of 25nmol/l in winter even without supplements (39). In the current study, participants reported that they typical spent at least 30 minutes outdoors every day of the week and as such would reach the necessary sunlight exposure for adequate vitamin D levels. Research has shown that light of even relatively low intensity (~180 lux) significantly phase-shifts the human circadian rhythm [40]. We therefore suggest that the current population are unlikely to be vitamin D deficient. The 'healthy, presumably non-deficient' status of the participants may have been another reason for the lack of effectiveness of the supplementation in the current study. In a recent study, it was reported that in an elderly population, most participants were unable to meet the recommended daily omega-3 (ω-3) intake without the use of fish oil supplements (41). Future studies need to recruit those lacking in physiological reserve and those more at risk of malnutrition/deficiencies e.g. older and/or injured persons. Future studies also need to assess endocrine markers, including serum EPA & DHA, 25(OH) D3 levels (biomarker for vitamin D status) and parathyroid hormone (PTH) to observe the effects of supplementation.

Blinding of ω-3 supplementation is sometimes an issue in studies due to the fact that it often repeats on participants. On completion of the study, participants were asked whether they were able to identify what supplement they believed they were taking. None of the participants correctly guessed the supplement they were taking and in fact, two participants receiving the placebo supplement believed they were taking an ω-3 supplement.

Conclusion

In accordance with previous research, we demonstrated a decrease in muscle thickness, arm girth and lean mass with short-term upper limb immobilisation. We also observed a significant decrease in BMC, with no observed effect of immobilisation on fat mass or BMD. At the current dosage, neither vitamin D nor EPA supplementation impacted on these parameters at a statistically significant level. Interestingly nonetheless, we have observed that in the case of sub-cutaneous adiposity on the biceps brachii, there is a protective effect of EPA supplementation against limb immobilisation. We propose that the model used in the current study could have relevance to a sporting population in which short-term immobilisation may be prescribed (e.g. treatment for minor injury) or in clinical populations (e.g. injury/surgery induced short-term immobilisation/bed-rest). A short-term model such as this is relatively less invasive and inconvenient for participants and therefore provides a model to be used to assess other supplements and treatments in future studies.

the guidelines in the Declaration of Helsinki and approved by the Ethics Committee of Manchester Metropolitan University.

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