

# **Sprint interval and traditional endurance training increase net intramuscular triglyceride breakdown and expression of perilipin 2 and 5**

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S.O. Shepherd<sup>1,4</sup>, M. Cocks<sup>1,4</sup>, K.D. Tipton<sup>2</sup>, A.M. Ranasinghe<sup>3</sup>, T.A. Barker<sup>3</sup>, J.G. Burniston<sup>4</sup>, A.J.M. Wagenmakers<sup>1,4</sup>, C.S. Shaw<sup>1,5</sup>

<sup>1</sup>*School of Sport & Exercise Sciences, University of Birmingham, Birmingham B15 2TT, UK*

<sup>2</sup>*Exercise and Health Sciences Research Group, University of Stirling, Scotland, UK*

<sup>3</sup>*School of Clinical and Experimental Medicine, Cardiovascular and Respiratory Sciences, University of Birmingham, UK*

<sup>4</sup>*Current address: Research Institute for Sport & Exercise Sciences, Liverpool John Moores University, Tom Reilly Building, Byrom Street Campus, Liverpool L3 3AF, UK*

<sup>5</sup>*Current address: Institute of Sport, Exercise & Active Living, Victoria University, Melbourne, Australia*

## **Address for correspondence:**

Professor Anton JM Wagenmakers

Research Institute for Sport & Exercise Sciences

Liverpool John Moores University

Tom Reilly Building, Byrom Street Campus

Liverpool L3 3AF, UK

Email: a.j.wagenmakers@ljmu.ac.uk

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**Non-technical summary:**

- Increases in aerobic capacity and intramuscular triglyceride (IMTG) utilization are well-described adaptations to endurance training (ET) and contribute to improvements in insulin sensitivity.
- Sprint interval training (SIT) also improves aerobic capacity and insulin sensitivity with a lower time commitment than ET.
- This study aimed to determine whether SIT also induces improvements in insulin sensitivity and net IMTG breakdown, and to investigate the underlying mechanisms.
- Six weeks of ET and SIT increased net IMTG breakdown during moderate-intensity cycling and improved insulin sensitivity. A greater concentration of lipid droplet-associated proteins, perilipin 2 and perilipin 5, was observed following both training modes and contributes to the increases in net IMTG breakdown following training.
- The results suggest a novel mechanism for the training-induced improvements in IMTG breakdown and insulin sensitivity, and clearly demonstrate that SIT is an alternative, time-efficient training strategy that induces similar beneficial metabolic adaptations.

## Abstract

Intramuscular triglyceride (IMTG) utilization is enhanced by endurance training (ET) and is linked to improved insulin sensitivity. This study first investigated the hypothesis that ET-induced increases in net IMTG breakdown and insulin sensitivity are related to increased expression of perilipin 2 (PLIN2) and perilipin 5 (PLIN5). Secondly, we hypothesized that sprint interval training (SIT) also promotes increases in IMTG utilization and insulin sensitivity. Sixteen sedentary males performed 6 weeks of either SIT (4-6, 30s Wingate tests per session, 3d.week<sup>-1</sup>) or ET (40-60 min moderate-intensity cycling, 5d.week<sup>-1</sup>). Training increased resting IMTG content (SIT 1.7-fold, ET 2.4-fold;  $P<0.05$ ) concomitant with parallel increases in PLIN2 (SIT 2.3-fold, ET 2.8-fold;  $P<0.01$ ) and PLIN5 expression (SIT 2.2-fold, ET 3.1-fold;  $P<0.01$ ). Pre-training, 60 min cycling at ~65% pre-training  $\text{VO}_{2\text{peak}}$  decreased IMTG content in type I fibres (SIT  $17\pm10\%$ , ET  $15\pm12\%$ ;  $P<0.05$ ). Following training, a significantly greater breakdown of IMTG in type I fibres occurred during exercise (SIT  $27\pm13\%$ , ET  $43\pm6\%$ ;  $P<0.05$ ), with preferential breakdown of PLIN2- and particularly PLIN5-associated lipid droplets. Training increased the Matsuda insulin sensitivity index (SIT  $56\pm15\%$ , ET  $29\pm12\%$ , main effect  $P<0.05$ ). No training x group interactions were observed for any variables. In conclusion, SIT and ET both increase net IMTG breakdown during exercise and similar increases in PLIN2 and PLIN5 protein expression. The data are consistent with the hypothesis that increases in PLIN2 and PLIN5 are related to the mechanisms that promote increased IMTG utilization during exercise and improvements in insulin sensitivity following 6 weeks SIT and ET.

## Abbreviations list:

ATGL, adipose triglyceride lipase; CGI-58, comparative gene identification-58; CHO, Chinese hamster ovary; DAG, diacylglycerol; DXA, Dual energy X-ray Absorptiometry; ET, endurance training; SIT, sprint interval training; HSL, hormone-sensitive lipase; IMTG, intramuscular triglyceride; LD, lipid droplet; OGTT, oral glucose tolerance test; PLIN2, perilipin 2; PLIN5, perilipin 5.

## Introduction

High intramuscular triglyceride (IMTG) concentrations are associated with insulin resistance in sedentary obese individuals and type 2 diabetes patients (Phillips *et al.*, 1996; Pan *et al.*, 1997; Goodpaster *et al.*, 2001). However, the athletes' paradox describes a state of elevated IMTG storage alongside high levels of insulin sensitivity in endurance trained athletes (Goodpaster *et al.*, 2001; van Loon *et al.*, 2004). Endurance training also increases oxidative capacity and promotes a shift towards greater IMTG utilization during exercise (Schrauwen *et al.*, 2002). Therefore, the capacity to oxidise IMTG as a fuel source is believed to be mechanistically important for the preservation of high insulin sensitivity in the face of elevated intramuscular lipid storage (Goodpaster *et al.*, 2001; Bruce *et al.*, 2003; van Loon & Goodpaster, 2006). The overriding current hypothesis is that high rates of IMTG oxidation during exercise allows the regular turnover of the intramuscular lipid pool and prevents the accumulation of fatty acid metabolites, such as long-chain acyl-CoA, diacylglycerol and ceramides, which are believed to blunt insulin sensitivity (van Loon & Goodpaster, 2006; Moro *et al.*, 2008; Shaw *et al.*, 2010). Therefore, understanding the mechanisms regulating IMTG lipolysis and IMTG-derived FA oxidation during exercise remain important.

Recent attention has focused on the role of the perilipins (PLINs), a family of lipid droplet (LD) proteins, of which perilipin 2 (PLIN2) and 5 (PLIN5) are expressed in skeletal muscle, but whose precise role is not fully understood. PLIN2 is ubiquitously expressed in the human body (Brasaemle *et al.*, 1997) and its content is ~2-fold higher in type I muscle fibres compared to type II fibres, which mirrors the fibre type distribution of IMTG (Shaw *et al.*, 2009). In non-muscle cells, PLIN2 expression regulates basal lipolytic rates *in vitro* by limiting the interaction of adipose triglyceride lipase (ATGL) with the LD (Listenberger *et al.*, 2007; Bell *et al.*, 2008). However, in intact rat skeletal muscle in response to lipolytic stimuli (electrically induced contractions and adrenaline) there is an increase in colocalisation of hormone sensitive lipase (HSL) with PLIN2 and of HSL with LDs (Prats *et al.*, 2006). Furthermore, we recently reported that PLIN2-containing LDs are depleted in human skeletal muscle during moderate intensity exercise (Shepherd *et al.*, 2012). PLIN5, on the other hand, is highly expressed in oxidative tissues (Wolins *et al.*, 2006a; Yamaguchi *et al.*, 2006; Dalen *et al.*, 2007). Cell culture studies demonstrate that PLIN5 expression limits basal lipolysis more effectively than PLIN2 (Wang *et al.*, 2011a). Interestingly, these studies also show that PLIN5 expression (but not PLIN2) recruits ATGL and its coactivator, CGI-58, to the LD surface under basal conditions, and it is believed that PLIN5 phosphorylation releases CGI-58 to bind ATGL to stimulate lipolysis in response to PKA-activation (Wang *et al.*, 2011a). Skeletal muscle PLIN5 expression is higher in athletes (Amati *et al.*, 2011) and increases following training in lean and obese individuals (Peters *et al.*, 2012). Collectively, the results from

the limited number of studies so far suggest that both PLIN2 and PLIN5 are important in regulating IMTG lipolytic rates, but that PLIN5 may be the key PLIN regulating IMTG lipolysis and net IMTG breakdown during exercise.

Sprint interval training (SIT) promotes skeletal muscle adaptations and improvements in insulin sensitivity comparable to those induced by ET (Burgomaster *et al.*, 2008; Gibala *et al.*, 2012). In particular, skeletal muscle oxidative capacity is enhanced following SIT (Burgomaster *et al.*, 2005; Burgomaster *et al.*, 2007; Burgomaster *et al.*, 2008; Little *et al.*, 2010; Hood *et al.*, 2011; Little *et al.*, 2011), and may partly explain the SIT-induced improvements in whole-body insulin sensitivity (Babraj *et al.*, 2009; Richards *et al.*, 2010; Hood *et al.*, 2011; Little *et al.*, 2011). However, it has yet to be investigated whether such improvements in insulin sensitivity occur alongside increases in net IMTG breakdown during exercise and enhanced skeletal muscle expression of PLIN2 and PLIN5.

Given the importance of studying IMTG metabolism in a fibre type specific manner (van Loon, 2004), in the current study we investigated IMTG metabolism and the content and localization of PLIN2 and PLIN5 following different training interventions using immunofluorescence microscopy. We hypothesized that increased expression of PLIN2 and PLIN5 plays a role in the well-established increase in net IMTG breakdown during exercise following ET. In addition, we investigated the hypothesis that SIT also increases the capacity to breakdown IMTG during moderate intensity exercise, the protein expression of PLIN2 and PLIN5 and insulin sensitivity.

## **Method**

### ***Ethical approval***

Sixteen healthy, sedentary males volunteered to take part in the study (for characteristics see Table 1), which was approved by the Black Country NHS Research Ethics Committee (West Midlands, UK) and conformed with the Declaration of Helsinki. Written, informed consent was obtained from volunteers following a verbal and written explanation of the nature and risks involved in the experimental procedure. All subjects were healthy (free of any known metabolic or cardiovascular disease) and engaged in less than two 30 min sessions of physical activity per week in the preceding year. Percutaneous muscle biopsies taken in this study before and after each training mode, under basal conditions and after 60 min of moderate-intensity exercise have been used both for the measurements described in this manuscript and for measurements of endothelial eNOS content, eNOS serine<sup>1177</sup> phosphorylation, NOX2 content and capillarization. The latter are reported in a parallel manuscript (M. Cocks *et al.*, under review). Measures such as insulin sensitivity and  $\text{VO}_{2\text{ peak}}$  also made in the volunteers are relevant for the interpretation of both studies and are presented in both manuscripts.

### ***Pre-experimental procedures***

At least 5 days prior to entering the study subjects performed a progressive exercise test to exhaustion on an electronically braked cycle ergometer (Lode BV, Groningen, The Netherlands) in order to determine peak oxygen uptake ( $\text{VO}_{2\text{ peak}}$ ) using an on-line gas collection system (Oxycon Pro, Jaeger, Germany). The test consisted of initially cycling at 95 W, followed by sequential increments of 35 W every 3 minutes until cadence was reduced to < 50rpm, at which point the test was terminated.  $\text{VO}_{2\text{ peak}}$  was taken as the highest value obtained in the last 30-seconds of the test. During this visit subjects were also allocated to their training intervention. The 16 subjects were divided into pairs, with the best possible match for age, BMI and  $\text{VO}_{2\text{ peak}}$ , with one member from each pair randomly assigned to SIT and the other to the ET group. As none of the volunteers were familiar with SIT exercise, the subjects in the SIT group were familiarised to this exercise mode through the performance of one 30 s all out effort (Wingate test, (Bar-Or, 1987)) on a cycle ergometer against a load equivalent to 0.075 kg per kg body mass.

### ***Experimental procedures***

Experimental procedures performed before (pre) and after (post) training were identical, and were undertaken across two consecutive days. Post-training experimental procedures were performed > 48h after the final exercise training session in order to minimize any acute effects of the last exercise training bout.

*Day 1:* All subjects reported to the laboratory after an overnight fast (> 10 h), having been instructed to refrain from performing vigorous exercise in the preceding 48 h period. On arrival, a 20G cannula was inserted into the antecubital vein of one arm and a 3-way stop cock attached to allow for multiple blood sampling and flushing of the cannula. After a 20 mL baseline sample was obtained, subjects consumed a beverage containing 75 g glucose (using 82.5g dextrose monohydrate obtained from Roquette, UK) dissolved in water made up to 300 ml. Further 5 mL blood samples were obtained after 30, 60, 90 and 120 minutes, and collected into EDTA-containing vacutainers. Isotonic saline (3 mL) (B Braun, UK) was used to keep the cannula patent every 15 minutes during the 2 h test. Plasma samples for each time point were obtained through centrifugation (10-minutes at 3500 rpm at 4°C) and stored at -80°C for subsequent analysis. Immediately following the oral glucose tolerance test (OGTT), body composition analysis was performed using Dual energy X-ray Absorptiometry (DXA). QDR software (Hologic Inc., MA, US) was used to analyse fat mass and fat-free mass in individual limbs and the trunk region of each subject. Relative fat mass was calculated using absolute fat mass as a proportion of total region mass.

*Day 2:* On arrival at the laboratory after an overnight fast (> 10 h), the thigh of one leg was prepared for muscle biopsy collection. Subjects were randomised as to which leg they received the biopsy from pre-training; the contralateral leg was sampled post-training. Briefly, local anaesthetic (1% lidocaine, B Braun, UK) was administered under the skin and over the fascia of the vastus lateralis before two incisions were made approximately 2 cm apart. A muscle biopsy (~100mg) was taken using the Bergström technique (Bergström, 1975) from the distal incision prior to exercise. Skeletal muscle samples were first blotted to remove excess blood, and visible fat and collagen were removed through dissection. A portion of the muscle tissue was then prepared for immunohistochemical analysis by embedding in Tissue-Tek OCT Compound (Sakura Finetek Europe, The Netherlands) and frozen in liquid nitrogen-cooled isopentane. The remaining muscle tissue (~40mg) was snap frozen in liquid nitrogen. Subsequently, subjects underwent 60 min of steady state exercise on an electronically braked cycle ergometer set at a workload equivalent to 65% pre-training  $\text{VO}_{2\text{ peak}}$ . Heart rate and rate of perceived exertion (RPE) were recorded every 5 minutes, while gas collection (5 min collection period) was performed at 15 minute intervals ( $t = 15, 30, 45, 60$  min) using an on-line gas collection system (Oxycon Pro, Jaeger, Germany), in order to determine rates of carbohydrate and fat oxidation. A second muscle biopsy was taken immediately post-exercise from the proximal incision.

For all experimental trials, subjects were provided with all food and drinks to be consumed in the preceding 24 h period. The diet was of a standard macronutrient composition (50% carbohydrate, 35% fat, 15% protein), but adjusted for each individuals habitual energy intake, assessed through the completion of 3 day diet diary. When completing diet diaries, subjects were provided with electronic scales in order to increase the accuracy of portion size determination. All subjects were asked to continue their habitual dietary and physical activity patterns during the training period, again completing a 3 day diet diary post-training in order to assess any differences in energy or macronutrient intake over the training period.

### ***Training interventions***

*Sprint Interval Training (SIT):* The SIT training protocol was similar to the protocol used by Burgomaster et al (Burgomaster *et al.*, 2008). Subjects performed 30 s 'all out' sprints (Wingate test) on a cycle ergometer against a load equivalent to 0.075 kg per kg body mass and with 4.5 minutes recovery between each test. During the recovery period subjects cycled against a small load (30 W) maintaining a cadence of below 50 rpm. All participants trained 3 times a week for 6 weeks, and were excluded from the study if they were absent from more than 2 sessions. Initially participants performed 4 Wingate tests per training session, increasing to 5 tests in weeks 3 and 4, and finishing with 6 tests per session in weeks 5 and 6.

*Endurance Training (ET):* Subjects in the ET group trained 5 times a week over the 6 week training period, and were excluded from the study if they were absent from more than 3 training sessions. All subjects cycled at a workload equivalent to ~65%  $\text{VO}_{2\text{ peak}}$  for 40 minutes in the first 2 weeks, increasing to 50 minutes in the following 2 weeks, and 60 minutes in the final 2 weeks.  $\text{VO}_{2\text{ peak}}$  was reassessed after 3 weeks of training and workload adjusted accordingly.

### ***Muscle sample analysis***

*Immunofluorescence staining:* Serial 5  $\mu\text{m}$  sections were cut at  $-30^{\circ}\text{C}$  on to ethanol-cleaned glass slides. Sections were fixed in 3.7% formaldehyde for 1 h, rinsed briefly in deionised water, and permeabilised in 0.5% Triton-X 100 for 5 min, followed by 3 x 5 min washes in PBS. Subsequently, slides underwent incubation with appropriate antibodies for 45 min, after which, a further 3 x 5 min PBS wash preceded incubation of sections with appropriately targeted secondary fluorescent conjugated antibodies for 30 min. IMTG (lipid droplets) were visualised using neutral lipid dye oil red O staining (Koopman *et al.*, 2001). As such, oil red O solution was applied to washed (3 x 5min PBS) sections for 30 min following secondary antibody incubation. Slides were then rinsed briefly in deionised water, followed by a 10 min rinse with slow



running tap water, after which coverslips were mounted with a glycerol and mowiol 4-88 solution in 0.2 M Tris buffer (pH 8.5) (including 0.1% DABCO anti-fade medium).

Cell border visualisation was achieved through incubation of sections with a wheat germ agglutinin Alexa Fluor 350 conjugate (Cat. #W11263, Invitrogen, Paisley, UK). Muscle fibre type was determined using a mouse anti-myosin antibody for slow twitch fibres (A4.840-c, DSHB, developed by Dr. Blau) followed by the application of Alexa Fluor goat anti-mouse IgM 488 or 350. Fibres containing positive staining were classified as type I fibres, while those with no staining were classified as type II fibres. The oil red O working solution was freshly produced for each staining procedure and consisted of 100 mg oil red O in 20 ml 60% triethylphosphate (Sigma-Aldrich, UK). Twelve ml of working solution was added to 8 ml deionised water and filtered twice to remove any oil red O crystals. Mitochondria were visualised using a mouse monoclonal anti-OxPhos Complex IV primary antibody (Cat. #459600, Invitrogen, Paisley, UK), followed by an Alexa Fluor goat anti-mouse IgG<sub>2a</sub> 594 secondary antibody. PLIN2 visualisation was achieved using a mouse monoclonal anti-adipophilin primary antibody (Cat. #03-610102, American Research Products, MA, USA), followed by an Alexa Fluor goat anti-mouse IgG<sub>1</sub> 594 secondary antibody. On occasions when IMTG staining with oil red O was undertaken in parallel with PLIN2, an Alexa Fluor goat-anti mouse IgG<sub>1</sub> 488 secondary antibody was used. PLIN5 was visualised using a guinea pig polyclonal anti-OLPAT primary antibody (Cat. #GP31, Progen Biotechnik, Germany) followed by application of an Alexa Fluor goat-anti guinea pig IgG 488 secondary antibody. All Alexa Fluor secondary antibodies were obtained from Invitrogen (Paisley, UK). The specificity of the PLIN5 antibody was confirmed in a competition experiment in which a PLIN5 recombinant peptide (Cat. #NB110-60511PEP, Novus Biologicals, Cambridge, UK) was incubated with the PLIN5 primary antibody, subsequently resulting in removal of the positive fluorescent signal for PLIN5 (Supplementary fig. 1).

*Image capture, processing and data analysis:* Images of cross-sectional orientated sections were used for the determination of fibre-type differences in the content of IMTG, mitochondria, and PLIN2 and PLIN5. Images were captured using a Nikon E600 microscope with a 40x 0.75 NA objective, coupled to a SPOT RT KE colour 3 shot CCD camera (Diagnostic Instruments Inc., MI, USA). Both DAPI UV (340-380 nm) and FITC (465-495 nm) excitation filters were used to view the Alexa Fluor 350 and 488 fluorophores, respectively. In addition, a Texas red (540-580 nm) excitation filter was used to view sections stained with oil red O or the Alexa Fluor 594 fluorophore. The use of 3 excitation filters and 1 dichroic and 1 emission filter ("Pinkel" Triple Set, Semrock, Kettering, UK) allowed for semi-automated sequential image capture. Digital

images to specifically assess the size and number of IMTG and PLIN2 and PLIN5 objects were obtained using an inverted confocal microscope (Leica DMIRE2, Leica Microsystems) using a 63x 1.4 NA oil immersion objective. An argon laser was used to excite the Alexa Fluor 488 fluorophore, while both the Alexa Fluor 594 fluorophore and oil red O was excited using a Helium-Neon laser. The same system was used to capture digital images and to assess IMTG and PLIN2 or PLIN5 colocalisation.

Image processing was undertaken using Image-Pro Plus 5.1 software (Media Cybernetics, MD, USA). Fibre-type distribution of IMTG, PLIN2 and PLIN5 and mitochondria was assessed using widefield images. To this end, between 6 and 10 widefield images were used per muscle section, resulting in  $104 \pm 5$  fibres analysed per muscle cross-section ( $42 \pm 3$  type I fibres,  $62 \pm 3$  type II fibres). Mitochondrial fluorescence staining intensity was used to indicate changes in mitochondrial content after training. An intensity threshold was uniformly selected to represent a positive signal for IMTG, and PLIN2 and PLIN5. The content of IMTG and PLIN2 and PLIN5 were expressed as the positively stained area fraction relative to the total area of each muscle fibre. Images captured using confocal microscopy were used to identify changes in mean IMTG, PLIN2 and PLIN5 size and density. Density was calculated as the number of IMTG objects relative to area. The area of individual IMTG (lipid droplets), PLIN2 or PLIN5 objects was used as a measure of size.

Colocalisation analysis was performed separately for PLIN2 or PLIN5 with IMTG. This analysis was restricted to type I fibres as a previous publication from our laboratory has shown that changes in colocalisation with IMTG do not occur in type II fibres in response to the exercise bout used in the present study (Shepherd *et al.*, 2012). In this respect, sequential images of either PLIN2 or PLIN5 and IMTG were captured using confocal microscopy (as described) with 8x digital zoom. Positive signal for either PLIN protein and IMTG was obtained through the selection of a uniform intensity threshold. Binary images were created based on the selected threshold and subsequently used for colocalisation analysis. A colocalisation map was generated, displaying the merged images, with the overlapping area subsequently extracted to create a separate binary image. The total number of objects in this image was expressed relative to the total number of PLIN2 (or PLIN5) as a measure of colocalisation (Fig. 4). The extracted objects were also used as a measure of the number of PLIN associated-LDs (PLIN-LDs), and the number of extracted objects were subsequently subtracted from the total number of LDs to quantify the number of LDs not associated with PLIN (PLIN-null-LDs). Several controls were performed to check for bleed through, non specific secondary antibody binding and autofluorescence before colocalisation analysis was performed. No positive staining was observed in the opposite channel when single staining (IMTG, PLIN2 and PLIN5) was performed and

omission of the primary or secondary antibody, and oil red O abolished all positive signal in the relevant channel discounting the influence of tissue autofluorescence or non-specific secondary antibody binding. In addition, we performed the same colocalisation analysis on a series of non-corresponding PLIN2 (or PLIN5) and IMTG images, in order to determine whether the level of random colocalisation was statistically different from that obtained for the true corresponding images, as previously described (Lachmanovich *et al.*, 2003).

*Western blot analysis:* Snap frozen muscle tissue was powdered in liquid nitrogen and transferred to eppendorf tubes containing lysis buffer (1x RIPA buffer, Cell Signalling, including 1 complete mini protease inhibitor tablet, Roche Diagnostics, Germany). Samples were mixed on ice for 2 hours and then homogenized on ice for ~30 s at slow speed (Polytron), followed by centrifugation for 20 min at 4°C and 10,000 G. The supernatant was removed and a small portion used to determine protein concentration (Pierce BCA assay kit). Samples were subsequently diluted to a final concentration of 3  $\mu\text{g} \cdot \mu\text{l}^{-1}$ , using homogenization buffer and Laemmli sample buffer (Sigma) and boiled for 5 min. Equal quantities of protein (45  $\mu\text{g}$ ) were separated by electrophoresis on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes at 25 V for 1.5 hours. Ponceau S staining was performed to assess transfer efficacy and subsequently imaged using a ChemiDoc XRS+ system (Bio-Rad, UK). This image was used to quantify the total protein stain of each lane, and this was used as a measure of equal loading when the inter-lane variation was less than 10%. Membranes were subsequently destained (0.1M NaOH) and blocked for 1 h in 5% non-fat dried milk (NFDM) in phosphate-buffered saline with 0.1% Tween (PBST) followed by incubation overnight at room temperature with either the anti-adipophilin (Cat. #03-610102, American Research Products, MA, USA) or anti-OXPAT (Cat. #GP31, Progen Biotechnik, Germany) primary antibody diluted in 3% NFDM in PBST. The membranes were then washed 3 times in PBST, blocked in 5% NFDM in PBST for 1 h, followed by incubation for 1 h at room temperature with appropriate horseradish peroxidase-conjugated IgG secondary antibodies diluted in 3% NFDM in PBST. Antibody binding was detected using enhanced chemiluminescence HRP detection reagent (GE Healthcare, UK) and imaging and band quantification was performed using a ChemiDoc XRS+ system (Bio-Rad, UK).

### **Blood Sample Analysis**

Plasma glucose concentrations were determined spectrophotometrically using an ILab-600 semi-automatic analyzer in combination with the glucose oxidase kit (Instrumentation Laboratory Ltd UK, Warrington, UK). Insulin concentrations during the OGTT were determined using a commercially available direct insulin ELISA

kit (Invitrogen, Paisley, UK). Insulin sensitivity index (Matsuda & DeFronzo, 1999) was calculated based on plasma glucose and insulin values.

### ***Calculations and statistical analysis***

Rates of whole body fat and carbohydrate oxidation ( $\text{g}\cdot\text{min}^{-1}$ ) were calculated from the  $\text{VO}_2$  and  $\text{VCO}_2$  values collected at 15, 30, 45 and 60 minutes during the steady state cycling exercise. The calculations were made assuming protein oxidation was negligible, and according to previously published equations (Jeukendrup & Wallis, 2005):

$$\text{Carbohydrate oxidation } (\text{g}\cdot\text{min}^{-1}) = 4.210 \cdot \text{VCO}_2 - 2.962 \cdot \text{VO}_2$$

$$\text{Fat Oxidation} = 1.695 \cdot \text{VO}_2 - 1.701 \cdot \text{VCO}_2$$

Insulin sensitivity was calculated using the Matsuda index (Matsuda & DeFronzo, 1999), using the following equation:

$$\text{Matsuda (ISI)} = \frac{10,000}{\sqrt{(\text{FPG} \times \text{FPI}) \times (\text{P}_{\square} \times \text{P}_{\square})}}$$

All data are expressed as means  $\pm$  S.E.M. Significance was set at the 0.05 level of confidence. Changes in body composition, exercise capacity, insulin sensitivity and substrate utilization were assessed using a 2-factor repeated measures ANOVA, with the between-subject factor '*group*' (SIT vs. ET) and the within-subject factor '*training*' (pre- vs. post-training). Changes in mitochondrial density and PLIN2 and 5 content were assessed using a mixed 3-factor repeated measures ANOVA using the within-subject factors '*fibre*' (type I versus type II fibres) and '*training*' and between-subject (*group*) factor used in the 2-factor analysis. To inspect differences in IMTG, we hypothesised *a priori* that IMTG content would be significantly different between type I and type II fibres. Thus, we used a 3-factor repeated measures ANOVA with the between-subject factor as '*group*', and the within-subject factor's '*training*' and '*time*' (pre- versus post-exercise). The same three-factor repeated measures ANOVA and between-subject and within-subject factors were used when assessing differences in colocalisation, following an *a priori* hypothesis that colocalisation would be significantly different between actual and randomised images, based on a previous study (Lachmanovich *et al.*, 2003). Significant main effects or interactions were assessed using Bonferroni adjustment post hoc analysis. Improvements in training performance (SIT group; e.g. mean power) were examined using a paired t test analysis. A Pearson's bivariate correlation analysis was used to investigate specific relationships between variables.

## Results

### ***Exercise capacity, body composition, substrate utilization and insulin sensitivity***

At baseline, no differences between groups were observed for any of the variables mentioned in Table 1. In response to training  $\text{VO}_{2\text{peak}}$  was significantly improved irrespective of training type (SIT  $7 \pm 2\%$ , ET  $15 \pm 3\%$ , main effect for condition,  $P < 0.001$ , Table 1). The difference between the modes of exercise did not reach statistical significance ( $P = 0.064$ ).  $W_{\text{max}}$  was also increased after training, with no difference between groups (main effect for condition,  $P < 0.001$ ). Fat free mass increased significantly post-training, with no difference between groups (main training effect,  $P = 0.039$ ). The observed decrease in relative fat mass (Table 1) did not reach statistical significance, with no difference between groups (main training effect,  $P = 0.075$ ). Glucose area under the curve (AUC) was reduced post-training (SIT  $17 \pm 2\%$ , ET  $12 \pm 2\%$ ; main training effect,  $P < 0.001$ , Table 1), with no difference between groups. Similarly, training reduced insulin AUC (SIT  $33 \pm 3\%$ , ET  $18 \pm 5\%$ ; main training effect,  $P < 0.001$ , Table 1), with no difference between groups. In accordance, training improved insulin sensitivity, calculated using the Matsuda index (Matsuda & DeFronzo, 1999) (SIT  $56 \pm 15\%$ , ET  $29 \pm 12\%$ ; main training effect,  $P < 0.01$ , Table 1), with no difference between groups. Carbohydrate (absolute and relative) and fat (relative only) oxidation, as well as RER, were all significantly altered compared to pre-training following ET only ( $P < 0.05$ , Table 2), with no effect apparent following SIT. RER tended to be lower in the SIT group ( $P = 0.055$ ) before training. When considering the effectiveness of SIT, the first and final training sessions were examined. Specifically, mean power output increased during training by  $14 \pm 3\%$  ( $P = 0.001$ ) and peak power output increased by  $21 \pm 6\%$  ( $P = 0.01$ ).

### ***Analysis of mitochondrial content***

Immunofluorescence images demonstrated that COX protein expression (fluorescence intensity), representing mitochondrial density, was greater in type I fibres than type II fibres pre and post-training (main effect of fibre;  $P < 0.001$ , Table 3). No group differences were apparent at baseline. A main effect of training was observed, with increases in mitochondrial density in both type I fibres (SIT  $39 \pm 4\%$ , ET  $46 \pm 16\%$ ) and type II fibres (SIT  $39 \pm 3\%$ , ET  $50 \pm 14\%$ ;  $P < 0.05$ , Table 3). No difference between groups was apparent following training.

### ***Intramuscular triglyceride analysis***

Our *a priori* hypothesis that IMTG content is greater in type I fibres compared to type II fibres meant that we examined IMTG fibre-type specific training effects separately. There were no differences in resting IMTG content between groups at baseline. A significant main effect of training was observed with increased resting

IMTG content in type I fibres after both interventions (expressed as % fibre stained) (SIT 1.9-fold, ET 2.9-fold, main training effect ;  $P < 0.05$ , Fig. 1E). This finding was attributed to a significant rise in IMTG density, expressed as the number of LDs per area, after SIT (pre-training,  $0.065 \pm 0.009$ ; post-training,  $0.087 \pm 0.010 \mu\text{m}^2$ ) and ET (pre-training,  $0.064 \pm 0.009$ ; post-training,  $0.109 \pm 0.012 \mu\text{m}^2$ ). A main effect of training was observed for IMTG content in type II fibres after both interventions (SIT 1.4-fold, ET 2.5-fold, main training effect,  $P < 0.05$ , Fig. 1F). This increase in IMTG content was again attributable to an increase in IMTG density. No changes in IMTG size were observed in response to training. There were no interaction effects between training and group in any of the measures of resting IMTG content.

Before training, absolute IMTG content in type I fibres decreased significantly in response to 60 min steady state cycling, with no difference between groups (SIT  $17 \pm 9\%$ , ET  $15 \pm 12\%$ , main effect of time;  $P < 0.05$ , Fig. 1E). Following training, net IMTG breakdown during exercise in type I fibres was also significantly increased (SIT  $27 \pm 13\%$ , ET  $43 \pm 5\%$ ; main effect of time;  $P < 0.05$ , Fig. 1E). In comparison to pre-training, net IMTG breakdown in type I fibres was significantly greater following training (training x time interaction;  $P < 0.05$ ) with no difference in net IMTG breakdown between groups. Both pre- and post-training, the reduction in IMTG content in type I fibres was attributed to decreases in IMTG density after SIT (pre-training,  $21 \pm 13\%$ ; post-training,  $38 \pm 7\%$ ) and ET (pre-training,  $20 \pm 17\%$ ; post-training,  $32 \pm 8\%$ ). No significant breakdown of IMTG occurred during the exercise bout in type II fibres either before or after training (Fig. 1F).

### ***PLIN2 and PLIN5 analysis***

Immunofluorescence images show that PLIN2 content (expressed as % fibre stained) in type I fibres was significantly greater than in type II fibres both before and following training, irrespective of intervention ( $P < 0.001$ , Fig. 2C & D). A main effect of training was observed, with an increase in PLIN2 content in both type I (SIT 2.1-fold, ET 3.4-fold, Fig. 2C & D) and type II fibres (SIT 2.5-fold, ET 2.2-fold, main training effect,  $P < 0.001$ ). This finding was attributed to an increase in PLIN2 density in both type I (SIT: pre  $0.057 \pm 0.008$ , post  $0.092 \pm 0.010 \mu\text{m}^2$ , ET: pre  $0.051 \pm 0.012$ , post  $0.100 \pm 0.009 \mu\text{m}^2$ ) and type II fibres (SIT: pre  $0.020 \pm 0.004$ , post  $0.044 \pm 0.009 \mu\text{m}^2$ , ET: pre  $0.012 \pm 0.002$ , post  $0.031 \pm 0.006 \mu\text{m}^2$ ) (main training effect,  $P < 0.001$ ). No significant condition and group interactions were observed for either PLIN2 content or density. PLIN5 content (expressed as % of fibre stained) was significantly greater in type I fibres than type II fibres at all time points, irrespective of intervention ( $P < 0.001$ , Fig. 3C & D). A main effect of training was observed for PLIN5 content, with significant increases occurring in both fibre types after training (type I fibres: SIT 2.2-fold, ET 2.8-fold, type II fibres: SIT 2.2-fold, ET 3.4-fold, main training effect;  $P < 0.001$ , Fig. 3C & D). This finding was

attributed to an increase in PLIN5 density in both type I (SIT: pre  $0.054 \pm 0.005$ , post  $0.079 \pm 0.007 \mu\text{m}^2$ , ET: pre  $0.039 \pm 0.003$ , post  $0.107 \pm 0.011 \mu\text{m}^2$ ) and type II fibres (SIT: pre  $0.009 \pm 0.001$ , post  $0.018 \pm 0.004 \mu\text{m}^2$ , ET: pre  $0.005 \pm 0.001$ , post  $0.025 \pm 0.005 \mu\text{m}^2$ ) (main training effect,  $P < 0.001$ ). Again, no significant condition and group interaction effects were observed.

We performed immunoblotting of whole muscle homogenates in order to confirm the finding of an increase in both PLIN2 and PLIN5 content in response to training using immunohistochemical methodology. Immunoblot analysis revealed a main effect of training for PLIN2 content (SIT 3.9-fold, ET 5.8-fold;  $P = 0.02$ , Fig 2E), with no difference between groups. PLIN5 protein expression was also increased following training (SIT 2.0-fold, ET 2.4-fold, main training effect;  $P = 0.01$ , Fig 3E), again with no difference between groups.

### ***Colocalisation of PLIN2 and PLIN 5 with IMTG***

Before training, the fraction of PLIN2 colocalised with IMTG in resting muscle was similar between groups (SIT  $0.58 \pm 0.04$ , ET  $0.61 \pm 0.04$ ;  $P = 0.96$ ). This relationship was unchanged in response to both training interventions. Pre-training, 60 min steady state exercise induced a significant reduction in the fraction of PLIN2 colocalised with PLIN2 (SIT  $30 \pm 6\%$ , ET  $23 \pm 4\%$ ; main training effect,  $P < 0.01$ ). This reduction was the result of a decrease in IMTG content and an unchanged PLIN2 content. Post-training, a significantly larger reduction in the fraction of PLIN2 associated with IMTG was observed in response to the exercise bout (SIT  $37 \pm 7\%$ , ET  $40 \pm 4\%$ ;  $P = 0.01$ ) with no difference between groups (data not shown). As a change in PLIN2 association with IMTG was observed following steady state exercise both before and after training, we investigated the number of LDs either associated (PLIN2-LD) or not associated (PLIN2-null-LD) with PLIN2 at each time point. Both before and after training, the number of PLIN2-LD was greater than the number of PLIN2-null-LD before exercise. Pre-training, 60 min steady state exercise induced a reduction in the number of PLIN2-LD (SIT  $22 \pm 10\%$ , ET  $28 \pm 6\%$ ;  $P = 0.01$ , Fig. 4E & F), whereas the number of PLIN2-null-LD was unchanged. In contrast, following training, exercise induced a significant reduction in both the PLIN2-LD (SIT  $32 \pm 6\%$ , ET  $39 \pm 6\%$ ;  $P < 0.001$ ) and the PLIN2-null-LD (SIT  $24 \pm 12\%$ , ET  $35 \pm 12\%$ ;  $P = 0.01$ , Fig. 4E & F). No group interactions were detected with either condition or time for variables of PLIN2 and IMTG association, indicating that observations were statistically similar between groups.

Prior to training, the fraction of PLIN5 colocalised with IMTG was similar between groups (SIT  $0.58 \pm 0.04$ , ET  $0.50 \pm 0.04$ ;  $P = 0.20$ ), and following both training interventions this relationship was unchanged. Pre-training, 60 min steady state exercise significantly reduced the fraction of PLIN5 associated with IMTG (SIT

36 ± 4%, ET 33 ± 2%; main effect of time,  $P < 0.01$ ), which was the result of a reduction in IMTG with no change in PLIN5 content. Following training, the exercise-induced reduction in the fraction of PLIN5 colocalised with IMTG tended to be larger compared to pre-training, which bordered on statistical significance (SIT 40 ± 3%, ET 41 ± 3%;  $P = 0.06$ ), and with no difference between groups (data not shown). As above, we subsequently investigated the absolute number of LDs either associated (PLIN5-LD) or not associated (PLIN5-null-LD) with PLIN5 at each time point. Pre-training, the number of PLIN5-LD was larger in the SIT than the ET group ( $P < 0.05$ ), but no difference in the number of PLIN5-null-LD was apparent. Despite this discrepancy, a similar reduction in PLIN5-LD was observed in response to exercise (SIT 32 ± 4%, ET 35 ± 4%;  $P < 0.01$ , Fig. 4G & H), with no change in the number of PLIN5-null-LD, and no difference between groups for either variable. Following training, exercise induced a reduction in the number of PLIN5-LD only (SIT 38 ± 3%, ET 41 ± 5%;  $P < 0.01$ , Fig. 4G & H), with no difference between groups.

### **Correlation analyses**

Due to the lack of group x training interactions in all variables, significant training effects were detected independent of exercise modality. Therefore, the data from the SIT and ET interventions (type I and type II fibres, pre and post training) were pooled for correlation analysis (Table 4). Both PLIN2 ( $R = 0.68$ ;  $P < 0.001$ ) and PLIN5 content ( $R = 0.72$ ;  $P < 0.001$ ) showed strong positive correlations with IMTG content. A very strong positive correlation was observed between pre-exercise IMTG concentrations and the decrease in IMTG content in response to 60 min steady state exercise ( $R = 0.81$ ,  $P < 0.001$ ). In addition, PLIN2 ( $R = 0.57$ ,  $P = 0.001$ ) and PLIN5 content ( $R = 0.63$ ,  $P < 0.001$ ) were strongly associated with net IMTG breakdown. Insulin sensitivity were modestly associated with PLIN2 ( $R = 0.40$ ;  $P = 0.02$ ) and PLIN5 content ( $R = 0.42$ ;  $P = 0.02$ ), as well as IMTG content ( $R = 0.53$ ;  $P = 0.01$ ).



## Discussion

This is the first study to report that greater protein expression of PLIN2 and PLIN5 is associated with larger IMTG stores and enhanced net IMTG breakdown during exercise in type I muscle fibres following a period of traditional ET. This observation is consistent with our first hypothesis. We also provide novel evidence that PLIN5-LDs are utilized during an acute bout of exercise irrespective of training status, whereas after a period of training both PLIN2-LDs and PLIN2-null-LDs are utilized during an acute bout of exercise. In support of our second hypothesis, we demonstrate for the first time that like traditional ET, SIT also enhances IMTG content and net breakdown during 60 min of moderate intensity exercise and leads to increases in the protein expression of PLIN2 and PLIN5. This common metabolic response following both SIT and ET is also evident in the utilization of PLIN5-LDs, PLIN2-LDs and PLIN2-null-LDs. Taken together, these adaptations may contribute to the mechanisms underlying the observed improvements in insulin sensitivity following SIT and ET.

We report that ET leads to increases in IMTG content in both type I and type II fibres, and is accompanied by increased mitochondrial density, corroborating the findings of previous studies (Schrauwen-Hinderling *et al.*, 2003; Pruchnic *et al.*, 2004; Tarnopolsky *et al.*, 2007; Dubé *et al.*, 2011). In addition before training, 60 min moderate-intensity cycling induced a small but significant decrease in IMTG in type I fibres. Importantly, we report that ET leads to increased rates of whole body fat oxidation during exercise at 65% pre-training  $VO_{2peak}$  and that this is accompanied by large increases in net IMTG breakdown primarily in type I fibres. Previous studies combining IMTG content and A-V balance measurements using  $[U-^{13}C]$ -palmitate demonstrate that oxidation of plasma fatty acids does not completely account for fat oxidation during exercise, indicating that other fat sources contribute to total fat oxidation (Sacchetti *et al.*, 2002; van Hall *et al.*, 2002; van Loon *et al.*, 2003). Therefore, it is reasonable to assume that fatty acids liberated through the net reduction in IMTG content contribute to total fat oxidation during exercise in lean but sedentary individuals and the IMTG contribution to total fat oxidation is increased following ET. The latter observation is consistent with a number of recent training studies also examining net IMTG breakdown in a fibre type specific manner (Van Proeyen *et al.*, 2011a; Van Proeyen *et al.*, 2011b).

Our data demonstrate that net IMTG breakdown during exercise is positively correlated with resting IMTG concentration. This corroborates the findings of several other reports in trained individuals (Coggan *et al.*, 2000; van Loon *et al.*, 2003; Stellingwerff *et al.*, 2007) and indicates that the ET-induced increase in IMTG content contributes to the increase in net IMTG breakdown during endurance type exercise. However, it is

worth noting that this is only one of a number of adaptations which likely explain the increase in IMTG breakdown during exercise following ET (van Loon, 2004; Shaw *et al.*, 2010). The increase in IMTG content was due to a greater number of LDs rather than an increased volume of pre-existing LDs. This conclusion is in agreement with a previous report using transmission electron microscopy (Tarnopolsky *et al.*, 2007). As smaller LDs maintain a higher surface area to volume ratio, they also provide a greater surface area for the interaction of lipolytic enzymes with the IMTG substrate and regulatory proteins on the LD surface. As a result, the mobilization of FA for oxidation during exercise would be enhanced.

Using immunofluorescence microscopy we previously reported that trained athletes display a ~two-fold greater PLIN2 concentration in type I compared to type II muscle fibres (Shaw *et al.*, 2009). The present data extends this finding to sedentary individuals, and further reveals a ~four-fold difference in PLIN5 protein expression when comparing type I and type II fibres. In addition, we report that ET enhances the intramuscular content of both PLIN2 and PLIN5, assessed through immunoblotting of whole muscle homogenates. The findings are partly in agreement with a recent study showing increased PLIN5, but not PLIN2, protein abundance in the muscle of lean and obese males following 12 weeks of endurance training (Peters *et al.*, 2012) and with a cross-sectional study that reported a ~seven-fold higher PLIN5 content in older athletes compared to age-matched sedentary controls (Amati *et al.*, 2011). However, our study is the first to examine changes in the intramuscular expression of the PLIN proteins in response to training interventions in a fibre type specific manner using immunofluorescence microscopy. Accordingly, we demonstrate that, like the changes in IMTG, the increase in PLIN2 and PLIN5 protein expression occurs in both type I and type II muscle fibres.

In agreement with data obtained in rodent (Minnaard *et al.*, 2009) and human skeletal muscle (Amati *et al.*, 2011; Peters *et al.*, 2012), both PLIN2 and PLIN5 expression positively correlated with IMTG content suggesting that the expression of these PLIN proteins is proportional to IMTG content in lean, healthy individuals. Future research will be required to clarify if the increase in PLIN expression is the primary event driving the increase in IMTG content (which is the mechanism that we propose) or the consequence of the increase in IMTG content. Furthermore, the fraction of IMTG associated with either PLIN2 or PLIN5 at rest was similar pre and post training in type I fibres (~0.64 and ~0.59, respectively). These values are consistent with previous reports in rat (Macpherson *et al.*, 2012) and human skeletal muscle (Shaw *et al.*, 2009; Shepherd *et al.*, 2012). Although the physiological significance of this partial colocalisation is currently unknown, this finding is in agreement with a number of observations demonstrating that the protein

composition of LDs may vary (Wolins *et al.*, 2005; Wolins *et al.*, 2006b; Ducharme & Bickel, 2008; Fujimoto *et al.*, 2008). To further investigate this relationship we assessed the colocalisation of these PLINs with LDs before and after 60 min moderate intensity endurance exercise. For each PLIN we describe two pools of LDs; 1) PLIN2-associated LDs (PLIN2-LD) and LDs that do not contain PLIN2 (PLIN2-null-LD) and 2) PLIN5-associated LDs (PLIN5-LD) and LDs that do not contain PLIN5 (PLIN5-null-LD). In the untrained state, 60 min moderate intensity endurance exercise induced a significant decrease in the number of PLIN2-LD and PLIN5-LD, with no change in the number of PLIN2-null-LD and PLIN5-null-LD. These observations confirm a recent report from our laboratory showing a preferential utilization of PLIN2-LD, and not PLIN2-null-LD, in response to moderate intensity endurance exercise in sedentary individuals (Shepherd *et al.*, 2012), and provides novel evidence to suggest that a similar relationship exists for PLIN5. After 6 weeks ET, a preferential decrease in the number of PLIN2-LD and PLIN5-LD also occurred in response to 60 min moderate intensity endurance exercise. Interestingly, in the trained state exercise also induced a decrease in the content of PLIN2-null-LD after 60 min moderate intensity endurance exercise, whereas the content of PLIN5-null-LD did not change. This is the first observation to suggest that PLIN5 is more important than PLIN2 in mediating the hydrolysis of intramuscular lipid stores during exercise and is in line with emerging evidence concerning the precise role of PLIN5 in lipolysis in cultured cells. Indeed, PLIN5 is the only PLIN thus far that has been shown to bind ATGL, and its coactivator, CGI-58. In this respect, overexpression of PLIN5 in cultured cells recruits ATGL and CGI-58 under basal conditions, while expression of PLIN2 does not have this effect (Wang *et al.*, 2011a). Furthermore, PLIN5 is phosphorylated in response to protein kinase A (PKA)-activation, and the expression and PKA-induced activation of PLIN5 stimulates triacylglycerol hydrolysis in Chinese hamster ovary cells, whereas rates of lipolysis are unaffected by PKA activation in cells overexpressing PLIN2 (Wang *et al.*, 2011a; Wang *et al.*, 2011b). It is also important to note that electrical stimulation and adrenaline stimulation of rat skeletal muscle (Prats *et al.*, 2006) has been reported to increase the colocalisation of HSL with PLIN2. However, it is not known whether this association occurs in man *in vivo* and whether it increases rates of lipolysis. It is also worth acknowledging that we only assessed colocalisation of either PLIN2 or PLIN5 with the LD's, and therefore cannot discriminate LDs containing both PLIN2 and PLIN5 from those LDs containing only PLIN2 or PLIN5. Nevertheless, the observation that the number of PLIN5-LD is reduced following exercise, while no reduction occurs in the number of PLIN5-null-LD appears to underpin the important role of PLIN5 in LD lipolysis and subsequent oxidation in skeletal muscle. In line with this conclusion, observations from various cell culture models and rat skeletal muscle preparations suggest that PLIN5 also recruits mitochondria to the surface of LD's, thus stimulating the formation of functional complexes between mitochondria and LDs (Bosma *et al.*, 2011; Wang

*et al.*, 2011b). Accordingly, PKA activation in alpha mouse liver 12 (AML12) cells expressing PLIN5 has recently been shown to promote triacylglycerol hydrolysis and efficient channeling of liberated fatty acids to the mitochondria for  $\beta$ -oxidation (Wang *et al.*, 2011b). IMTG and mitochondria in skeletal muscle of endurance athletes are observed in close proximity (Shaw *et al.*, 2008), and ET enhances the spatial interaction between IMTG and mitochondria (Tarnopolsky *et al.*, 2007). Collectively these observations suggest that increased expression and PKA activation of PLIN5 may also contribute to an increased net IMTG breakdown during moderate intensity endurance exercise by mediating stronger interactions between IMTG and mitochondria.

It is worth noting that there was no net IMTG breakdown in type II fibres during 60 min moderate intensity exercise pre or post 6 weeks of training. This observation is in line with previous studies in well-trained athletes performing 2-3 h moderate-intensity exercise (van Loon *et al.*, 2003; De Bock *et al.*, 2005; Stellingwerff *et al.*, 2007). However, PLIN2 and PLIN5 protein expression was significantly increased in type II fibres, raising the question as to why IMTG breakdown was not enhanced in parallel as seen in the type I fibres. However, as the exercise was performed at a moderate intensity, it is likely that only a small proportion of the type II fibres were recruited, and this therefore may explain why no net IMTG breakdown was observed in type II fibres. In addition the higher PLIN2 and PLIN5 content after training in both fibre types may also serve other metabolic roles such as to mediate IMTG storage under non-exercise conditions.

Both PLIN2 and PLIN5 also appear to play an important role in limiting rates of lipolysis in the basal state, since expression of either PLIN2 or PLIN5 in cultured cells reduces basal lipolytic rates compared to when PLIN2 is knocked down or PLIN5 is not expressed (Listenberger *et al.*, 2007; Bell *et al.*, 2008; Wang *et al.*, 2011a). Specifically, PLIN2 expression reduces the association of ATGL with the LD (Listenberger *et al.*, 2007; Bell *et al.*, 2008), and PLIN5 binds ATGL under basal conditions thereby reducing its activity (Wang *et al.*, 2011a). Furthermore, it has recently been shown that PLIN5 expression in AML12 cells reduces basal triacylglycerol hydrolysis and promotes palmitate incorporation into LD triacylglycerol (Wang *et al.*, 2011b). If such mechanisms exist *in vivo*, these observations suggest that PLIN5 and/or PLIN2 may play a key role in the coupling of TAG hydrolysis to the metabolic demand for fatty acids. As mentioned, ET increases the net breakdown of IMTG during exercise, therefore during an ET intervention IMTG concentrations are reduced on a regular basis. In addition, IMTG synthesis is upregulated in the period following IMTG-depleting exercise (Schenk & Horowitz, 2007). Therefore, undertaking repeated bouts of exercise during an exercise training programme likely results in high rates of IMTG synthesis (Bergman *et al.*, 2010). As a result of this

combination of training adaptations in the enzymes controlling IMTG metabolism, insulin resistance-inducing-FA metabolites have been suggested to be maintained at a lower concentration in skeletal muscle, thereby preserving higher insulin sensitivity in trained individuals. (Dubé *et al.*, 2008; Dubé *et al.*, 2011).

The most well-documented adaptations to SIT are an improved  $\text{VO}_{2\text{peak}}$  and an enhanced skeletal muscle oxidative capacity (reviewed Gibala *et al.*, 2012), both of which are increased by a similar magnitude as ET (Burgomaster *et al.*, 2008). We also demonstrate that SIT and ET improve  $\text{VO}_{2\text{peak}}$  in sedentary individuals as previously described (Burgomaster *et al.*, 2008), although in the present study the improvement in  $\text{VO}_{2\text{peak}}$  tended to be greater in the ET group ( $P = 0.064$ ). Like ET, SIT also increased mitochondrial density, as previously reported (Burgomaster *et al.*, 2008), and we provide novel data showing that SIT leads to higher IMTG content in type I and type II muscle fibres. In contrast to ET, no changes in whole body fat oxidation rates were observed following SIT. This finding is in contrast to the findings of Burgomaster *et al.* (2008), who reported an increase in fat oxidation during exercise following SIT. The SIT group tended to exhibit a lower RER than the ET group pre-training, suggesting that rates of fat oxidation were higher in the SIT group before training, despite the groups being matched for physical activity level, aerobic capacity and fat mass, and diet was controlled in the 48 h prior to the exercise bout. Nevertheless, we observe clear increases in IMTG breakdown in type I fibres during moderate-intensity exercise in response to SIT, suggesting that the source of oxidised fat is shifted from plasma fatty acids towards IMTG. Importantly, the expression of both PLIN2 and PLIN5 correlated with IMTG content, net IMTG breakdown in type I fibres during exercise, and insulin sensitivity. Therefore, the data suggests that the increase in IMTG content and net breakdown during exercise that results from the increased expression of PLIN2 and PLIN5 likely maintains low intramuscular fatty acid metabolite concentrations leading to improvements in insulin sensitivity following both ET and SIT. Accordingly, the present study adds to the growing body of evidence that SIT is a time-efficient exercise intervention to improve insulin sensitivity in healthy individuals and patient populations (Babraj *et al.*, 2009; Richards *et al.*, 2010; Hood *et al.*, 2011; Little *et al.*, 2011).

It is important to note that the primary aim of the study was to investigate exercise training-induced increases in net IMTG breakdown during 60 min moderate-intensity exercise and PLIN2 and PLIN5 protein expression. However, it is likely that the number of subjects in the present study is not sufficiently high to detect between groups differences for some of the measured variables. Therefore, although many of the training effects are of a similar magnitude, larger-scale studies are required to determine which exercise mode is superior in increasing the main metabolic outcome measures (IMTG breakdown during exercise and PLIN2 and PLIN5

expression) and/or in increasing whole body insulin sensitivity and aerobic exercise capacity. Studies involving a larger number of participants should be completed before advice can be given about which exercise training mode is the best for the general population and for specific patient populations.

In conclusion, PLIN2 and PLIN5 expression are upregulated in response to ET, and the upregulation of these proteins appears to be important to facilitate the enhanced rates of net IMTG breakdown during exercise. In addition, PLIN2 and PLIN5 expression may contribute to an increase in IMTG content, which likely results in lower fatty acid metabolite concentrations, and in turn may drive the ET-induced improvements in insulin sensitivity. Despite the large differences in duration and energy expenditure between SIT and ET, we provide novel evidence indicating that SIT induces similar improvements in net IMTG breakdown, PLIN2 and PLIN5 protein expression, aerobic exercise capacity and insulin sensitivity as traditional ET. This study, therefore, generates novel information on the mechanism by which net IMTG breakdown during endurance type exercise increases following both modes of exercise training, and provides evidence that SIT provides a time-efficient exercise alternative to achieve improvements in aerobic fitness and insulin sensitivity.

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**Author contributions:**

SOS: Design of the experiments. Collection, analysis and interpretation of the data. Drafting and final revisions of the manuscript. MC: Design of the experiments. Collection, analysis and interpretation of the data. KDT: Design of the experiments. Collection of the data. Critical revision of the manuscript for intellectual content. AMR: Collection of the data. Critical revision of the manuscript for intellectual content. TAB: Collection of the data. Critical revision of the manuscript for intellectual content. JGB: provided technical expertise and access to his laboratory for western blotting analysis. Critical revision of the manuscript for intellectual content. AJMW: Design of the experiments. Analysis and interpretation of the data. Drafting and final revisions of the manuscript. CSS: Design of the experiments. Analysis and interpretation of the data. Drafting and final revisions of the manuscript.

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### **Translational Perspective**

It is well-established that individuals who perform 3-5 hours of continuous moderate-intensity exercise per week maintain a higher physical fitness level and have a reduced risk of developing metabolic and cardiovascular diseases. However, these current physical activity guidelines are not met by the majority of the UK population, with 'a lack of time' cited as the primary reason for failure to engage in regular exercise. Sprint interval training (SIT) may provide an effective alternative to traditional endurance-type exercise training (ET), with a substantial reduction in time commitment. Indeed, SIT is reported to induce similar increases in  $VO_{2max}$ , mitochondrial enzyme abundance, and whole-body insulin sensitivity. Shepherd and colleagues show that like ET, 6 weeks of SIT increases the capacity to oxidise intramuscular triglyceride (IMTG) during exercise. Furthermore, they show that this adaptation may be mediated by an upregulation of PLIN2 and PLIN5, proteins known to be involved in regulating IMTG breakdown. The depletion and subsequent replenishment of the IMTG pool during exercise and recovery periods is postulated to prevent the accumulation of insulin resistance-inducing fatty acid metabolites. Therefore increasing IMTG oxidation is an important training adaptation contributing to the improvement insulin sensitivity following both ET and SIT. These findings add to the evidence that SIT could provide an effective exercise training mode to improve fitness and metabolic health in obese individuals, and elderly individuals with sarcopenia, type II diabetes and cardiovascular disease. Long term, large-scale studies investigating the clinical benefits of SIT in such populations are now warranted.

## Tables

**Table 1.** Subject characteristics

	SIT		ET	
	<i>Pre</i>	<i>Post</i>	<i>Pre</i>	<i>Post</i>
Age (yrs)	22 ± 1		21 ± 1	
Height (m)	1.74 ± 0.02		1.77 ± 0.03	
Body mass (kg)	75.1 ± 3.0	75.2 ± 3.1	70.8 ± 4.4	70.8 ± 4.5
BMI (kg.m <sup>-2</sup> )	24.8 ± 0.8	24.8 ± 0.9	22.6 ± 1.6	22.6 ± 1.2
VO <sub>2 peak</sub> (l.min <sup>-1</sup> ) *	3.13 ± 0.15	3.37 ± 0.18	2.93 ± 0.30	3.40 ± 0.38
VO <sub>2 peak</sub> (l.min <sup>-1</sup> .kg <sup>-1</sup> ) *	41.9 ± 1.8	45.1 ± 2.3	41.7 ± 4.1	48.2 ± 5.0
W <sub>max</sub> (W) *	221 ± 11	241 ± 14	218 ± 11	253 ± 16
FFM (kg) *	52.5 ± 1.9	53.6 ± 1.8	50.7 ± 1.5	51.1 ± 2.7
FM (kg)	14.2 ± 1.6	13.6 ± 1.5	12.3 ± 1.5	12.2 ± 1.9
Insulin AUC (μIU.ml <sup>-1</sup> .min <sup>-1</sup> ) *	8847 ± 1140	5792 ± 688	8434 ± 880	6813 ± 711
Glucose AUC (mg.dl <sup>-1</sup> .min <sup>-1</sup> ) *	17634 ± 710	14551 ± 398	16835 ± 992	14783 ± 950
ISI-Matsuda *	3.9 ± 0.3	5.8 ± 0.4	3.7 ± 0.5	4.7 ± 0.7

Data provided are means ± S.E. ( $n = 8$  per group). *BMI* body mass index, *W<sub>max</sub>* maximum workload, *OGTT* oral glucose tolerance test, *FFM* fat free mass, *FM* fat mass, *AUC* area under the curve, *ISI* insulin sensitivity index. \* Indicates main effect for training ( $P < 0.05$ ), such that Pre ≠ Post. No interaction effects (training x group) were observed for any of the variables.

**Table 2.** Substrate utilization during 60 min cycling at ~65% pre-training  $\text{VO}_{2\text{ peak}}$ 

	SIT		ET	
	<i>Pre</i>	<i>Post</i>	<i>Pre</i>	<i>Post</i>
Heart rate ( $\text{b}\cdot\text{min}^{-1}$ ) *	164 $\pm$ 3	147 $\pm$ 3	165 $\pm$ 3	138 $\pm$ 4
$\text{VO}_2$ ( $\text{l}\cdot\text{min}^{-1}$ ) *	2.22 $\pm$ 0.11	2.12 $\pm$ 0.12	2.19 $\pm$ 0.14	2.10 $\pm$ 0.13
$\text{VCO}_2$ ( $\text{l}\cdot\text{min}^{-1}$ ) *	1.94 $\pm$ 0.09	2.01 $\pm$ 0.13	1.97 $\pm$ 0.11	1.81 $\pm$ 0.10
CHO oxidation				
( $\text{g}\cdot\text{min}^{-1}$ )	1.61 $\pm$ 0.07	1.56 $\pm$ 0.13	1.80 $\pm$ 0.12	1.37 $\pm$ 0.07 <sup>†</sup>
(% of total oxidation)	62.3 $\pm$ 2.0	63.2 $\pm$ 4.4	70.9 $\pm$ 4.3	56.6 $\pm$ 3.1 <sup>†</sup>
Fat oxidation				
( $\text{g}\cdot\text{min}^{-1}$ )	0.46 $\pm$ 0.04	0.42 $\pm$ 0.06	0.36 $\pm$ 0.07	0.49 $\pm$ 0.05
(% of total oxidation)	39.5 $\pm$ 1.9	38.6 $\pm$ 4.3	31.0 $\pm$ 4.3	45.1 $\pm$ 3.1 <sup>†</sup>
RER	0.88 $\pm$ 0.01	0.88 $\pm$ 0.01	0.90 $\pm$ 0.01	0.86 $\pm$ 0.01 <sup>†</sup>

Values obtained during 60 min of moderate-intensity cycling at ~65% pre-training  $\text{VO}_{2\text{ peak}}$ . Data provided are means  $\pm$  S.E. ( $n = 8$  per group). *RER* respiratory exchange ratio, *CHO* carbohydrate. \* Main effect for training ( $P < 0.05$ ) such that Pre  $\neq$  Post (no significant differences between training interventions). <sup>†</sup> Interaction effect (training x group;  $P < 0.05$ ), such that the effect of ET was significantly different from the effect of SIT.

**Table 3.** Effect of 6 weeks of SIT or ET on fibre type specific mitochondrial content

COX expression (fluorescence intensity)	SIT		ET	
	<i>Pre</i>	<i>Post</i>	<i>Pre</i>	<i>Post</i>
Type I fibres	54.1 ± 4.6	74.4 ± 4.0 <sup>†</sup>	60.1 ± 7.7	81.5 ± 5.9 <sup>†</sup>
Type II fibres*	41.1 ± 3.0	56.8 ± 3.5 <sup>†</sup>	42.4 ± 4.8	60.4 ± 5.1 <sup>†</sup>

Mitochondrial density, quantified from immunofluorescence images of COX in type I and type II fibres obtained before and after 6 weeks of SIT or ET. Values are presented as means ± S.E. ( $n = 8$  per group). \*

Main fibre effect ( $P < 0.05$  vs. type I fibres). † Main training effect ( $P < 0.05$  vs. pre-training).

**Table 4.** Bivariate correlation analysis

	IMTG	Net IMTG breakdown	Insulin sensitivity
PLIN2	0.68 <sup>‡</sup>	0.57 <sup>‡</sup>	0.40*
PLIN5	0.72 <sup>‡</sup>	0.63 <sup>‡</sup>	0.42*
IMTG		0.81 <sup>‡</sup>	0.53 <sup>‡</sup>

IMTG, PLIN2 and PLIN5 represent resting content. Net IMTG breakdown refers to absolute change in IMTG concentration from pre to post-exercise. Values represent Pearson's squared correlation coefficients (R values) for relationships between variables. Due to the lack of group x training interactions in all variables, it was assumed that the exercise training was driving the changes independent of exercise modality. Therefore, the data from both intervention groups was pooled for correlation analysis. Significant correlations: \* $P < 0.05$ , <sup>†</sup> $P < 0.01$ , <sup>‡</sup> $P < 0.001$ .



## Figure legends

### **Figure 1. Effect of 6 weeks of SIT and ET on fibre type specific IMTG content and net IMTG breakdown during exercise.**

Representative immunofluorescence images of IMTG (stained red) in combination with WGA to identify the cell border (stained blue) in skeletal muscle, pre (A) and post (B) 6 weeks of SIT. Type I fibres are indicated with a “I”, all other fibres are assumed type II fibres. White bar = 50µm. Images (C) and (D) display the ORO signal obtained after the intensity threshold was applied during quantitation (showing the LDs in white), before and after SIT, respectively. IMTG content, quantified from immunofluorescence images of oil red O-stained muscle sections, before (*Pre-Training*) and after (*Post-Training*) 6 weeks of SIT (closed bars) or ET (open bars). IMTG content was measured before (*PreEx*) and after (*PostEx*) 60 min steady state cycling, and net IMTG breakdown calculated (*Breakdown*), in type I (E) and type II fibres (F). Values are presented as means ± S.E. ( $n = 8$  per group). \* Main effect of training intervention ( $P < 0.05$  vs. pre-training).

### **Figure 2. Effect of 6 weeks of SIT and ET on fibre type specific PLIN2 expression.**

Representative immunofluorescence images of PLIN2 (stained red) in combination with WGA to identify the cell border (stained blue) in skeletal muscle, pre (A) and post (B) 6 weeks of SIT. Type I fibres are indicated with a “I”, all other fibres are assumed type II fibres. White bar = 50µm. PLIN2 expression, quantified from immunofluorescence images of PLIN2, in type I and type II fibres obtained before (C) and after (D) 6 weeks of SIT (closed bars) or ET (open bars). PLIN2 expression quantified from immunofluorescence images correlated with PLIN2 expression determined following immunoblotting of whole muscle homogenates (E). Values are presented as means ± S.E. ( $n = 8$  per group). \* Main effect of fibre type ( $P < 0.05$  vs. type I fibres). † Main effect of training intervention ( $P < 0.05$  vs. pre-training).

### **Figure 3. Effect of 6 weeks of SIT and ET on fibre type specific PLIN5 expression.**

Representative immunofluorescence images of PLIN5 (stained green) in combination with WGA to identify the cell border (stained blue) in skeletal muscle, pre (A) and post (B) 6 weeks of SIT. Type I fibres are indicated with a “I”, all other fibres are assumed type II fibres. White bar = 50µm. PLIN5 expression, quantified from immunofluorescence images of PLIN5, in type I and type II fibres obtained before (C) and after (D) 6 weeks of SIT (closed bars) or ET (open bars). PLIN5 expression quantified from immunofluorescence images correlated with PLIN5 expression determined following immunoblotting of

whole muscle homogenates (*E*). Values are presented as means  $\pm$  S.E. ( $n = 8$  per group). \* Main effect of fibre type ( $P < 0.05$  vs. type I fibres). † Main effect of training intervention ( $P < 0.05$  vs. pre-training).

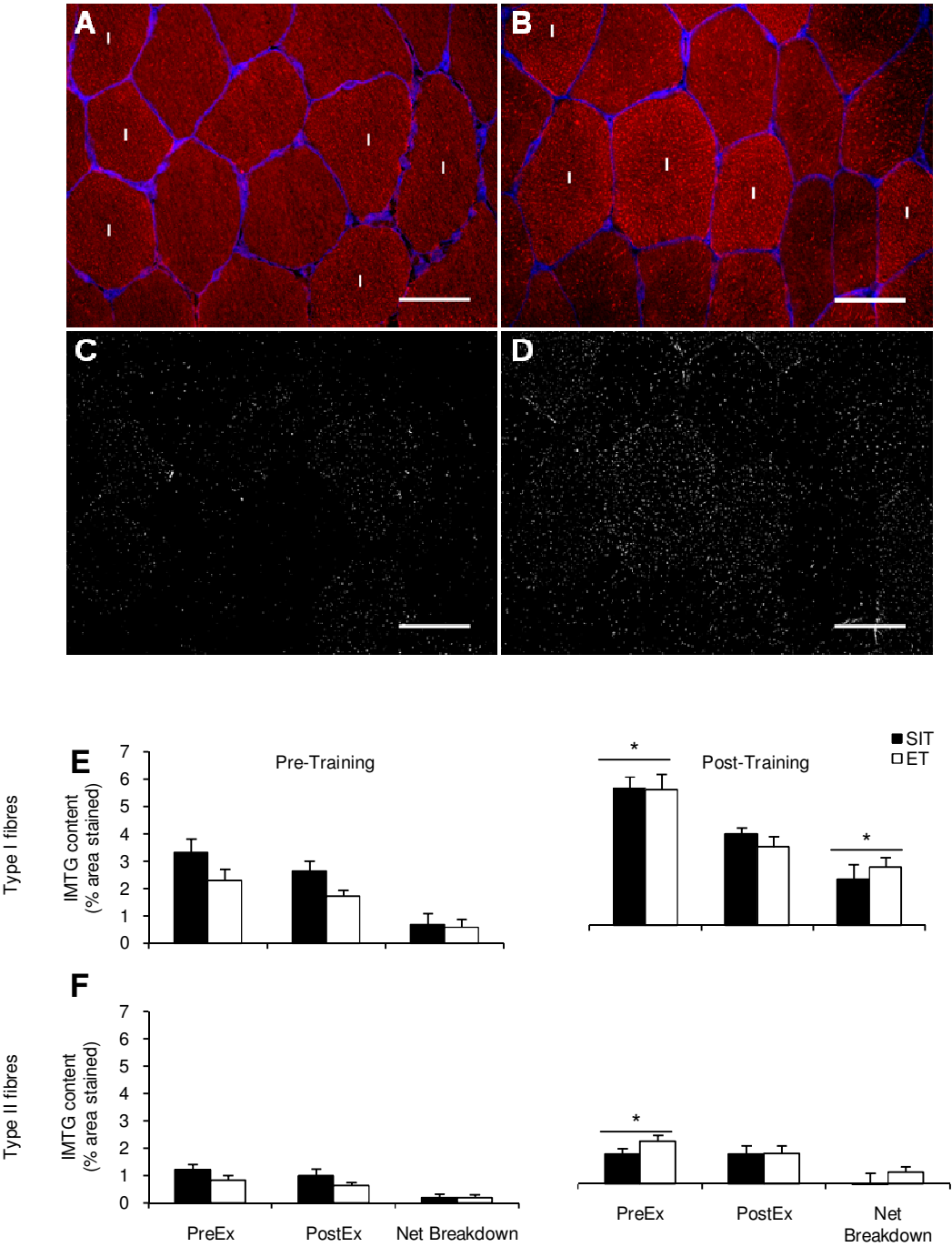
**Figure 4. Analysis of the colocalisation of PLIN2 and PLIN5 with LDs pre and post 60 min steady state cycling, before and after 6 weeks of SIT or ET.**

Representative immunofluorescence images of PLIN5 (*A*), IMTG (*B*), and the merged images (*C*) with the yellow areas describing regions of overlap between PLIN5 and IMTG images. The yellow objects were then extracted (to measure the number of PLIN5-LDs) (*D*), and the number subtracted from the total number of LDs to quantify the number of PLIN5-null-LD. The same procedure was used to obtain values of colocalisation between PLIN2 and IMTG. White bar = 5 $\mu$ m. The number of PLIN2-LD (closed bars) and PLIN2-null-LD (open bars) were quantified before (PreEx) and after exercise (PostEx), prior to (*E*) and following (*F*) training in both the SIT and ET groups. The same analysis was repeated for PLIN5 for pre (*G*) and post training (*H*). \* Main effect of exercise bout ( $P < 0.05$  vs. pre-exercise).

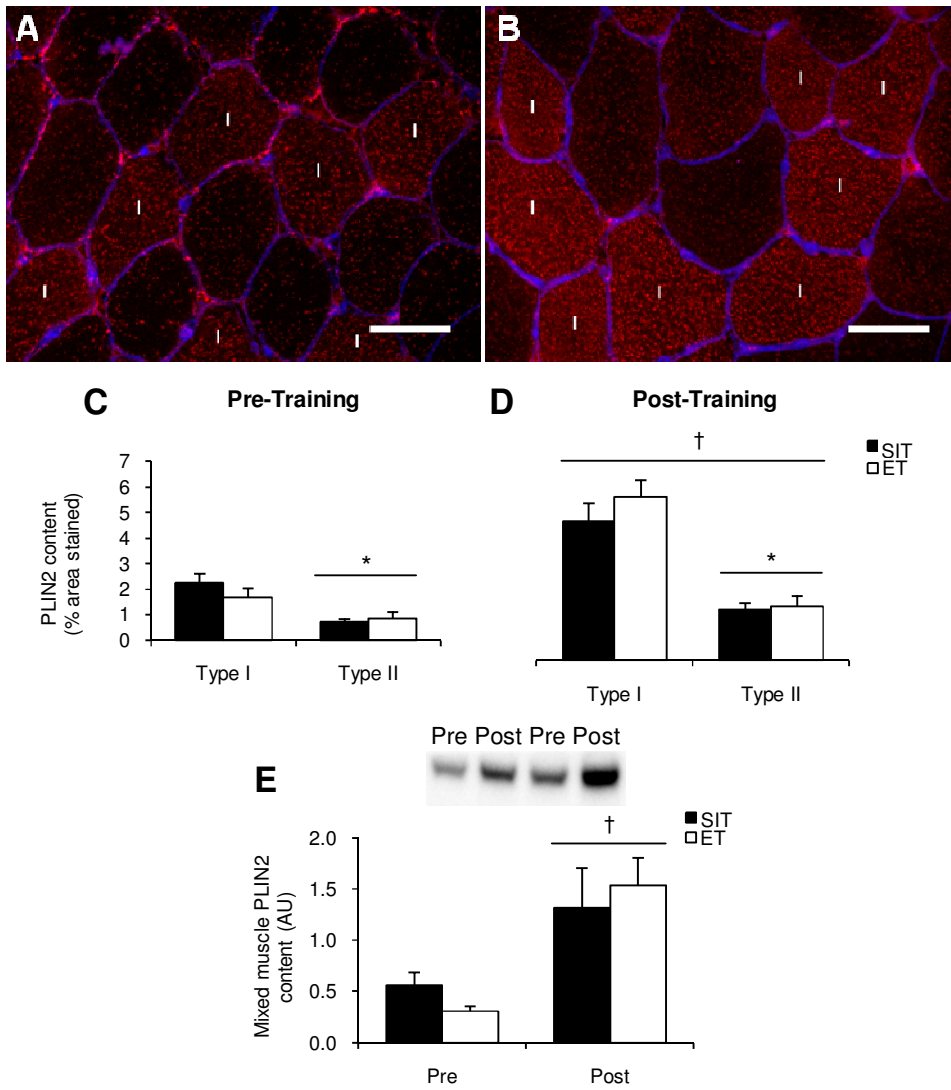
**Supplementary figure 1. Confirmation of PLIN5 antibody specificity.**

The specificity of the PLIN2 antibody was confirmed in a competition experiment where a PLIN5 recombinant peptide (Novus Biologicals, Cambridge, UK) was incubated with the antibody, and subsequently the punctate pattern typical of PLIN5 positive staining (*A*) was removed (*B*). White bar = 50 $\mu$ m.

Figure 1



**Figure 2**



**Figure 3**

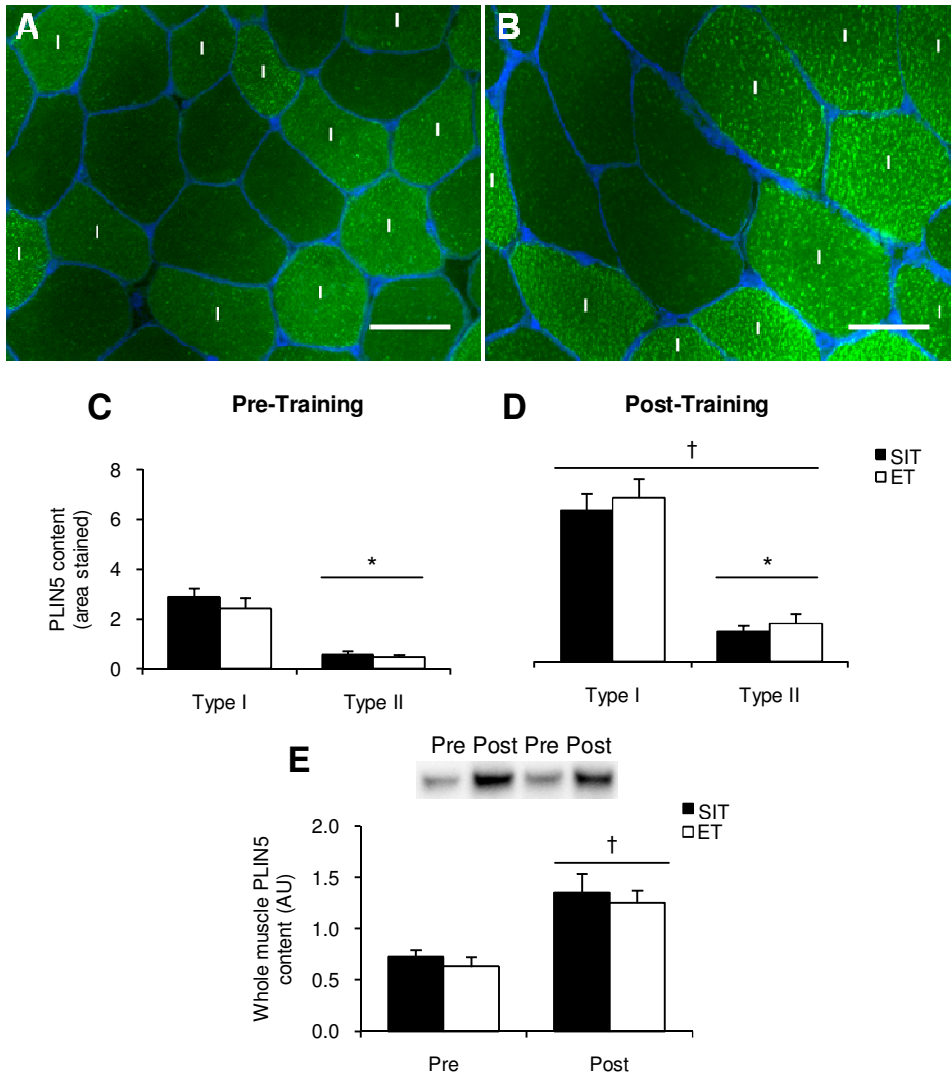
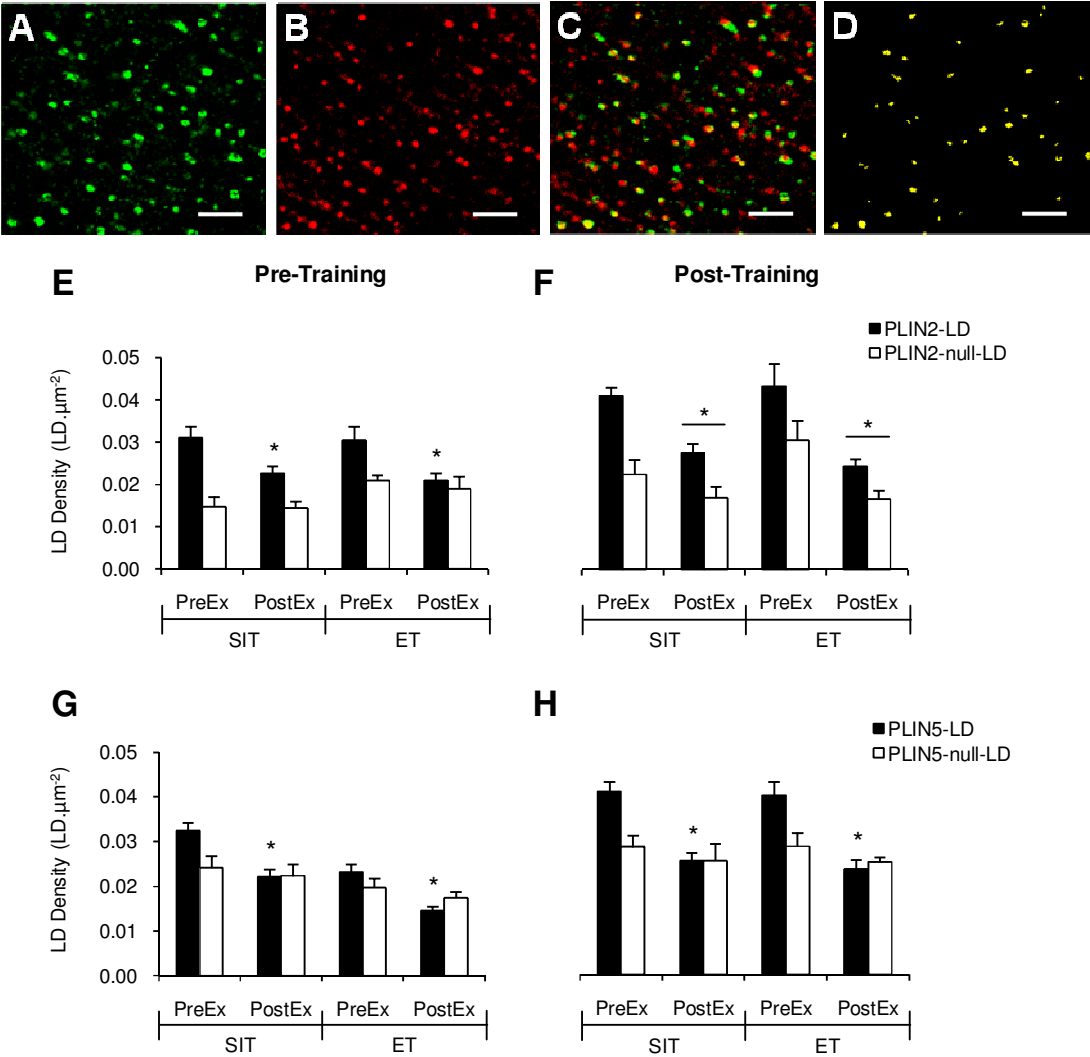


Figure 4



**Supplementary figure 1.**

