

**Training alters the distribution of perilipin proteins in muscle following  
acute free fatty acid exposure**

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**Running head:** Muscle lipid droplet response to lipid infusion

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## Key points summary

- The lipid droplet (LD)-associated perilipin (PLIN) proteins promote IMTG storage, but whether the abundance and association of the PLIN proteins with LDs is related to the diverse lipid storage in muscle between trained and sedentary individuals is unknown.
- We show that lipid infusion augments IMTG content in type I fibres of both trained and sedentary individuals. Most importantly, despite there being no change in PLIN protein content, lipid infusion did increase the number of LDs connected with PLIN proteins in trained individuals only.
- We conclude that trained individuals are able to redistribute the pre-existing pool of PLIN proteins to an expanded LD pool during a lipid infusion, and through this adaptation may support storage of fatty acids in IMTG.

## Abstract

Because the lipid droplet (LD)-associated perilipin (PLIN) proteins promote intramuscular triglyceride (IMTG) storage, we investigated the hypothesis that differential protein content of PLINs and their distribution with LDs may be linked to the diverse lipid storage in muscle between trained and sedentary individuals. Trained ( $n=11$ ) and sedentary ( $n=10$ ) subjects, matched for age, sex and BMI, received either a 6-h lipid or glycerol infusion in the setting of a concurrent hyperinsulinaemic-euglycaemic clamp. Sequential muscle biopsies (0-h, 2-h, 6-h) were analysed using confocal immunofluorescence microscopy for fibre type-specific IMTG content and PLIN associations with LDs. In both groups lipid infusion increased IMTG content in type I fibres (trained: +62%, sedentary: +79%;  $P<0.05$ ), but did not affect PLIN protein content. At baseline, PLIN2 (+65%), PLIN3 (+105%) and PLIN5 (+53%; all  $P<0.05$ ) protein content was higher in trained compared to sedentary individuals. In trained individuals, lipid infusion increased the number of LDs associated with PLIN2 (+27%), PLIN3 (+73%) and PLIN5 (+40%; all  $P<0.05$ ) in type I fibres. In contrast, in sedentary individuals lipid infusion only increased the number of LDs not associated with PLIN proteins. Acute FFA elevation, therefore, induces a redistribution of PLIN proteins to an expanded LD pool in trained individuals only, and this may be part of the mechanism which enables fatty acids to be stored in IMTG.

83    **Keywords**

84    Intramuscular triglyceride, perilipin 2, perilipin 3, perilipin 5, insulin sensitivity

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87    **Abbreviations:**

88    Diacylglycerol (DAG)

89    Free fatty acid (FFA)

90    Glucose infusion rate (GIR)

91    Intramuscular triglyceride (IMTG)

92    Lipid droplet (LD)

93    Perilipin (PLIN)

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## Introduction

Large intramuscular triglyceride (IMTG) stores in skeletal muscle of sedentary individuals are strongly associated with insulin resistance and an elevated risk of developing type 2 diabetes (Kelley *et al.*, 1999; Goodpaster *et al.*, 2001). Even larger IMTG stores are observed in endurance-trained athletes but this occurs in the presence of high insulin sensitivity, a phenomenon termed the “athlete’s paradox” (Goodpaster *et al.*, 2001; van Loon *et al.*, 2004). Research addressing this concept suggests that lipid metabolites, such as diacylglycerols (DAGs) and ceramides (van Loon & Goodpaster, 2006; Samuel & Shulman, 2012), rather than IMTG per se, are mechanistically linked to insulin resistance in sedentary individuals, due to their ability to directly impair insulin signalling (Itani *et al.*, 2002; Adams *et al.*, 2004). Therefore, the metabolic consequences of a lipid overload seems to depend on whether fatty acids taken up by muscle accumulate as DAGs and/or ceramides or are stored as IMTG, as occurs in trained athletes. In support, when trained and sedentary individuals underwent an Intralipid® infusion to acutely raise plasma free fatty acid (FFA) concentrations during a hyperinsulinaemic euglycaemic clamp (Chow *et al.*, 2014), training status modified how the fatty acids were stored in skeletal muscle (Chow *et al.*, 2014). Specifically, fatty acids reflecting the composition of the Intralipid® infusion appeared in IMTG in trained individuals, whereas they accumulated in IMTG and DAGs in sedentary individuals (Chow *et al.*, 2014). The mechanisms by which trained individuals preferentially accumulate IMTG rather than DAGs in the setting of FFA elevation warrants further investigation.

In skeletal muscle, IMTG are stored within cytosolic lipid droplets (LD) which have over 300 proteins incorporated into their phospholipid monolayer (Zhang *et al.*, 2011), the most abundant of which are the perilipin (PLIN) family of proteins. Much of our knowledge of the PLIN proteins in human skeletal muscle is generated from studies in which muscle samples are obtained from overnight-fasted participants under resting conditions. This approach has revealed that the PLIN protein content is higher in type I compared to type II muscle fibres (Shaw *et al.*, 2009; Shepherd *et al.*, 2013; Pourteymour *et al.*, 2015), and that PLIN2 and PLIN5 are observed at both the LD and non-LD locations (Shepherd *et al.*, 2012, 2013). Furthermore, exercise training augments protein levels of PLIN2, PLIN3 and PLIN5 in skeletal muscle alongside a greater IMTG content (Shaw *et al.*, 2012; Shepherd *et al.*, 2013; Shepherd

*et al.*, 2014). This suggests that during exercise training interventions the increase in PLIN protein content is proportional to the increase in IMTG levels. It may also imply that the increase in PLIN protein content plays a mechanistic role in the increased IMTG content in trained individuals. In support, IMTG accumulates in muscle cells overexpressing PLIN3 (Kleinert *et al.*, 2016), and PLIN5 overexpression in primary human myotubes promotes IMTG storage by restricting basal lipolytic rates (Laurens *et al.*, 2016). Moreover, myotubes overexpressing PLIN5 exhibit reduced DAG and ceramide accumulation in response to a palmitate overload (Laurens *et al.*, 2016), and similarly when rats are fed a high-fat diet, muscle-specific overexpression of PLIN2 (Bosma *et al.*, 2012a) or PLIN5 (Bosma *et al.*, 2012a; Bosma *et al.*, 2013) promotes IMTG storage with no accumulation of DAG. Collectively, these data suggest that the PLIN proteins may play an important role in enabling excess fatty acids to be stored in IMTG.

Studying the PLIN proteins under ‘dynamic’ conditions, where IMTG levels are altered independently of changes in PLIN protein content, may provide further insight into their potential roles. In accordance, we have shown that LDs containing either PLIN2 (Shepherd *et al.*, 2012) or PLIN5 (Shepherd *et al.*, 2012, 2013) are preferentially used during moderate-intensity exercise, thereby highlighting a potential role for the PLIN proteins in the breakdown and oxidation of IMTG. Gemmink *et al.* (Gemmink *et al.*, 2016) recently reported that prolonged fasting in trained individuals augmented IMTG content and increased the quantity of PLIN5 in contact with LDs, suggesting that the pre-existing PLIN5 pool is redistributed across the LD pool when it expands. Furthermore, only an increase in the number and size of LDs that contained PLIN5 occurred (Gemmink *et al.*, 2016), suggesting a role for PLIN5 in mediating IMTG storage. This could be one mechanism for IMTG storage in muscle, which could be modified by endurance training. It is yet to be investigated if a similar redistribution of other PLIN proteins occurs under conditions of elevated FFA exposure during a hyperinsulinaemic euglycaemic clamp.

In order to further elucidate the roles of the PLIN proteins in skeletal muscle, the present study aimed to determine the effect of endurance training, compared to a sedentary condition, on the dynamic

behaviour of LDs and PLIN proteins during acute, moderate FFA elevation concurrent to a hyperinsulinaemic-euglycaemic clamp. Specifically, we used our previously described method (Shepherd *et al.*, 2012, 2013) to identify changes in LDs that either contained (PLIN+ LDs) or were devoid of PLIN (PLIN- LDs) during the lipid infusion. Lipid accumulation in muscle is not uniform across fibre types and therefore all analyses were performed on a fibre-type specific basis. Importantly, the lipid infusion was undertaken in the setting of hyperinsulinaemia to maximise skeletal muscle fatty acid uptake (Dyck *et al.*, 2001; Chabowski *et al.*, 2004), suppress lipase activity (Holm *et al.*, 2000) and drive TAG synthesis (Muoio *et al.*, 1999; Dyck *et al.*, 2001). Therefore, we investigated the hypothesis that in response to simultaneous infusion of Intralipid<sup>®</sup> and insulin, the increase in IMTG storage in trained individuals would be accompanied by a redistribution of the cellular pool of PLIN proteins across the expanded LD pool.

## Materials and Methods

### *Participants and ethical approval*

The samples used in this study were collected as part of a previous study (Chow *et al.*, 2012; Chow *et al.*, 2014) and therefore a portion of the demographic data in Table 1 has been previously presented (Chow *et al.*, 2012; Chow *et al.*, 2014). In this study, twenty one young, healthy, lean participants who were either trained (n=11) or sedentary (n=10) (see Table 1 for characteristics) were included and matched for sex, age ( $\pm 5$  yr) and BMI ( $\pm 1.5$  kg.m<sup>2</sup>). The International Physical Activity Questionnaire was used to classify individuals as sedentary (30 minutes or less of exercise per week) or trained (history of aerobic training, preferably running, at  $\geq 45$  min/day,  $\geq 5$  days/wk) (Craig *et al.*, 2003). The study protocol adhered to the Declaration of Helsinki and was approved by the University of Minnesota Institutional Review Board and written, informed consent was obtained from all participants.

### *Experimental protocol*

The protocol for the study has been described in detail previously (Chow *et al.*, 2012; Chow *et al.*, 2014). Briefly, after undergoing assessments of aerobic exercise capacity (VO<sub>2max</sub>), body composition (dual energy X-ray absorptiometry) and insulin sensitivity (3 h hyperinsulinaemic euglycaemic clamp), participants attended the Masonic Clinical Research Unit at the University of Minnesota on a separate day and consumed a standardised evening meal (41% carbohydrate, 32% fat, 27% protein) and remained on bed rest at the unit until study completion the following day. Following an overnight fast, a muscle biopsy (Bx1) was obtained from the *vastus lateralis* under local anaesthesia, after which participants underwent 6 h infusion of either lipid (20% Intralipid<sup>®</sup> at 90 ml.h<sup>-1</sup>) or glycerol (2.25 g.100 ml<sup>-1</sup> at 90 ml.h<sup>-1</sup>). The glycerol infusion matched the glycerol content of the lipid infusion. Participants received either a lipid or glycerol infusion to maintain matching between activity groups. Simultaneous to the lipid or glycerol infusion, a 6 h hyperinsulinaemic euglycaemic clamp was initiated (1.5 mIU.kg free fat mass<sup>-1</sup>.min<sup>-1</sup>, KPO<sub>4</sub> at 50 ml.h<sup>-1</sup>, dextrose titrated to maintain glucose at 4.7-5.3 mmol.l<sup>-1</sup>). After starting the lipid or glycerol infusion, a second muscle biopsy (Bx2; 120 min) was obtained from a proximal incision of the same leg, with a third biopsy (Bx3; 360 min) being obtained from the contralateral leg. Each muscle biopsy was dissected free of fat and connective tissue before being



embedded in Tissue-Tek OCT Compound (Sakura Finetek Europe) and frozen in liquid nitrogen-cooled isopentane for immunohistochemical analyses.

## ***Muscle analysis***

### *Immunohistochemistry*

Serial 5 µm cryosections were cut at -30°C and transferred on to ethanol-cleaned glass slides, fixed in 3.7% formaldehyde and permeabilized for 5 min in 0.5% Triton-X 100, followed by washing in phosphate-buffered saline (PBS) and then incubated for 1 h with primary antibodies targeting either PLIN2, PLIN3, PLIN4 or PLIN5 (see antibody section for details) in combination with a myosin antibody for slow-twitch fibres. After washing with PBS, sections were subsequently incubated with appropriate Alexa Fluor secondary antibodies for 30 min, washed in PBS again, and then incubated with BODIPY 493/503 (Invitrogen, Paisley, UK) in order to image and quantify IMTG. Cover slips were mounted with Vectashield mounting medium (H-1000, Vector Laboratories, Burlingame, CA, USA) and sealed with nail varnish. Fibre type-specific protein expression of PLIN2, PLIN3, PLIN4 and PLIN5 was assessed using the same protocol but with the omission of BODIPY 493/503 from the procedure, as previously described (Shepherd *et al.*, 2012, 2013).

### *Antibodies*

The following primary antibodies were used: guinea pig polyclonal anti-XPAT (PLIN5) and guinea pig polyclonal anti-S3-12 (PLIN4: both Progen Biotechnik, Germany), mouse monoclonal anti-adipophilin (PLIN2: American Research Products, MA, USA), rabbit polyclonal anti perilipin 3/TIP-47 (PLIN3: Novus Biologicals, Cambridge, UK), mouse anti-myosin antibody for slow twitch fibres (A4.840-c, DSHB, developed by Dr. Blau), wheat germ agglutinin Alexa Fluor 350 conjugate (Invitrogen, Paisley, UK). Appropriate Alexa Fluor secondary antibodies were obtained from Invitrogen (Paisley, UK).

### *Image capture, processing and data analysis*

Images of cross-sectionally orientated sections were used for determining fibre type-specific differences in the protein expression of IMTG, PLIN2, PLIN3, PLIN4, and PLIN5. Images were captured using an inverted confocal microscope (Zeiss LSM710, Carl Zeiss AG, Oberkochen, Germany) with a 40x 0.75 NA oil immersion objective. An argon laser was used to excite the Alexa Fluor 488 fluorophore and BODIPY, whilst a helium-neon laser excited the Alexa Fluor 546 and 633 fluorophores. Images to assess LD characteristics and co-localisation with either PLIN2, PLIN3, PLIN4 or PLIN5 were captured with the same system but using an 8x digital zoom. When assessing fibre-specific content of IMTG and individual PLIN proteins, fibres stained positively for myosin heavy chain type I were classified as type I fibres, whereas those with no staining were classified as type II fibres.

Image processing was undertaken using Image-Pro Plus 5.1 Software (Media Cybernetics, MD, USA). To assess fibre type distribution of IMTG and each PLIN protein, between 8 and 12 images were used per muscle section, resulting in a similar proportion of fibres being analysed for each trained ( $97 \pm 10$  type I fibres,  $118 \pm 15$  type II fibres) and sedentary participant ( $105 \pm 17$  type I fibres,  $125 \pm 17$  type II fibres). An intensity threshold was uniformly selected to represent a positive signal for IMTG and each PLIN protein. The content of IMTG and each PLIN protein was expressed as the positively stained area fraction relative to the total area of each muscle fibre. Images captured at 8x digital zoom were used to identify changes in LD density (number of LDs expressed relative to area) and LD size (mean area of individual LDs).

Co-localisation analysis was performed separately for each PLIN protein with IMTG, as described previously for PLIN2 and PLIN5 (Shepherd *et al.*, 2013). Briefly, a positive signal for the PLIN protein of interest and IMTG in sequential images was obtained by selecting a uniform intensity threshold (Fig. 1A-C). Based on the selected threshold, binary images were created and subsequently used for co-localisation analysis. A co-localisation map displaying the merged images was generated (Fig. 1D), with the overlapping regions subsequently extracted to a separate image (Fig. 1E). First, the total number of extracted objects in this image as a proportion of the total number of PLIN2 objects (or

PLIN3, PLIN4 or PLIN5 objects, depending on the analysis performed) was used as a measure of co-localisation. The number of extracted objects was expressed relative to area and therefore represents the density of PLIN-associated LDs (PLIN+ LDs). In addition, the number of extracted objects was subtracted from the total number of LDs, and expressed relative to area, to quantify the density of LDs not associated with PLIN (PLIN- LDs). Finally, the number of extracted objects was subtracted from the total number of PLIN objects (an expressed relative to area) to determine the density of “free PLIN”. The PLIN proteins do not always form a continuous structure around the LD, and are therefore observed as several discrete structures on the LD. Consequently, it is possible that two or more extracted objects are derived from the same LD leading to an overestimation of the PLIN+ LDs. This possibility was accounted for during the colocalisation analysis. As previously described (Shepherd *et al.*, 2012, 2013), several controls were performed to check for bleed through, non-specific secondary antibody binding and autofluorescence before co-localisation analysis was performed.

### ***Statistical analysis***

All baseline data are reported as means  $\pm$  S.E.M. A two-tailed t-test was used to determine significant differences at baseline between groups. Multiple group comparisons of LD, PLIN protein expression and LD-PLIN co-localisation variables were performed as follows: 1) trained lipid vs. trained glycerol, 2) sedentary lipid vs. sedentary glycerol, 3) trained lipid vs. sedentary lipid, and 4) trained glycerol vs. sedentary glycerol. Linear mixed effects models, with random intercepts to account for repeated measurements within subjects, were used to examine these group differences, as well as differences over the infusion (across biopsies) and between fibre types. LD, PLIN protein expression and LD-PLIN co-localisation variables are reported as least square means  $\pm$  S.E.M. Pairwise differences between biopsies were performed using post hoc tests. Significance was set at the 0.05 level of confidence. All analyses were conducted with SAS (version 9.2; SAS Institute, Cary, NC).

## Results

### *Baseline characteristics*

As expected due to matching, there was no differences between sedentary and trained participants for age and BMI (Table 1). Trained participants displayed lower body fat, and higher free-fat mass,  $\text{VO}_{2\text{max}}$  and glucose infusion rate (GIR) compared to sedentary participants (Table 1).

At baseline, all PLIN proteins displayed a fibre type-specific distribution, such that PLIN protein content (expressed as % area stained; Fig. 2) was significantly greater in type I fibres compared with type II fibres, irrespective of training status ( $P<0.05$ ). Compared to sedentary participants, trained participants had higher PLIN2, PLIN3, and PLIN5 protein content ( $P<0.05$ ) in both type I and type II fibres (Fig. 2). A trend for greater PLIN4 protein content in type I fibres of trained participants ( $P=0.06$ ; Fig. 2C) compared to sedentary participants was also observed, whereas no differences between groups was evident for PLIN4 content in type II fibres. IMTG content was significantly greater in type I fibres compared with type II fibres in both trained and sedentary participants ( $P<0.001$ ), but overall IMTG content was similar between groups (Fig. 3A).

### *Lipid droplet responses to lipid infusion*

During the hyperinsulinaemic euglycaemic clamp, lipid infusion elevated FFA concentrations, although this occurred to a greater degree in sedentary ( $932\pm105 \mu\text{mol.L}^{-1}$ ) compared to trained participants ( $600\pm86 \mu\text{mol.L}^{-1}$ ;  $P=0.03$ , Table 1). The glycerol infusion combined with the hyperinsulinaemic euglycaemic clamp led to comparable suppression of FFA concentrations between groups ( $P=0.91$ ). Lipid infusion significantly increased IMTG content in type I fibres of both trained (+62%;  $P=0.001$  for Bx3 vs. Bx1) and sedentary participants (+79%;  $P=0.02$  for Bx3 vs. Bx1), with no differences between groups (Fig. 3A). In trained participants, the greater IMTG content following lipid infusion was attributed to an increase in both LD density (+97%;  $P=0.01$  for Bx3 vs. Bx1) and LD size (+22%;  $P=0.03$  for Bx3 vs. Bx1), whereas in type I fibres of sedentary participant's lipid infusion only led to an increase in LD density (+64%;  $P=0.03$  for Bx3 vs. Bx1; Fig. 3B & C). Interestingly, lipid infusion also increased LD size in type II fibres of trained participants (+64%;  $P=0.03$  for Bx3 vs. Bx1; Fig. 3C),

but this did not result in a significant overall increase in IMTG content in type II fibres. Furthermore, IMTG content was not elevated in type II fibres of sedentary participants following lipid infusion.

#### ***Lipid droplet and PLIN protein co-localisation***

Lipid infusion had no effect on the protein content of any of the PLINs in either trained or sedentary participants ( $P>0.05$ ; Fig. 2). We next investigated the association between LD and each PLIN protein by expressing the number of overlapping objects relative to the total number of PLIN protein objects (Fig. 1). Further, we also determined the number of LDs that either contained (PLIN+ LD) or were devoid of each PLIN protein (PLIN- LD) in response to the combined lipid and insulin infusion. The results of these analyses are detailed below.

***PLIN2:*** At baseline, the fraction of PLIN2 co-localised with IMTG was similar between the groups in type I fibres (Trained:  $0.65\pm0.02$ , Sedentary  $0.68\pm0.02$ ), and lipid infusion did not change this relationship. However, lipid infusion did lead to an increase in the number of PLIN2+ LDs in trained participants (+27%;  $P=0.01$  for Bx3 vs. Bx1; Fig. 4A) but no changes occurred in the sedentary group. In contrast, the number of PLIN2- LDs was elevated by lipid infusion by a similar degree in both groups (Trained: +69%, Sedentary: +69%;  $P<0.05$  for Bx3 vs. Bx1 for both groups; Fig. 4B). In type II fibres, the fraction of PLIN2 co-localised with IMTG was similar between the groups at baseline (Trained:  $0.59\pm0.03$ , Sedentary  $0.65\pm0.04$ ). Lipid infusion increased PLIN2 co-localisation with IMTG from baseline ( $0.59\pm0.03$ ) to post-infusion ( $0.71\pm0.03$ ) in type II fibres in trained participants only (+21%;  $P=0.001$  for Bx3 vs. Bx1). This was accounted for by a 33% significant decrease in free PLIN2 in response to lipid infusion ( $0.006\pm0.002$  vs.  $0.005\pm0.001$  PLIN2 objects. $\mu\text{m}^2$  for Bx1 and Bx3, respectively;  $P=0.004$ ).

***PLIN3:*** There was no difference between trained and sedentary groups when comparing the fraction of PLIN3 co-localised with IMTG in both type I (Trained:  $0.67\pm0.04$ , Sedentary  $0.64\pm0.04$ ) and type II fibres (Trained:  $0.62\pm0.05$ , Sedentary:  $0.61\pm0.05$ ;  $P>0.05$ ), and this relationship was unchanged by lipid

infusion. However, lipid infusion did lead to an increase in the number of PLIN3+ LDs in type I fibres of trained participants (+73%;  $P=0.004$  for Bx3 vs. Bx1; Fig. 5A), whereas no changes occurred in the sedentary group. In contrast, in sedentary participants lipid infusion augmented the number of PLIN3- LDs (+133%;  $P<0.001$  for Bx3 vs. Bx1; Fig. 5B) in type I fibres, with no changes in the trained group. Furthermore, no changes in the number of PLIN3+ or PLIN3- LDs occurred in type II fibres in either group in response to lipid infusion.

*PLIN4*: A similar fraction of PLIN4 co-localised with IMTG when comparing trained and sedentary groups at baseline in both type I (Trained:  $0.74\pm0.05$ , Sedentary  $0.70\pm0.06$ ) and type II fibres (Trained:  $0.66\pm0.06$ , Sedentary  $0.66\pm0.07$ ;  $P>0.05$ ). Lipid infusion had no effect on this relationship, and no increase in PLIN4+ LDs was observed in either group (Fig. 6A & C). However, the number of PLIN4- LDs was elevated in type I fibres following lipid infusion in both trained (+55%;  $P=0.005$  for Bx3 vs. Bx1) and sedentary participants (+94%;  $P=0.02$  for Bx3 vs. Bx1; Fig. 6B).

*PLIN5*: The fraction of PLIN5 co-localised with IMTG was similar between trained and sedentary groups at baseline in both type I (Trained:  $0.64\pm0.04$ , Sedentary  $0.58\pm0.04$ ;  $P>0.05$ ) and type II fibres (Trained:  $0.63\pm0.03$ , Sedentary  $0.61\pm0.04$ ;  $P>0.05$ ). Lipid infusion led to an increase in PLIN5 co-localised with IMTG from baseline ( $0.58\pm0.04$ ) to post-infusion ( $0.71\pm0.03$ ) in type I fibres of sedentary participants only (+21%;  $P=0.013$  for Bx3 vs. Bx1). This occurred alongside a trend for a decrease in free PLIN5 in response to lipid infusion ( $0.011\pm0.002$  vs.  $0.008\pm0.002$  for Bx1 and Bx3, respectively;  $P=0.089$ ). Notably, however, the number of PLIN5+ LDs was only augmented by lipid infusion in type I fibres of trained participants (+40;  $P=0.006$  for Bx3 vs. Bx1; Fig. 7A), whereas the number of PLIN5- LDs was elevated by lipid infusion only in type I fibres of sedentary participants (+123%;  $P=0.03$  for Bx3 vs. Bx1; Fig. 7B).

## Discussion

This study examined the effect of training on the muscle LD pool and the association of the PLIN proteins with LDs in response to acute FFA elevation (through infusion of Intralipid<sup>®</sup>) alongside a hyperinsulinaemic-euglycaemic clamp. The major novel observations are that acutely elevating FFA concentrations alongside hyperinsulinaemia: 1) augments IMTG content in type I fibres of both trained and sedentary individuals, but that increases in the number and/or size of LDs are dependent on training status, 2) increased the number of LDs associated with PLIN2, PLIN3 and PLIN5 in trained individuals, and 3) led to the accumulation of LDs that were not associated with any of the PLIN proteins in sedentary individuals. Together, and in line with our hypothesis, these data indicate that the ability to redistribute PLIN proteins to the expanded LD pool under conditions stimulating IMTG synthesis is unique to trained individuals.

The first novel finding of the present study was that acute FFA elevation and insulin infusion led to an increase in IMTG content that was specific to type I fibres in both trained and sedentary individuals. Previously, IMTG content was not significantly elevated by acute FFA elevation when analysed in whole muscle homogenates (Chow *et al.*, 2014), highlighting the importance of considering fibre type-specific responses when investigating adaptations to the intramuscular lipid pool. Hyperinsulinaemia will increase fatty acid uptake into muscle (Dyck *et al.*, 2001; Chabowski *et al.*, 2004), suppress intramuscular lipase activity (Holm *et al.*, 2000), and enhance fatty acid triacylglycerol esterification (Muoio *et al.*, 1999; Dyck *et al.*, 2001), and when combined with acute FFA elevation it is likely that these conditions underpin the net increase in IMTG content. It is notable that the increase in IMTG content in trained individuals was attributable to both a greater LD number and size, whereas only an increase in LD number could explain the higher IMTG content following acute FFA elevation in sedentary participants. An increase in LD number could be deemed advantageous since this would maintain a high LD surface area to volume ratio, thereby providing a greater surface area available for LD regulatory proteins (such as PLINs) to support fatty acid storage and mobilisation relative to metabolic demand. A combined increase in LD number and size in trained individuals was reported in a recent study using prolonged fasting to physiologically raise plasma FFA concentrations (Gemink

*et al.*, 2016). As well as synthesising new LDs, expanding the size of LDs, may be an additional mechanism by which trained individuals are able to sequester excess plasma FFA into IMTG.

Under resting conditions, the expression of PLIN2 and PLIN5 is closely related to IMTG content (Minnaard *et al.*, 2009; Amati *et al.*, 2011; Peters *et al.*, 2012; Shepherd *et al.*, 2013), although not all PLIN2 or PLIN5 is associated with the IMTG pool (Shepherd *et al.*, 2012, 2013). The results of the present study now show that this partial co-localisation with IMTG also exists for PLIN3 and PLIN4. In response to acute FFA elevation, IMTG levels were increased independent of changes in PLIN protein content. We therefore investigated whether acute FFA elevation altered the fraction of each PLIN associated with IMTG, and for each PLIN protein we describe two pools of LDs: (1) PLIN-associated LDs (PLIN+ LD), and (2) LDs that do not contain PLIN (PLIN- LDs). The major novel finding of the present study was that in trained individuals, acute FFA elevation led to an increase in the number of PLIN2+, PLIN3+ and PLIN5+ LDs specifically in type I fibres. Furthermore, this occurred in the absence of a change in the fraction of any PLIN protein associated with IMTG. Gemmink *et al.* (2016) recently showed that in trained individuals elevating plasma FFA concentrations through prolonged fasting also led to a greater number of PLIN5+ LDs, but this was accompanied by an increase in the fraction of PLIN5 associated with IMTG. Since PLIN5 protein expression was unaltered by fasting in this study, the authors concluded that prolonged fasting led to a redistribution of cytosolic PLIN5 to the LD surface (Gemmink *et al.*, 2016). We now provide evidence that in trained individuals PLIN2, PLIN3 and PLIN5 are all redistributed across the expanded LD pool following acute FFA elevation. However, as we did not observe a change in the fraction of any PLIN protein associated with IMTG, it appears that PLIN2, PLIN3 and PLIN5 are redistributed from pre-existing PLIN+ LDs to either newly-synthesised LDs and/or pre-existing PLIN- LDs. As a result, the proportion of the expanded LD pool containing these PLIN proteins was maintained.

The redistribution of PLIN2, PLIN3 and PLIN5 in trained individuals may be an important adaptation to enable FFA storage as IMTG in LDs. In support, palmitate incorporation into IMTG is reduced when PLIN2 is knocked-down in cultured muscle cells (Bosma *et al.*, 2012a), whereas PLIN3 overexpression



in muscle cells increases palmitate incorporation into IMTG (Kleinert *et al.*, 2016). Furthermore, muscle-specific overexpression of PLIN2 (Bosma *et al.*, 2012a) or PLIN5 (Bosma *et al.*, 2013) enhances IMTG storage in rats fed a high-fat diet. PLIN5 overexpression in primary human myotubes also leads to an increase in IMTG content by restricting rates of basal lipolysis (Laurens *et al.*, 2016). In this regard, there is a large body of evidence obtained in a number of different cell types demonstrating that the PLIN proteins support triacylglycerol storage by regulating basal lipolytic rates (reviewed in MacPherson & Peters, 2015). Through this role, PLIN5 (and PLIN2 and PLIN3) may lead to enlargement of LDs, and could therefore explain the observed increase in LD size in trained individuals. PLIN2 also has been observed to cluster at specific locations in the endoplasmic reticulum membrane where LD biogenesis occurs (Robenek *et al.*, 2006). In trained individuals, part of the PLIN2 protein pool may therefore be redistributed to the membrane of the endoplasmic reticulum in response to acute FFA elevation to support the synthesis of new LDs. Maintaining the proportion of the LD pool that contains PLIN2, PLIN3 and PLIN5 may also be important to support mobilisation and oxidation of IMTG-derived FAs when metabolic demand increases. We have shown that both PLIN2+ and PLIN5+ LDs (Shepherd *et al.*, 2013) are preferentially targeted for breakdown during exercise. This is in line with studies in cultured cells demonstrating that PLIN5 overexpression enhances triacylglycerol hydrolysis and fat oxidation, possibly by recruiting LDs to the mitochondrial network (Wang *et al.*, 2011; Bosma *et al.*, 2012b; Laurens *et al.*, 2016). A positive association is also reported between PLIN3 expression and both whole-body fat oxidation (Covington *et al.*, 2014) and *ex vivo* palmitate oxidation (Covington *et al.*, 2014; Covington *et al.*, 2015), and PLIN3 is observed in the mitochondrial fraction of sedentary and endurance-trained rats (Ramos *et al.*, 2015), suggesting that PLIN3 plays a role in IMTG oxidation. Taken together, these data suggest that redistributing PLIN2, PLIN3 and PLIN5 in response to acute FFA elevation would confer a metabolic advantage by maintaining a metabolically flexible intramuscular LD pool.

In contrast to the trained group, acute FFA elevation led to an increase in the number of PLIN2-, PLIN3-, PLIN4-, and PLIN5- LDs in sedentary individuals, suggesting that no redistribution of these proteins occurred. The protein content of all PLINs was lower in sedentary individuals compared to the trained

group, and therefore it is possible that sedentary individuals have a reduced capacity to redistribute PLINs when the muscle LD pool expands. Knockdown of PLIN2 in cultured muscle cells leads to increased palmitate incorporation into DAG (Bosma *et al.*, 2012a), whereas rats fed a high-fat diet accumulated IMTG, with no changes in DAG, but only when PLIN2 (Bosma *et al.*, 2012a) or PLIN5 (Bosma *et al.*, 2013) was overexpressed in muscle. PLIN2 and PLIN5 therefore appear to be important in channelling fatty acids into IMTG. The lack of a redistribution and/or lower abundance of these proteins in the present study may explain our previous finding that fatty acids reflecting the composition of an Intralipid® infusion appeared in IMTG and DAGs in sedentary individuals and only IMTG in trained individuals (Chow *et al.*, 2014).

Although IMTG content was unchanged in type II fibres, LD size did increase in trained individuals following acute FFA elevation. In addition, the fraction of PLIN2 associated with IMTG was increased, and therefore PLIN2 might also be redistributed to LDs in type II fibres, possibly to ensure that the surface coverage of larger LDs is maintained. Although PLIN2 is considered to be predominantly found at the LD surface (Prats *et al.*, 2006; Wolins *et al.*, 2006; Bell *et al.*, 2008), PLIN2 has also been observed at the endoplasmic reticulum (Robenek *et al.*, 2006), and may cycle between the cytosolic and LD fractions (Robenek *et al.*, 2006; Wang *et al.*, 2009). Since there was a significant decrease in PLIN2 that was unbound to LDs (free PLIN2) following acute FFA elevation, it is possible therefore that in type II fibres PLIN2 that was not previously associated with LDs became connected to the LD pool. Similarly, in sedentary individuals acute FFA elevation led to an increase in the fraction of PLIN5 associated with LDs in type I fibres. Like the aforementioned changes in PLIN2 in type II fibres of trained individuals, it is possible that PLIN5 that was previously not associated with LDs was recruited to the LD pool in type I fibres of sedentary individuals, especially as there was a tendency for PLIN5 that was unbound to LDs to decrease. However, because in sedentary individuals only an increase in the number of PLIN5- LDs was observed, it is likely that previously unbound PLIN5 was recruited to LDs already coated with PLIN5.

Little is known about the role of PLIN4 in skeletal muscle, although we report for the first time here that PLIN4 expression is ~2-fold greater in type I compared to type II fibres, and that trained individuals exhibit higher protein expression of both PLIN4 compared to sedentary individuals. These observations are in accordance with the fibre type distribution of the other PLINs in skeletal muscle, and findings of greater PLIN2, PLIN3 and PLIN5 expression in muscle in response to chronic training (Peters *et al.*, 2012; Shaw *et al.*, 2012; Louche *et al.*, 2013; Shepherd *et al.*, 2013; Shepherd *et al.*, 2014). Despite the higher PLIN4 expression in trained individuals, we only observed an increase in PLIN4- LDs suggesting that no redistribution of PLIN4 occurred in response to acute FFA elevation. Future studies will determine the precise role of PLIN4 in human skeletal muscle.

The use of validated immunofluorescence microscopy techniques (Shepherd *et al.*, 2012, 2013) to examine fibre type-specific changes in LD characteristics and the associations of PLIN proteins with LDs in response to acute FFA elevation is a clear strength of this study. Applying these techniques to samples obtained under ‘dynamic’ conditions has provided further insight into the potential role of the PLIN proteins in muscle. Our colocalisation assays, however, only permit fibre-specific analysis of the association between LDs and a single PLIN protein. This is important to acknowledge because acute FFA elevation increased PLIN4- LDs in trained and sedentary individuals, and increased both PLIN2+ and PLIN2- LDs in trained individuals. One possibility is that PLIN- LDs are actually newly-formed LDs that have not yet acquired sufficient PLIN protein to exceed the lower detection limit of the microscope. It is also possible that PLIN4- LDs and PLIN2- LDs were in fact coated with PLIN3 and/or PLIN5. Co-localisation analysis of PLIN2 and PLIN5 in rat skeletal muscle demonstrated only a partial overlap between the two proteins (Macpherson *et al.*, 2012), suggesting that some, but not all LDs, have both of these proteins associated with them. Whether there are distinct pools of LDs in skeletal muscle that have all, some, or none of the PLIN proteins associated remains to be determined.

Although exercise capacity was significantly different between the trained and sedentary participants, the mean  $\text{VO}_{2\text{max}}$  for the trained group was lower than that reported for previously published ‘trained’ groups (van Loon *et al.*, 2004; Amati *et al.*, 2011), which may explain the lack of difference in baseline

IMTG between trained and sedentary participants that we observed compared with previous literature (Goodpaster *et al.*, 2001; Amati *et al.*, 2011). We consider this a strength of the study because it demonstrates that only a small increase in exercise capacity, as can be achieved using exercise training, can improve the ability to redistribute PLIN proteins to LDs during acute FFA elevation and channel fatty acids into IMTG. In addition, since we had matched for age, gender and BMI, the trained participants consistently had a higher  $\text{VO}_{2\text{max}}$  than their matched counterparts and therefore our findings were still consistent with a training effect. A further strength of the study is the use of a glycerol infusion as a control, as this approach enabled the specific examination of the effect of acute FFA elevation in trained and sedentary individuals. Previous studies conducted in trained individuals using a saline infusion lacked such control (Matzinger *et al.*, 2002; Schenk *et al.*, 2009).

In conclusion, this study has generated novel evidence that acute FFA elevation concurrent to a hyperinsulinaemic-euglycaemic clamp does not change PLIN protein content in skeletal muscle, but rather leads to a redistribution of PLIN2, PLIN3 and PLIN5 to an expanded LD pool in trained individuals only. In contrast, no redistribution of PLIN proteins occurs in sedentary individuals. This may be part of the mechanism by which trained individuals are able to channel fatty acids into IMTG.

**Additional information**

***Competing interests***

The authors have no conflicts of interest to disclose.

***Author contributions***

SOS, QW and LSC: conception and design of the experiments. SOS, JAS, QW, DGM and LSC: collection, analysis and interpretation of data. SOS, JAS, QW, JJD, BG, DGM and LSC: drafting and revising the manuscript. All authors have read and approved the final submission.

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## Tables

**Table 1.** *Baseline characteristics of trained and sedentary participants*

	Trained (n=11)	Sedentary (n=10)	<i>P</i> value
Sex (males/females)	6/5	4/6	0.98
Age (y)	23 ± 1	21 ± 1	0.26
BMI (kg.m <sup>-2</sup> )	22.2 ± 0.6	21.3 ± 0.6	0.31
FFM (kg)	50.8 ± 3.7	40.9 ± 2.3	0.04
Body fat (%)	19.9 ± 2.0	27.4 ± 3.5	0.07
VO <sub>2max</sub> (ml.kg <sup>-1</sup> .min <sup>-1</sup> )	47.8 ± 2.0	38.0 ± 1.6	<0.01
Baseline GIR (μmol glucose infused.kg FFM <sup>-1</sup> .min <sup>-1</sup> )	66.1 ± 4.7	48.3 ± 5.7	0.03
FFA at end of 6 hr lipid infusion (μmol.L <sup>-1</sup> )	600 ± 86	932 ± 105	0.03

Values are means ± S.E.M. BMI, body mass index; FFM, free fat mass; GIR, glucose infusion rate; FFA, free fatty acids.

## Figure Legends

**Figure 1.** Representative immunofluorescence images co-stained for IMTG and PLIN5 that were used for colocalisation analysis from a trained participant. Images for colocalisation analysis were obtained at 8x zoom from the central region of a cell (indicated by the white box; A). IMTG were stained with BODIPY 493/503 (green; B), PLIN5 was stained red (C) and the subsequent merged images (D) were used to calculate colocalisation. Image E shows the extracted overlying area and was used to calculate the relative association of PLIN5 with IMTG, and determine the number of PLIN5+ and PLIN5- LDs. Note that PLIN5 is associated with the majority, but not all, LDs. Images were obtained at 8x zoom (white bar = 5  $\mu$ m), except for A (2x zoom; white bar = 25  $\mu$ m). All images were obtained with the same resolution (1024 x 1024 pixels). The same method was used for colocalisation analysis for PLIN2, PLIN3 and PLIN4.

**Figure 2.** A 6-h Intralipid infusion did not alter fibre-specific PLIN2 (A), PLIN3 (B), PLIN4 (C) and PLIN5 (D) protein expression in trained and sedentary individuals. Fibre type-specific content of PLIN2, PLIN3 (E), PLIN4 (F) and PLIN5 was quantified from immunofluorescence images, where myosin heavy chain I (MHC I) (stained red) was combined with wheat germ agglutinin Alexa Fluor 350 (WGA) to identify the cell border (stained blue) in skeletal muscle (G & H). Positively stained fibres (red) are type I fibres, all other fibres are assumed to be type II fibres. White bars represent 50  $\mu$ m. \*Significantly different compared to sedentary group ( $P<0.05$ ). <sup>†</sup>Significantly different than type I fibres ( $P<0.001$ ). There was a trend ( $P=0.06$ ) for a difference in PLIN4 content between trained and sedentary participants in type I fibres only.

**Figure 3.** A 6-h Intralipid infusion alters fibre-specific IMTG content (A), LD density (B) and LD size (C). Fibre type-specific content of IMTG was quantified from immunofluorescence images of muscle sections obtained at baseline (Bx1; D), and after 120 min (Bx2; E) and 360 min (Bx3; F) of lipid or glycerol infusion. Panels G-I are corresponding images of myosin heavy chain I (MHC I) (stained red) in combination with wheat germ agglutinin Alexa Fluor 350 (WGA) to identify the cell border (stained

blue) in skeletal muscle. Positively stained fibres (red) are type I fibres, all other fibres are assumed to be type II fibres. White bars represent 50  $\mu$ m. \*Significantly different from Bx1 given fitness category and infusion status ( $P<0.05$ ). <sup>†</sup>Significantly different than type I fibres ( $P<0.001$ ).

**Figure 4.** A 6-h lipid infusion alters the number of LDs with PLIN2 associated (PLIN2+ LDs) or not associated (PLIN2- LDs) differently between trained and sedentary individuals. Analysis was performed in type I (A, B) and type II fibres (C, D). \*Significantly different from Bx1 given fitness category and infusion status ( $P<0.05$ ). <sup>†</sup>Significantly different between glycerol and lipid infusion within fitness category for equivalent biopsies.

**Figure 5.** A 6-h lipid infusion alters the number of LDs with PLIN3 associated (PLIN3+ LDs) or not associated (PLIN3- LDs) differently between trained and sedentary individuals. Analysis was performed in type I (A, B) and type II fibres (C, D). \*Significantly different from Bx1 given fitness category and infusion status ( $P<0.05$ ). <sup>†</sup>Significantly different between glycerol and lipid infusion within fitness category for equivalent biopsies.

**Figure 6.** A 6-h lipid infusion does not alter the number of LDs with PLIN4 associated (PLIN4+ LDs) but does increase the number of LDs without PLIN4 associated (PLIN4- LDs) in trained and sedentary individuals. Analysis was performed in type I (A, B) and type II fibres (C, D). \*Significantly different from Bx1 given fitness category and infusion status ( $P<0.05$ ).

**Figure 7.** A 6-h lipid infusion alters the number of LDs with PLIN5 associated (PLIN5+ LDs) or not associated (PLIN5- LDs) differently between trained and sedentary individuals. Analysis was performed in type I (A, B) and type II fibres (C, D). \*Significantly different from Bx1 given fitness category and infusion status ( $P<0.05$ ). <sup>†</sup>Significantly different between glycerol and lipid infusion within fitness category for equivalent biopsies.















