

**Lipid droplet remodelling and reduced muscle ceramides following sprint interval and moderate-intensity continuous exercise training in obese males**

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## Abstract

**Background:** In obesity, improved muscle insulin sensitivity following exercise training has been linked to the lowering of diacylglycerol (DAG) and ceramide concentrations. Little is known, however, about how improved insulin action with exercise training in obese individuals relates to lipid droplet (LD) adaptations in skeletal muscle. In this study we investigated the hypothesis that short-term sprint interval training (SIT) and moderate intensity continuous training (MICT) in obese individuals would increase perilipin (PLIN) expression, increase the proportion of LDs in contact with mitochondria and reduce muscle concentrations of DAGs and ceramides.

**Methods:** Sixteen sedentary obese males performed 4 weeks of either SIT (4-7x 30s sprints at 200%  $W_{max}$ , 3 days.week<sup>-1</sup>) or MICT (40-60 min cycling at ~65%  $VO_{2peak}$ , 5 days.week<sup>-1</sup>), and muscle biopsies were obtained pre- and post-training.

**Results:** Training increased PLIN2 (SIT 90%, MICT 68%) and PLIN5 (SIT 47%, MICT 34%) expression in type I fibres only, and increased PLIN3 expression in both type I (SIT 63%, MICT 67%) and type II fibres (SIT 70%, MICT 160%) (all  $P<0.05$ ). Training did not change LD content but increased the proportion of LD in contact with mitochondria (SIT 12%, MICT 21%,  $P<0.01$ ). Ceramides were reduced following training (SIT -10%, MICT -7%,  $P<0.05$ ), but DAG was unchanged. No training  $\times$  group interactions were observed for any variables.

**Conclusions:** These results confirm the hypothesis that SIT and MICT results in remodelling of LDs and lowers ceramide concentrations in skeletal muscle of sedentary obese males.

## Introduction

Intramuscular triglyceride (IMTG) accumulation in sedentary and obese individuals is associated with low skeletal muscle insulin sensitivity and an increased risk of developing type 2 diabetes<sup>1,2</sup>. However, endurance-trained athletes store even larger quantities of IMTG while exhibiting high levels of insulin sensitivity. This phenomenon is termed the athlete's paradox<sup>1,3</sup>. Subsequently, it has been proposed that lipid metabolites such as diacylglycerols (DAG) and ceramides play a key role in the development of insulin resistance rather than IMTG<sup>4,5</sup>. Indeed, evidence from cell culture and animal models demonstrates that elevated DAG<sup>6</sup> or ceramides<sup>7</sup> directly impair insulin signalling. Evidence from human studies is less clear, with some<sup>7-11</sup>, but not all<sup>12-15</sup> studies reporting higher DAG and ceramide concentrations in skeletal muscle of insulin resistant obese and/or type 2 diabetes patients compared to healthy, insulin-sensitive individuals. Investigations exploring the degree of saturation and specific fatty acid composition of these lipids may unravel their specific role in the development of insulin resistance.

To date, few studies have evaluated the effect of longitudinal exercise training interventions on muscle DAGs and ceramides. A number of studies have reported reductions in ceramide concentrations<sup>16-18</sup>, whereas the concentration of DAGs has been shown to decrease<sup>17,18</sup> or remain unchanged<sup>16,19</sup> following training. Importantly, these studies have only investigated the effect of moderate-intensity continuous training (MICT). Sprint interval training (SIT) has gained increased attention as it can increase skeletal muscle oxidative capacity and improve whole-body insulin sensitivity with substantial reductions in time commitment and total training workload, compared to MICT<sup>20-23</sup>. Therefore, SIT offers promise as an alternative to continuous moderate-intensity exercise training to prevent and treat metabolic disease. This is the first study to investigate whether improvements in insulin sensitivity following SIT occur alongside reductions in DAG and ceramide concentrations.

IMTG are stored in lipid droplets (LD), the majority of which are in close spatial contact with the mitochondrial network in endurance-trained individuals<sup>24,25</sup>. Over 300 proteins are associated with the LD<sup>26</sup>, the most abundant of which are the perilipin (PLIN) family of proteins. It is generally accepted

that PLIN2 and PLIN5 promote IMTG storage<sup>27</sup>, since overexpression of either protein augments IMTG content in skeletal muscle while keeping insulin sensitivity high<sup>28, 29</sup>. PLIN5 knock-out on the other hand results in elevated ceramide concentrations and lower insulin-mediated glucose disposal rates<sup>30</sup>, highlighting the importance of PLIN5 for maintaining high levels of insulin sensitivity. We recently reported that improvements in insulin sensitivity in sedentary lean individuals following SIT occur alongside increases in IMTG storage and greater expression of PLIN2 and PLIN5<sup>22</sup>. At present, however, it is not known whether exercise training-induced increases in the abundance of the PLIN proteins contributes to improvements in insulin sensitivity, via decreases in DAG and ceramide content, in obese individuals at risk of developing metabolic syndrome.

Less attention has been paid to PLIN3, despite this protein also being highly expressed in skeletal muscle<sup>31</sup>. Fatty acid packaging in LD is reduced when PLIN3 is ablated in fibroblastic cells<sup>32</sup>, suggesting that PLIN3 is important for triacylglycerol storage. PLIN3 may also play a role in triacylglycerol oxidation, since skeletal muscle PLIN3 content is positively associated with whole-body fat oxidation<sup>33</sup> and *ex vivo* palmitate oxidation<sup>33, 34</sup>. Data on the effect of MICT on skeletal muscle PLIN3 content, measured through immunoblotting of whole muscle homogenates, are contradictory with both an increase<sup>19</sup> and no change<sup>35</sup> being reported in obese individuals. Part of this contradiction could be the consequence of muscle fibre type differences. Using quantitative immunofluorescence microscopy methods in combination with identification of muscle fibre type<sup>36</sup> we previously reported that 6 months of MICT in type 2 diabetes patients increased PLIN2 expression in type 1 fibres only<sup>37</sup>, highlighting the importance of considering fibre type when investigating exercise training adaptations.

In the current study, we used muscle fibre type-specific methods to investigate the hypothesis that SIT and MICT in obese individuals would augment protein expression of PLIN2, PLIN3, and PLIN5. We also employed transmission electron microscopy to assess mitochondrial density and the proportion of LD in direct contact with mitochondria within the muscle fibre. Finally, measurements of total and subspecies of DAGs and ceramides were made to test the hypothesis that SIT and MICT would also lower the concentration of these lipid metabolites in muscle.

## Materials and Methods

### *Participants and ethical approval*

The samples collected in this study have been used in a previous publication which focused on the effects of SIT and MICT on microvascular adaptations in skeletal muscle<sup>38</sup>. Brief subject characteristics, including body composition, exercise capacity and insulin sensitivity are also presented in Table 1 in this paper. Sixteen young, sedentary obese males ( $25 \pm 1$  y,  $34.8 \pm 0.9$  kg.m<sup>-2</sup>), who were engaging in less than one hour of structured physical activity per week, and were free of known metabolic or cardiovascular disorders, as determined by a medical professional during a pre-study screening visit, provided written informed consent. Two participants had impaired fasting glucose (fasting plasma glucose  $\geq 6.1$  mmol.L<sup>-1</sup>; SIT  $n=1$ , MICT  $n=1$ ) and four subjects had impaired fasting glucose and impaired glucose tolerance (2 h oral glucose tolerance concentration between 7.8 and 11.1 mmol.L<sup>-1</sup>; SIT  $n=2$ , MICT  $n=2$ ). The study protocol adhered to the Declaration of Helsinki and was approved by the South Birmingham NHS Research Ethics Committee.

### *Pre- and post-training experimental procedures*

Experimental procedures, including measures of aerobic capacity (VO<sub>2peak</sub>), body composition and insulin sensitivity, were undertaken before and after training (>48 h following the final exercise training session and identical in all respects to pre-training) as previously described<sup>38</sup>. Muscle samples (~100 mg) were obtained from the *vastus lateralis* under local anaesthesia using the Bergström technique<sup>39</sup> following an overnight fast. The biopsy was separated and preserved for immunofluorescence microscopy, transmission electron microscopy, and analysis of lipid species. Following the pre-training experimental procedures the 16 subjects were divided into pairs matched for age, BMI and VO<sub>2peak</sub>, with one member from each pair randomly assigned to either the SIT or MICT group.

### *Training procedures*

*Sprint interval training (SIT)*: Subjects performed repeated 30 s sprints on a cycle ergometer against a constant load equivalent to 200% W<sub>max</sub>, interspersed with 2 min recovery during which subjects cycled against a small load (30 W) maintaining a cadence of <50 rpm. Subjects trained three times per week

for four weeks. Exclusion criteria stated that subjects would be excluded if they were absent from more than two sessions. However, no subjects needed to be excluded on this basis. Four sprints per training session were performed in week one, after which an additional sprint was included on each consecutive week, such that seven sprints were performed per training session in week four. Heart rate was recorded throughout each session.

*Moderate-intensity continuous training (MICT):* Subjects in the MICT group trained 5 times per week over the 4 week training period, and were excluded from the study if they were absent from more than two training sessions. All subjects cycled at a workload equivalent to ~65%  $\text{VO}_{2\text{peak}}$  for 40 min during the first seven sessions, increasing to 50 min during sessions 8 to 14, and 60 minutes during sessions 15 to 20.  $\text{VO}_{2\text{peak}}$  was reassessed after two weeks of training and workload adjusted accordingly.

#### ***Immunofluorescence microscopy***

Muscle tissue was prepared for immunohistochemical analysis by embedding in Tissue-Tek OCT Compound (Sakura Finetek Europe, The Netherlands) and freezing in liquid nitrogen-cooled isopentane. Serial 5  $\mu\text{m}$  cryosections were cut at  $-30^{\circ}\text{C}$  and transferred on to ethanol-cleaned glass slides. The neutral lipid dye oil red O was used to image and quantify IMTG<sup>40</sup>. All primary and secondary antibodies for immunofluorescence microscopy have been used previously<sup>22, 24, 36</sup> except for the PLIN3 primary antibody (guinea pig polyclonal anti-TIP47, GP30, Progen Biotechnik, Germany). Training-induced changes in fibre-specific PLIN2, PLIN3 and PLIN5 content, and COX expression (as a marker of muscle oxidative capacity) were assessed using established and validated widefield and confocal immunofluorescence microscopy techniques<sup>22, 24, 36</sup>. Image processing was undertaken using Image-Pro Plus 5.1 software (Media Cybernetics, MD, USA). A total of  $97 \pm 9$  cross-sectionally orientated fibres from a muscle section ( $38 \pm 4$  type I fibres,  $59 \pm 6$  type II fibres) were analysed for each variable. Fluorescence staining intensity was used to indicate training-induced changes in COX expression. IMTG, PLIN2, PLIN3 and PLIN5 content were expressed as the positively stained area fraction relative to the total area of each muscle fibre. For all fibre type-specific analyses, fibres stained

positively for myosin heavy chain type 1 were classified as type I muscle fibres, whereas all other fibres were assumed to be type II muscle fibres.

### ***Transmission electron microscopy***

Fresh muscle tissue was immediately fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) for 24 h, followed by four rinses in 0.1 M sodium cacodylate buffer. Secondary fixation was undertaken with 1% osmium tetroxide (Agar Scientific, Essex, UK) for 1 h, followed by two rinses in 0.1 M sodium cacodylate buffer. Muscle tissue was subsequently dehydrated using increasing concentrations of alcohol (50, 70, 90, and 100%), followed by exposure to propylene oxide (Agar Scientific, Essex, UK). Dehydrated tissue was incubated in a propylene oxide/resin (1:1) and then embedded in 100% Mollenhauer resin (polymerised for >16 h at 60°C) (Agar Scientific, Essex, UK). Resin blocks (with tissue embedded) were trimmed and 1 µm sections were prepared to check fibre orientation. Longitudinal orientated ultra-thin sections (100 nm) were obtained using an ultramicrotome (Reichert Jung Ultracut, Vienna, Austria) fitted with a diamond blade and collected on to a formvar coated microscope grid (200 copper mesh size), followed by staining with uranyl acetate and lead citrate.

Sections were viewed and photographed at x10,000 magnification using a Jeol 1200 Ex transmission electron microscope (TEM) (Jeol, Tokyo, Japan) with a Megaview III FW camera. Four micrographs of the intermyofibrillar region per fibre from four different muscle fibres per time point (i.e. 16 micrographs per time point per person, 32 micrographs in total) were obtained in a randomized systematic manner. Micrograph analysis was undertaken using Image-Pro Plus 5.1 software (Media Cybernetics, MD, USA). A grid with squares of 500x500 nm (0.25 µm<sup>2</sup>) was superimposed on to each micrograph, and the number of points (two intersecting grid lines) that were in contact with mitochondria or LD was summed and expressed relative to the total number of points on the grid (corresponding to a total of 784 points). This grid size was selected in line with a recent study that aimed to standardise the grid size used across studies<sup>41</sup>. This process was undertaken for each micrograph and the values averaged to calculate mitochondria or LD volume density (expressed as %

area of muscle occupied by mitochondria or LDs). Individual mitochondria and LDs were isolated using the 'area of interest' tool in the Image-Pro Plus software and mean individual mitochondria and LD size ( $\mu\text{m}^2$ ) was subsequently determined. The total number of mitochondria or LDs was expressed per square micrometer of tissue ( $\#.\mu\text{m}^2 \text{ tissue}^{-1}$ ). The number of LDs in contact with mitochondria was manually counted and expressed as a percentage of the total number of LDs per micrograph. Acquisition and analysis of all micrographs was performed blinded to subject, condition and time-point.

### ***Lipid composition analysis***

Approximately 20 mg of muscle tissue was used for the lipid composition analysis. Muscle lipids were extracted using a single-phase chloroform/methanol extraction. Triacylglycerol (TAG), DAG, ceramide, monohexosylceramide (MHC) dihexosylceramide (DHC), trihexosylceramide (THC), sphingomyelin (SM), gangliomyelin (GM) were analysed using electrospray ionization-tandem mass spectrometry, as previously described<sup>42</sup>. The concentration of each lipid specie was expressed relative to the concentration of total PC<sup>43</sup>, and was then used to calculate a fold change (relative to the value for MICT Pre<sup>43</sup>).

### ***Statistics***

Training-induced changes in TEM LD and mitochondria measures and muscle lipid species were assessed using a two-factor repeated measures ANOVA. A three-factor repeated measures ANOVA was used to examine fibre-specific training-induced changes in IMTG, PLIN2, PLIN3, PLIN5 and COX content. Significant interactions obtained during repeated measures ANOVA were investigated using Bonferroni adjustment post hoc analysis. Pearson's bivariate correlation analysis was used to investigate specific relationships between variables. All data are expressed as means  $\pm$  S.E.M. The sample size was deemed sufficient to detect a within-group training effect of  $f=0.30$  (representative of a medium-effect size<sup>44</sup>) for PLIN protein content, adopting an alpha of 0.05 and a power of 80%. This was calculated using G\*Power 3.1 software (G\*Power Software Inc., Kiel, Germany), and was based on our previous observation that PLIN protein content was significantly increased following 6 weeks of SIT and MICT in lean sedentary individuals<sup>22</sup>.



## Results

### *Exercise capacity, body composition and insulin sensitivity (Table 1)*

As we have previously reported<sup>38</sup>,  $\text{VO}_{2\text{peak}}$  was increased by both SIT (13%) and MICT (10%), with no difference between groups (main training effect;  $P=0.002$ ). Fat mass (-5%) and percentage body fat (-4%) were both reduced by MICT only (training  $\times$  group interaction;  $P<0.05$ ), with no change observed following SIT. Insulin sensitivity, as measured by the Matsuda index, was increased by both SIT (11%) and MICT (24%), with no significant difference between groups.

### *Immunofluorescence microscopy analysis*

Muscle fibre type-specific COX expression (expressed as mean fluorescence intensity) was greater in type I compared to type II fibres both pre and post-training ( $P<0.01$ , Fig. 1A-B & Supplementary Fig. S1). Training increased COX expression in both type I (SIT 29%, MICT 36%) and type II fibres (SIT 49%, MICT 36%; main training effect,  $P<0.01$ , Fig. 1A-B & Supplementary Fig. S1), with no difference between groups. IMTG content (expressed as % area stained) was higher in type I versus type II muscle fibres both pre and post-training ( $P<0.01$ , Fig. 1C-D & 2). The increase in IMTG content in response to training both in type I (SIT 26%, MICT 22%) and type II fibres (SIT 35%, MICT 26%; Fig. 1C-D & 2) did not reach statistical significance ( $P=0.1$ ). PLIN2, PLIN3 and PLIN5 content (expressed as % area stained) was greater in type I compared to type II fibres ( $P<0.01$ , Fig. 1E-J & 2) across all time points. In response to training PLIN2 content increased, although this only reached significance in type I fibres (SIT 90%, MICT 68%; training  $\times$  fibre interaction,  $P<0.001$ , Fig. 1E & 2). In contrast, PLIN3 content increased following training in both type I (SIT 63%, MICT 67%, Fig. 1G & 2) and type II fibres (SIT 70%, MICT 160%; main training effect,  $P<0.01$ , Fig. 1H & 2). Training led to an increase in PLIN5 content in type I fibres only (SIT 47%, MICT 34%; training  $\times$  fibre interaction,  $P<0.01$ , Fig. 1I & 2). PLIN2, PLIN3 and PLIN5 expression was not different between the two training interventions at any time point.

### ***Transmission electron microscopy analysis***

Training induced a significant increase in mitochondrial volume density (expressed as % area of muscle) (SIT 46%, MICT 88%; main training effect,  $P<0.01$ , Fig. 3D), with no difference between groups. This increase was attributed to a greater number of mitochondria (SIT 33%, MICT 20%; main training effect,  $P=0.01$ , Fig. 3E) and increased mitochondrial size (SIT 27%, MICT 51%; main training effect,  $P<0.01$ , Fig. 3F), with no difference between groups. In contrast, no significant changes were observed in LD volume density, LD number or LD size in response to training ( $P>0.05$ , Fig. 3H-J). However, there was an increase in the proportion of LDs in contact with mitochondria following training (SIT 12%, MICT 21%; main training effect,  $P<0.01$ , Fig. 1G), with no difference between groups. Examples of LDs in contact and not in contact with mitochondria are provided in Fig. 3B-C.

### ***Muscle TAG, DAG and sphingolipids***

Training did not change the concentration of total TAG ( $P>0.05$ , Fig. 4) or specific TAG subspecies (data not shown). There was also no change in total DAG concentrations with training ( $P>0.05$ , Fig. 4), however there was a significant increase in DAG 18:1/18:2 (main training effect,  $P=0.02$ ) and DAG 18:0/18:2 (main training effect,  $P=0.04$ , Fig. 5A). Training induced a significant decrease in total ceramide content (SIT -10%, MICT -7%; main training effect,  $P=0.03$ , Fig. 4), with no difference between groups. Further examination of the specific ceramide species revealed that training led to a significant decrease in Cer 18:0 ( $P=0.01$ , Fig. 5C). We also examined the effect of training on other complex ceramides and sphingomyelin, and found that training led to an increase in total trihexosylceramide (THC) concentration (SIT 32%, MICT 10%; main training effect,  $P=0.02$ , Fig. 5B), with no difference between groups. The THC species THC 24:0 and THC 24:1 were the only species that significantly increased in response to training (both  $P<0.01$ , Fig. 5D).

### ***Correlation analysis***

Given the lack of group  $\times$  training interactions in muscle variables, significant training effects were detected independent of exercise modality. Therefore, the data from the two intervention groups were pooled for correlation analysis. The proportion of LDs touching mitochondria correlated positively

272 with expression of PLIN2 ( $R=0.67$ ;  $P<0.001$ ), PLIN3 ( $R=0.39$ ;  $P=0.049$ ) and PLIN5 ( $R=0.43$ ;  $P=0.028$ )  
273 in muscle. Muscle ceramide levels were also related to PLIN expression, demonstrating a moderate  
274 negative correlation with PLIN2 ( $R=-0.41$ ;  $P=0.029$ ), PLIN3 ( $R=-0.49$ ;  $P=0.008$ ) and PLIN5 expression  
275 ( $R=-0.39$ ;  $P=0.039$ ). Finally, a significant negative relationship between muscle Cer18:0 levels and  
276 PLIN2 expression ( $R=-0.44$ ;  $P=0.036$ ) was observed, but this did not reach significance when muscle  
277 Cer18:0 levels were related to PLIN3 ( $R=-0.39$ ;  $P=0.068$ ) or PLIN5 ( $R=-0.41$ ;  $P=0.052$ ) content.  
278 Whole-body insulin sensitivity was not significantly related to any muscle variables.

## Discussion

In the present study we demonstrate for the first time that in previously sedentary obese males, a short-term SIT and MICT intervention both lead to 1) a reduction in muscle ceramide concentrations, 2) an increase in the number of LDs in contact with mitochondria, and 3) muscle fibre-specific increases in PLIN2, PLIN3 and PLIN5. Importantly, these adaptations were comparable in response to both SIT and MICT interventions.

The first novel finding of the present study was that 4 weeks of either SIT or MICT significantly reduced muscle ceramide concentrations in obese individuals. Reductions in muscle ceramide concentrations have previously been reported following 8-16 weeks endurance training in obese individuals<sup>16, 18</sup>, and we now extend the ceramide-lowering effect of exercise training to include SIT. Further analysis revealed that training specifically lowered Cer18:0. This may be an important adaptation, since it has recently been reported in humans that the muscle concentration of Cer18:0 is inversely related to insulin sensitivity<sup>45</sup>. Ceramides are elevated in the muscle of insulin resistant populations<sup>7, 9-12</sup> and are a potent inhibitor of muscle insulin action through the activation of the phosphatase PP2A and subsequent dephosphorylation and inhibition of Akt<sup>46</sup>. Therefore, it is feasible that the reduction in ceramide concentrations, and particularly Cer18:0, observed following training may contribute to the improved whole-body insulin sensitivity observed in response to SIT and MICT<sup>38</sup>. We also observed a significant increase in total skeletal muscle THC concentration, with increases occurring in the THC 24:0 and THC 24:1 species. The relevance of THC to insulin sensitivity is not clear and warrants further investigation.

Contrary to our hypothesis, we did not detect a change in total DAG content but rather an increase in specific DAG species following training (DAG 18:1/18:2 and DAG 18:0/18:2). Although previous studies and reviews have heavily implicated DAG in the pathogenesis of insulin resistance<sup>4, 6, 8</sup>, the relationship between muscle DAG and insulin sensitivity has proved complex. Our results are in line with a number of studies that have demonstrated a disconnect between muscle DAG content and insulin sensitivity (reviewed by <sup>47</sup>). Most notably, Amati et al.<sup>12</sup>, demonstrated that trained individuals displayed higher DAG levels than sedentary lean and obese individuals. Therefore, it appears that

changes in DAG concentrations may not be directly relevant for the improvements in insulin sensitivity following exercise training.

The second novel finding of the study is that short-term SIT led to an increase in the number of LDs that are in contact with mitochondria. In line with this observation, it has been reported previously that 6-12 weeks of MICT augments the number of LDs in contact with mitochondria in both lean<sup>25</sup> and obese<sup>48</sup> individuals. This is likely to be an important adaptation, because a large proportion of extracellular FAs flux through the IMTG pool prior to oxidation in the mitochondria<sup>49</sup>, and therefore the contact of LDs with mitochondria is important to efficiently channel FA released by IMTG lipolysis into the mitochondrial reticulum for oxidation<sup>50</sup>. Thus, the greater number of LDs in contact with mitochondria will aid in the efficient transfer of LD-derived FAs to the mitochondria and contribute to the well described increase in IMTG-derived FA oxidation following exercise training. The greater COX expression following training would also support this by providing a greater oxidative phosphorylation capacity in both type I and type II fibres. Such an improvement in the channelling of FA's towards oxidation rather than other lipid pathways, such as ceramide synthesis, may also provide an explanation behind the lowered concentration of ceramides observed post-training.

Previous investigations into the PLIN proteins in human skeletal muscle have predominantly focused on PLIN2 and PLIN5. Less attention, however, has been paid to PLIN3, which is also expressed in skeletal muscle<sup>31</sup>. Studies report either an increase<sup>19</sup> or no change<sup>35</sup> in PLIN3 protein content in whole muscle homogenates following MICT in obese individuals. Here, we report for the first time that SIT and MICT leads to greater PLIN3 protein expression in both type I and type II fibres in obese individuals. Like PLIN2 and PLIN5, acute endurance-type exercise in mice leads to an increase in PLIN3 mRNA abundance<sup>51</sup>. In addition, PLIN3 expression in skeletal muscle was recently shown to be positively regulated by AMPK activity<sup>52</sup>, suggesting that exercise training will lead to greater PLIN3 protein expression. We also observed greater expression of both PLIN2 and PLIN5 in response to SIT and MICT in obese individuals, but interestingly the elevated expression of these proteins was specific to type I fibres. With regards to PLIN2, it is notable that other studies investigating the effect of MICT

specifically in obese individuals have failed to observe an increase in the expression of this protein<sup>19,35</sup>. This may be explained by the methodology used, since immunoblotting of whole muscle homogenates (as used in previous studies) does not take into account fibre type differences. In line with the results of the present study, we previously reported that PLIN2 expression is upregulated specifically in type I fibres following MICT in obese type 2 diabetes patients<sup>37</sup>. The increase in PLIN5 is consistent with other studies showing greater PLIN5 expression following MICT in obese individuals<sup>19,35</sup>, and with our previous study demonstrating that SIT and MICT augment PLIN5 in lean individuals<sup>22</sup>. Despite elevations in muscle PLIN protein abundance post-training, IMTG content and LD number did not increase significantly in the present study. It is possible that the short duration of the training period may explain the lack of a significant increase in IMTG content, since 10 consecutive days of endurance-type training also failed to increase IMTG content in obese individuals<sup>53</sup>. Increases in IMTG content in obese individuals do occur following 12 to 16 weeks of MICT<sup>17, 18, 54</sup>. Importantly, the fact that SIT and MICT augmented PLIN protein expression while IMTG content did not increase significantly, suggests that the increased PLIN abundance may be an early adaptation to exercise training in obese individuals and type 2 diabetes patients<sup>37</sup>.

Greater PLIN expression following training is likely important to support a dynamic intramuscular lipid pool that can respond appropriately to changes in metabolic demand. Indeed, evidence is accumulating to suggest that the PLIN proteins play a dual role in skeletal muscle, by 1) promoting IMTG storage under conditions of low metabolic demand, and 2) facilitating IMTG lipolysis and FA oxidation when metabolic demand increases. Under basal conditions the PLIN proteins restrict lipolytic rates<sup>27</sup>, and for PLIN5 this is suggested to occur by preventing the binding of ATGL with its co-activator, CGI-58, on the LD surface<sup>55</sup>. In accordance, overexpression of PLIN2 or PLIN5 in skeletal muscle augments TAG storage<sup>28, 29</sup>, whereas knockdown or knockout of PLIN2 or PLIN5 results in smaller and fewer LD, elevated rates of basal lipolysis, and a reduction in FA-induced TAG accumulation<sup>28, 30, 56</sup>. PLIN3 may also play a similar role in muscle, since overexpression of PLIN3 in cultured myotubes exposed to palmitate augments IMTG storage<sup>52</sup>. Evidence in the literature also points to a role for PLIN3 and PLIN5, and to a lesser extent PLIN2, in supporting IMTG lipolysis and FA oxidation under conditions

of increased metabolic demand. Accordingly, myotubes overexpressing PLIN5 not only exhibit reduced rates of lipolysis and FA oxidation under basal conditions, but demonstrate greater rates of FA oxidation (compared to control cells) following stimulated lipolysis via either forskolin or contraction<sup>57</sup>. The suggestion that PLIN3 plays a role in IMTG oxidation comes from human studies reporting a positive association between PLIN3 expression and both whole-body fat oxidation<sup>33</sup> and ex vivo palmitate oxidation<sup>33, 34</sup>. Furthermore, in human muscle we have previously shown that PLIN2<sup>36</sup> and PLIN5-containing LD<sup>22</sup> are preferentially broken down during moderate-intensity exercise compared to LDs with no PLIN2 or PLIN5 associated. The PLIN proteins may also play a mechanistic role in the functional linkage between LDs and the mitochondrial network, since PLIN5 recruits mitochondria to the LD surface in cultured non-muscle cells leading to increased rates of mitochondrial  $\beta$ -oxidation in response to protein kinase A activation<sup>58</sup>. In addition, both PLIN3 and PLIN5 have been reported to be present within mitochondria<sup>59, 60</sup> and electrical contraction of rat skeletal muscle increases the content of PLIN5 in the mitochondrial fraction<sup>60</sup>. We also observed a correlation between the number of LDs in contact with mitochondria and PLIN2, PLIN3 and PLIN5 expression in the present study. Therefore, although the exact mechanisms involved in the functional linkage of LDs and mitochondria following exercise training remains unresolved, our data are supportive of a key role of the PLIN proteins in this process.

The level of PLIN protein expression may also be important in mediating the concentration of muscle ceramides. In support, PLIN2, PLIN3 and PLIN5 expression all were inversely related to muscle ceramides in the present study, and PLIN2 expression also demonstrated an inverse relationship with levels of Cer18:0. There is evidence that the increase in PLIN5 expression may contribute to this, because PLIN5 overexpression in myotubes suppresses palmitate-induced ceramide accumulation<sup>57</sup>, whereas whole-body PLIN5 knock-out in mice leads to elevations in muscle ceramide<sup>30</sup>. Therefore, our data suggest that PLIN5, and perhaps PLIN2 and PLIN3 are important for facilitating storage of FAs entering skeletal muscle into IMTG, rather than the FAs being used to synthesise DAG and/or ceramides. This would theoretically improve skeletal muscle insulin sensitivity, and is in line with evidence that PLIN2 or PLIN5 overexpression improves (PLIN2) or maintains (PLIN5) insulin

sensitivity in rats fed a high-fat diet<sup>28, 29</sup>. Furthermore, when PLIN2 was overexpressed, IMTG content was increased, but importantly no changes in DAG concentrations were observed<sup>28</sup>. The increased expression of the PLIN proteins in the present study may therefore contribute to the lower ceramide concentrations observed following training.

The observed changes in LD remodelling and reductions in Cer18:0 coincided with improvements in whole-body insulin sensitivity<sup>38</sup>. Based on existing mechanistic studies<sup>30, 46</sup>, it is logical to link LD remodelling and reductions in ceramide levels with exercise training to the improvements in insulin action. However, neither the changes in total ceramide or Cer18:0 correlated with the improvements in whole-body insulin sensitivity in the present study. While it is important to note that the current study is limited by the assessment of whole-body insulin sensitivity from an OGTT rather than direct assessment of muscle insulin sensitivity, our data do not support a direct link between lowered muscle ceramide concentrations and improvements in whole-body insulin sensitivity. Therefore, further work is required to determine the contribution of exercise-mediated adaptations in lipid handling to the improvements in skeletal muscle insulin sensitivity in obese individuals.

In conclusion, this study has generated novel data that 4 weeks of exercise training increased the abundance of PLIN2, PLIN3 and PLIN5, led to greater spatial contact between LDs and the mitochondrial network, and reduced muscle ceramide concentrations. These results add to the growing body of evidence that SIT and MICT improve lipid handling in skeletal muscle and counteracts the metabolic impairments that result from obesity and a sedentary lifestyle, with SIT offering a time-efficient and effective alternative to MICT.



**Additional information**

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***Competing interests***

The authors have no conflicts of interest to declare.

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## Figure legends

### **Figure 1. Training-induced changes in fibre-specific COX expression, IMTG, PLIN2, PLIN3 and PLIN5 content**

Immunofluorescence images of muscle sections were used to quantify muscle fibre type-specific COX expression (expressed as whole cell mean fluorescence intensity) (A, B). Fibre-type specific content of IMTG (C, D), PLIN2 (E, F), PLIN3 (G, H) and PLIN5 content (expressed as % area stained) (I, J) was quantified from immunofluorescence images of muscle sections following selection of a uniform intensity threshold representative of positive signal. Fibres stained positively for myosin heavy chain type I were classified as type I fibres, while those with no staining were classified as type II fibres. Values are presented as means  $\pm$  S.E.M. ( $n=8$  per group). \*Main fibre effect ( $P<0.05$  vs. type I fibres). <sup>†</sup>Main training effect ( $P<0.05$  vs. pre-training). <sup>\*</sup>Significant training effect within fibre ( $P<0.05$  vs. pre-training).

### **Figure 2. Immunofluorescence images of IMTG, PLIN2, PLIN3 and PLIN5**

Representative images of IMTG, stained with oil red O, and PLIN2, PLIN3 and PLIN5 before and after 4 weeks of SIT. Right panels are corresponding images of myosin heavy chain I (MHC I) (stained green or red) in combination with wheat germ agglutinin Alexa Fluor 350 (WGA) to identify the cell border (stained blue) in skeletal muscle. Positively stained fibres (green or red) are type I fibres, all other fibres are assumed to be type II fibres. White bars represent 50  $\mu\text{m}$ .

### **Figure 3. Effect of 4 weeks SIT or MICT on TEM-derived measures of mitochondria and lipids**

A: Representative image from one muscle fibre at x10,000 magnification, with light arrows indicating a lipid droplet and dark arrows indicating mitochondrial fragments. Scale bar represents 2  $\mu\text{m}$ . Representative image indicating a lipid droplet not in contact with a mitochondria fragment (B) and a lipid droplet in contact with a mitochondria fragment (C). B and C are at x40,000 magnification. Scale bar represents 0.5  $\mu\text{m}$ . Mitochondria and lipid droplet physical characteristics, quantified from electron

microscopy images obtained before and after 4 weeks of SIT or MICT (D-J). Values are means  $\pm$  S.E.M. (SIT  $n=6$ , MICT  $n=7$ ). \*Main training effect ( $P<0.05$  vs. pre-training).

#### **Figure 4. Effect of 4 weeks SIT or MICT on muscle lipids**

Changes in muscle total TAG, DAG and ceramide concentrations in muscle in response to 4 weeks of SIT or MICT. The concentration of TAG, DAG and ceramide was expressed relative to the concentration of phosphatidylcholine (PC), and was then used to calculate a fold change (relative to the value for MICT Pre) ( $n=7$  per group). \*Main training effect ( $P<0.05$  vs. corresponding Pre-Training value).

#### **Figure 5. Effect of 4 weeks SIT or MICT on DAG and ceramides species**

Changes in individual DAG species (A), complex ceramides (*MHC* monohexosylceramide, *DHC* dihexosylceramide, *THC* trihexosylceramide, *GM* ganglioside) and sphingomyelin (SM) concentrations in muscle in response to 4 weeks of SIT or MICT (B). Changes in the composition of individual ceramide (C) and THC (D) species were also quantified in response to SIT or MICT. The concentration of each lipid species was expressed relative to the concentration of phosphatidylcholine (PC), and was then used to calculate a fold change (relative to the value for MICT Pre) ( $n=7$  per group). \*Main training effect ( $P<0.05$  vs. corresponding Pre-Training value). <sup>†</sup>Trend for training effect ( $P=0.06$  vs. corresponding Pre-Training value).

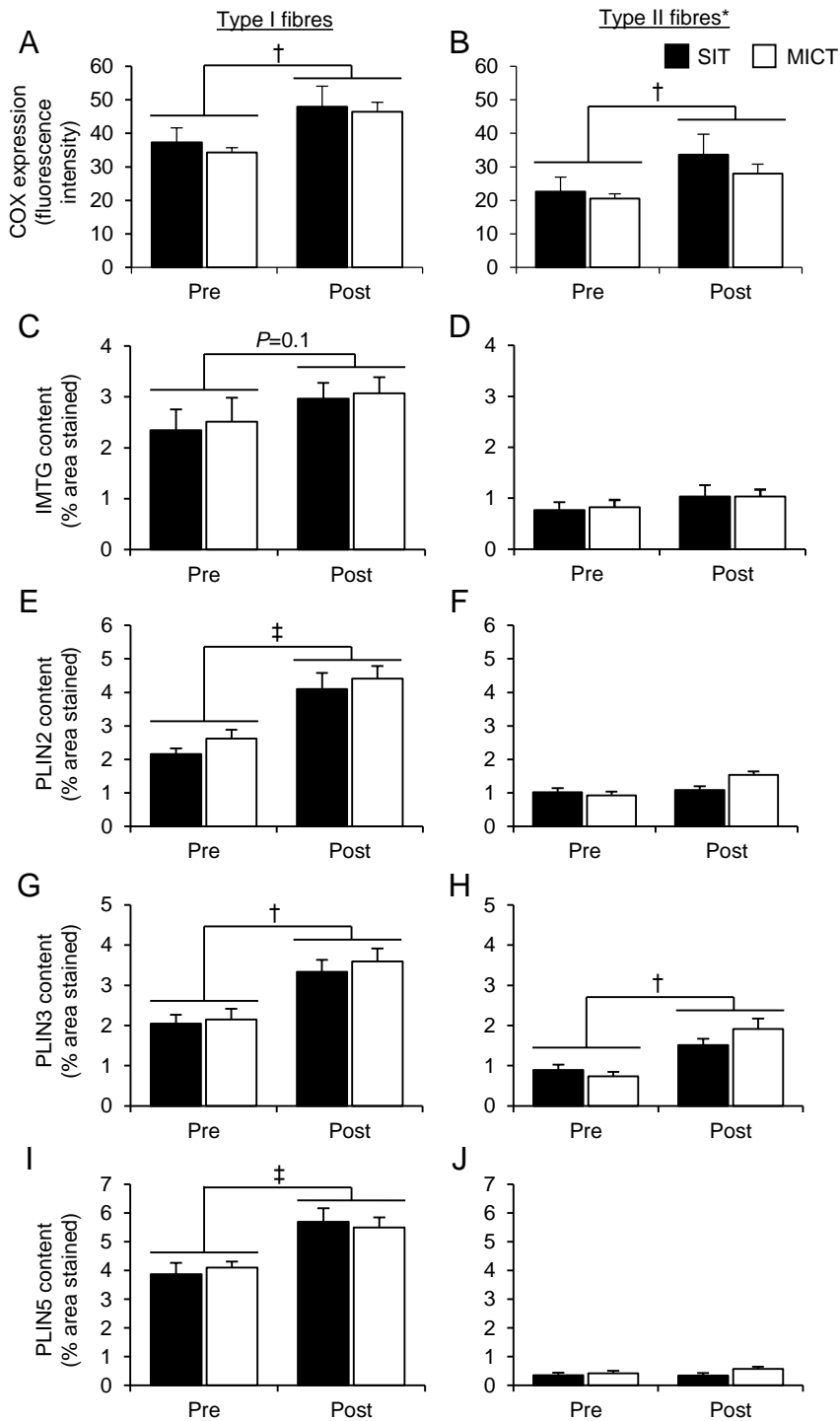


**Supplementary Figure 1. Effect of 4 weeks SIT or MICT on COX expression**

Representative images of COX staining before and after 4 weeks of SIT. Right panels are corresponding images of myosin heavy chain I (MHC I) (stained green) in combination with wheat germ agglutinin Alexa Fluor 350 (WGA) to identify the cell border (stained blue) in skeletal muscle. Positively stained fibres (green or red) are type I fibres, all other fibres are assumed to be type II fibres. White bars represent 50  $\mu$ m.

**Supplementary Figure 2. Phosphatidylcholine concentrations before and after 4 weeks SIT or MICT**

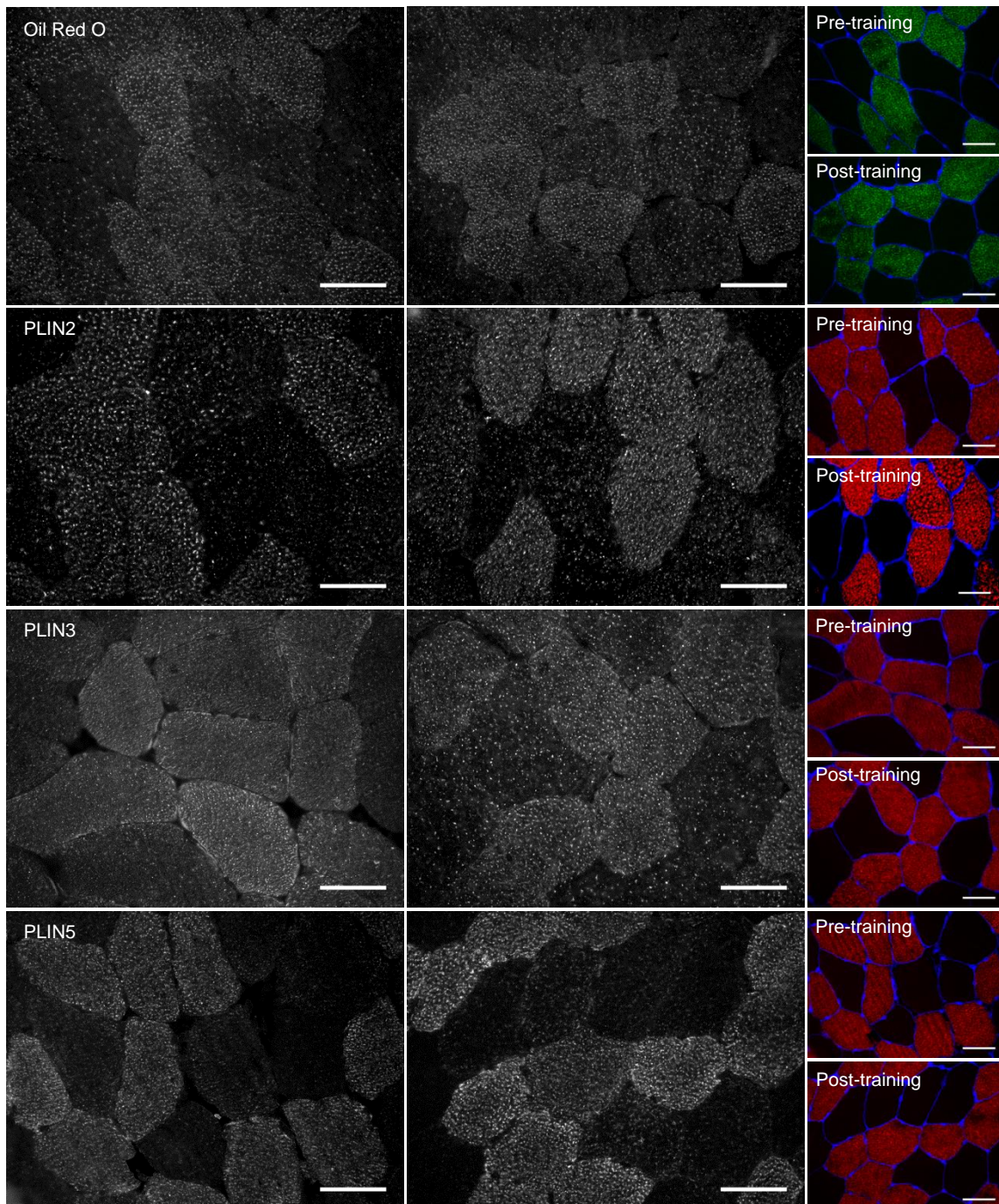
Values are means  $\pm$  S.E.M. (SIT  $n=6$ , MICT  $n=7$ ). Phosphatidylcholine (PC) concentration did not change from pre to post-training following either SIT or MICT.

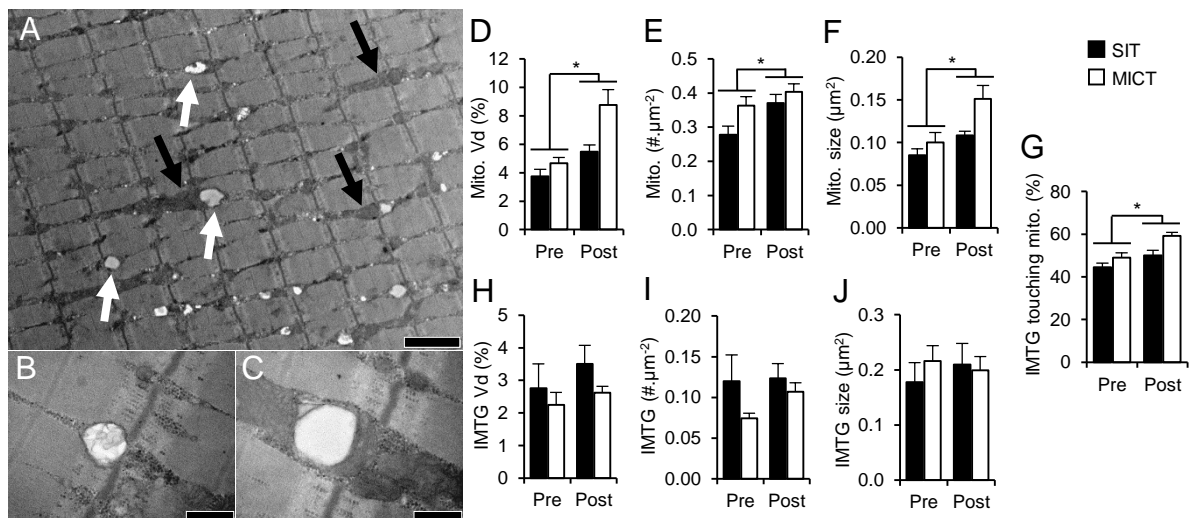


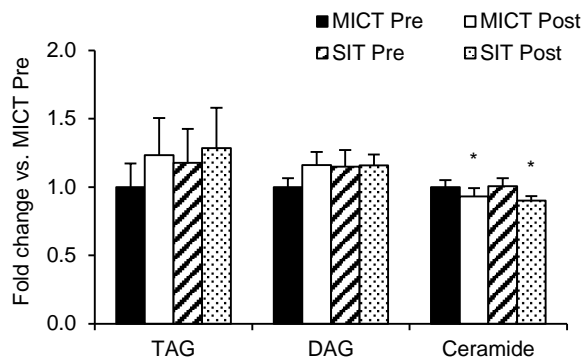
Pre-training

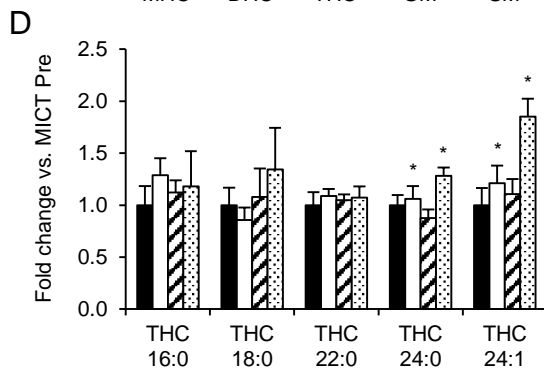
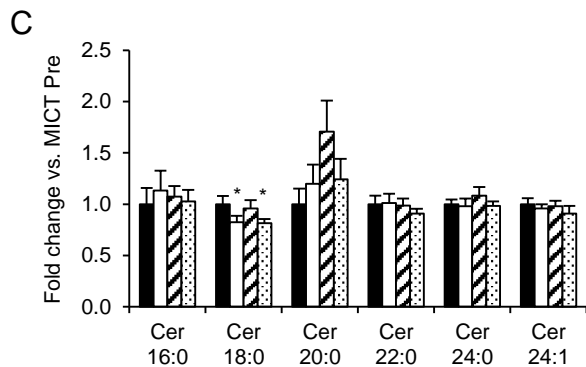
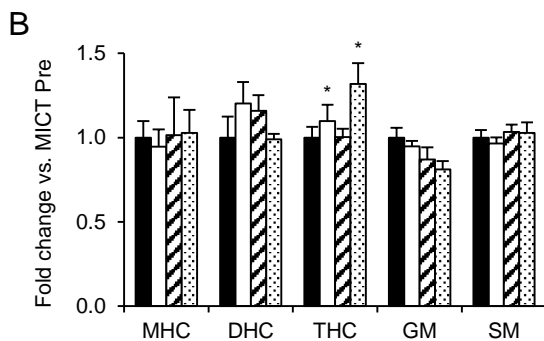
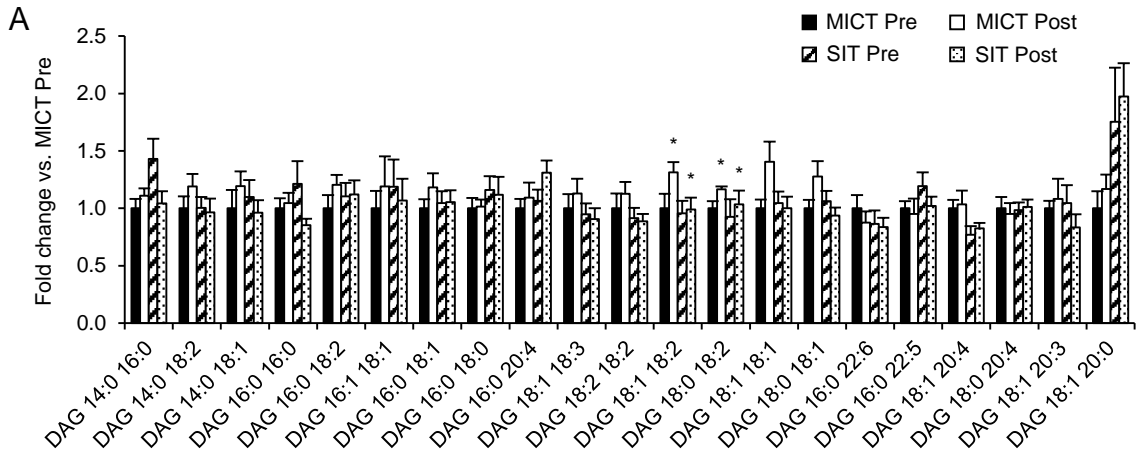
Post-training

MHC I &amp; WGA

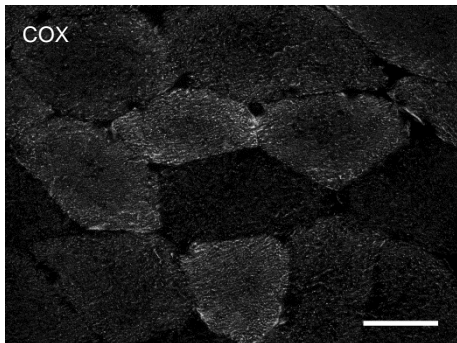




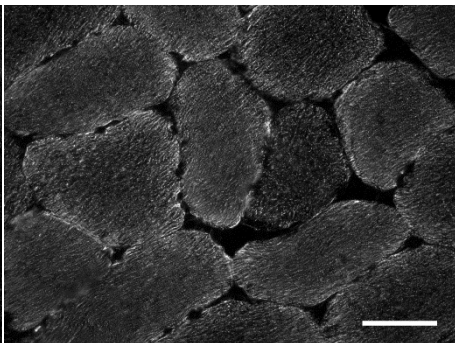




Pre-training



Post-training



MHC I & WGA

