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Whole-body & muscle responses to aerobic exercise training and withdrawal in ageing & COPD

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Whole-body & muscle responses to aerobic exercise training and withdrawal in ageing & COPD

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Take home message

Muscle mitochondrial function is not impaired in age or COPD. Whole-body and mitochondrial exercise training adaptations are robust in young, evident in older, and deficient in COPD. COPD adaptation may require high intensity exercise training programmes.

ABSTRACT

COPD patients exhibit lower peak oxygen consumption ($\dot{V}O_2^{PEAK}$), altered muscle metabolism and impaired exercise tolerance compared with age-matched controls. Whether these traits reflect muscle level deconditioning (impacted by ventilatory constraints) and/or dysfunction in mitochondrial ATP production capacity is debated. By studying aerobic exercise training (AET) at a matched relative intensity and subsequent exercise withdrawal period (EW) we aimed to elucidate the whole-body and muscle mitochondrial responsiveness of healthy-young (HY), healthy-older (HO) and COPD volunteers to whole-body exercise.

The HY (n=10), HO (n=10) and COPD (n=20) volunteers were studied before, after eight-weeks AET (65% $\dot{V}O_2^{PEAK}$) and after four-weeks EW. $\dot{V}O_2^{PEAK}$, muscle maximal mitochondrial ATP production rates (MAPR), mitochondrial content, mitochondrial DNA copy number and abundance of 59 targeted fuel metabolism mRNAs were determined at all time-points.

Muscle MAPR (normalised for mitochondrial content) was not different for any substrate combination in HO, HY and COPD at baseline, but mitochondrial DNA copy number relative to a nuclear-encoded house-keeping gene was greater in HY (mean$\pm$SD) (804$\pm$67) than in HO (631$\pm$69), p=0.041. AET increased $\dot{V}O_2^{PEAK}$ in HO (17%,p=0.002) and HY (21%,p<0.001) but not COPD (*p*=0.603). Muscle MAPR for palmitate increased with training in HO (57%, *p*=0.041) and HY (56%,*p*=0.003) and decreased with EW in HO (-45%,*p*=0.036) and HY (-30%,*p*=0.016), but was unchanged in COPD (*p*=0.594). Mitochondrial DNA copy number increased with AET in HY (66%,*p*=0.001) but not HO (*p*=0.081) or COPD (*p*=0.132). The observed changes in muscle mRNA abundance were similar in all groups after AET and EW.

Intrinsic mitochondrial function was not impaired by ageing or COPD in the untrained state. Whole-body and muscle mitochondrial responses to AET were robust in HY, evident in HO, but deficient in COPD. All showed robust muscle mRNA responses. Higher relative exercise intensities during whole-body training may be needed to maximise whole-body and muscle mitochondrial adaptation in COPD.

INTRODUCTION

Compared to age-matched healthy volunteers, patients with COPD exhibit lower whole-body maximal oxygen consumption (1-3) and abnormal skeletal muscle metabolic characteristics. Altered muscle fibre composition (4, 5); lower fibre cross-sectional area (6) and reduced capillarity (7) are apparent in COPD. At a mitochondrial level, lower ATP production capacity (reflected by lower maximal activities of mitochondrial enzymes (1)); altered mitochondrial efficiency (8); and both lower mitochondrial DNA (mtDNA) copy number and greater prevalence of mtDNA deletions (9) have been described in COPD. Collectively, these differences influence muscle metabolic responses to acute exercise, for example greater non-mitochondrial ATP production and adenine nucleotide loss (10), accentuating peripheral muscle fatigue development and reduced exercise capacity in COPD.

It is debated to what extent differences in muscle energy metabolism in COPD reflect muscle level deconditioning (associated with reduced mitochondrial volume and/or number) and/or a disease-specific COPD mitochondropathy (characterised by impaired intrinsic capacity of mitochondrial units for respiration or ATP production (8, 9, 11-15)). Similarly, chronological ageing has been linked with lower muscle mitochondrial protein content (16) and lower mitochondrial intrinsic capacity (17, 18) such that the aetiology of mitochondrial dysfunction in COPD could be age and/or disease related.

Aerobic exercise training robustly increases several markers of muscle mitochondrial content and function in young (19) and older (16) volunteers, but whether similar mitochondrial responses to aerobic exercise training occur in patients with COPD is uncertain, not least because ventilatory limitation in COPD can prevent skeletal muscle from being adequately challenged, thereby reducing training adaptation (20, 21).

Importantly, as far as we are aware, limited data are available depicting the impact of aerobic exercise training on markers of mitochondrial abundance and highly sensitive, direct measures of mitochondrial ATP production capacity in relation to whole-body cardiorespiratory adaptation in healthy, young and older volunteers and patients with COPD training concurrently. Similarly, these responses on return to habitual physical activity following training have not been depicted. Such data would provide insight regarding the aetiology of mitochondrial decline with age and COPD, and the mechanisms of exercise intervention in countering age and COPD related muscle decline and exercise intolerance. Such insight would also be beneficial to appreciate the utility of the mitochondrion as a therapeutic target.

We therefore aimed to determine the impact of matched relative intensity aerobic exercise training over an 8 week aerobic training regimen at 65% peak oxygen uptake ($\dot{V}O_2^{PEAK}$) and again 4 weeks following exercise withdrawal in healthy young and older volunteers and patients with COPD. Outcome measures include whole-body cardiorespiratory responses, markers of muscle mitochondrial content, maximal rates of muscle mitochondrial ATP production (MAPR), and the expression of mRNAs intimately linked to muscle fuel utilisation. We hypothesised that the response of MAPR and $\dot{V}O_2^{PEAK}$ to exercise intervention and withdrawal would differ between young and older healthy groups and between older healthy and COPD groups due to the impact of advanced age and disease pathology on muscle function and the potential impact of ventilatory limitation to exercise intensity in COPD.

METHODS

Due to limited article length additional detailed information for all methods is provided in the online supplement.

Subjects

Three groups of sedentary volunteers were recruited; patients with COPD (and significant exercise limitation due to breathlessness; MRC Grade $\geq$3) aged 60-80 years (COPD), age-matched healthy older (HO), and healthy younger (HY) subjects aged 18-35 years (inclusion criteria are in Table S1).

Study Design

A prospective, non-randomised interventional cohort study was performed. Anthropometry, pulmonary function and quadriceps strength were assessed at baseline and two symptom-limited incremental (first being a familiarization) and one submaximal cycling cardiopulmonary exercise tests (CPET) performed. Seven-day physical activity levels were assessed (accelerometry) before a fasted state resting quadriceps muscle biopsy was obtained (22). Participants commenced eight-weeks fully supervised cycling aerobic exercise training (AET; 3 sessions of 30 minutes per week) at a workload corresponding to 65% $\dot{V}O_2{}^{PEAK}$. This intensity was chosen to elicit increases in muscle lipid and carbohydrate flux (23, 24). Training intensity was reset at week four if workload at $\dot{V}O_2{}^{PEAK}$ had increased. After eight weeks training, participants were instructed to resume their pre-training habitual physical activity levels and were followed up after four weeks (week 12). CPET were repeated at weeks 4, 8 and 12. Further resting muscle biopsies were performed after eight-weeks (24 hours after preceding training session) and four weeks post exercise withdrawal

(week 12). The study design is outlined in Fig S1 of the online supplement. Ethical approval was granted by the NHS National Research Ethics Service and the trial registered at https://doi.org/10.1186/ISRCTN10906292. All participants provided written informed consent.

Muscle mitochondrial preparation

Muscle samples were immediately subjected to a homogenisation, centrifugation and resuspension protocol and kept on ice immediately prior to quantification of MAPR.

Maximal mitochondrial ATP production rates (intrinsic mitochondrial function)

MAPR was assessed utilising the bio-luminometric method described by Wibom et al (25). Briefly, diluted mitochondrial suspension, ADP, and an ATP monitoring reagent (firefly luciferase) were combined with the following substrate combinations: glutamate and succinate; glutamate and malate; pyruvate and malate; palmitoyl-l-carnitine and malate; succinate; and double-distilled water. All values were normalised to muscle citrate synthase activity.

Citrate synthase activity

Muscle citrate synthase (CS) activity, a robust surrogate marker of mitochondrial content (26) was determined spectrophotometrically using the isolated mitochondrial suspension (27).

Relative mitochondrial DNA copy number

The abundance of mitochondrial encoded (mtDNA) NADH:ubiquinone oxidoreductase core subunit 1 was established using real-time PCR and expressed relative to genomically encoded (nDNA) hydroxymethylbilane synthase as previously described (28).

Targeted muscle mRNA expression

mRNA was extracted from snap-frozen muscle as previously described (29), and quantitative real-time PCR performed. The abundance of 59 transcripts involved in muscle fuel metabolism was assessed (supplement and Table S2), and Ingenuity Pathway Analysis (IPA; QIAGEN, Hilden, Germany) was used to interrogate those differentially regulated.

Statistical analysis

Data are presented as mean ± SD (baseline) or SEM (change over time). Assumptions of normality were assessed using normal probability plots and Shapiro-Wilk testing. Between-group mean differences at baseline were assessed using one-way ANOVA and within-group changes across time were tested using one-way repeated measures ANOVA with post hoc Fishers LSD using IBM SPSS Statistics for Windows, version 21 (IBM Corp., Armonk, N.Y., USA). A calculation of statistical power can be found in the online supplement. For clarity, COPD vs HY comparisons are not reported due to lack of relevance to study aims.

RESULTS

Baseline

Baseline subject characteristics are reported in Table 1. Body mass index and fat-free mass index were not statistically different between groups at baseline. Whilst all groups were non-active, the mean step-count in COPD patients was lower than HO (p=0.046) and below the sedentary lifestyle index for adults of 5,000 steps/day (30).

Table 1. Baseline subject characteristics

	HO (n=10)	**HY (n=10)**	**COPD (n=20)**	***p* 3-way ANOVA**	***p* HO vs HY**	***p* HO vs COPD**
Age, *years*	70.7 (5.1)	28.0 (5.1)	70.2 (5.9)	<0.001	<0.001	0.834
Female, *number* †	5	6	14	0.556	-	-
FEV1, *% predicted*	98.3 (9.2)	112.6 (20.6)	55.6 (16.2)	<0.001	0.054	<0.001
FEV_1/FVC, ratio	74.5 (4.4)	81.6 (9.3)	44.6 (12.1)	<0.001	0.126	<0.001
RV, *% predicted*	98.4 (19.2)	107.9 (48.9)	157.3 (44.9)	0.001	0.603	0.001
TLC, *% predicted*	104.3 (11.9)	107.6 (15.0)	121.3 (19.1)	0.010	0.667	0.007
RV/TLC, *ratio*	38.4 (4.2)	26.8 (9.7)	53.5 (9.4)	<0.001	0.005	<0.001
TLCO, *% predicted*	98.3 (12.7)	95.6 (13.9)	61.5 (18.7)	<0.001	0.714	<0.001
Smoking history, *Current : Never : Ex* †	0 : 6 : 4	0 : 10 : 0	0 : 0 : 20	<0.001		
Smoking, *pack years* ‡	18.3 (21.5) (n = 4)	-	38.5 (15.4)	0.036	-	0.036
BMI, *kg/m^2*	28.5 (3.3)	26.0 (7.6)	29.0 (6.4)	0.430	-	-
FFMI, *kg/m^2*	18.1 (1.5)	16.5 (2.8)	17.2 (2.7)	0.379	-	-
QMVC isometric strength, *Nm*	129.7 (36.8)	162.8 (72.5)	98.6 (36.1)	0.021	0.573	0.058

Step count, *8 hr*	6007 (2088)	6180 (3449)	4012 (1864) (n=19)	0.040	0.879	0.046

HO, Healthy Older group; HY, Healthy Young group. Values are mean (SD). FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; RV, residual volume; TLC, total lung capacity; TLCO, transfer factor for the lung of carbon monoxide; BMI, body mass index; FFMI, fat-free mass index; QMVC, quadriceps maximum voluntary contraction. Percent predicted values are calculated from normal values (31). † differences in distribution between groups tested with Pearson Chi-square test; ‡ , pack year average for ex-smokers only.

Muscle CS maximal activity (mitochondrial content), MAPR (intrinsic mitochondrial function), and mitochondrial DNA copy number at baseline are shown in Table 2. There was no statistical difference in CS activity between groups (p=0.070). Muscle mitochondrial DNA copy number was less in HO than in HY (p=0.041, Table 2). Intrinsic mitochondrial function (MAPR) was not statistically different in HO compared to HY or COPD at baseline for any substrate combinations used (all p>0.05, Table 2).

Table 2. Baseline measures of quadriceps mitochondrial content and function

	HO (n=10)	**HY (n=10)**	**COPD (n=20)**	***p* 3-way ANOVA**	***p* HO vs HY**	***p* HO vs COPD**
CS activity, *mmol acetyl CoA. min^{-1}. l mitochondrial $suspension^{-1}$*	0.512 (0.132)	0.520 (0.146)	0.397 (0.172)	0.070	-	-
MAPR (Glutamate and Succinate), *µmol ATP $mmol^{-1}$ acetyl-CoA min^{-1}*	1235 (193)	1249 (142)	1148 (147)	0.884	-	-
MAPR (Glutamate and Malate), *µmol ATP $mmol^{-1}$ acetyl-CoA min^{-1}*	975 (340)	963 (355)	806 (277)	0.269	-	-
MAPR (Pyruvate and Malate), *µmol ATP $mmol^{-1}$ acetyl-CoA min^{-1}*	658 (245)	570 (161)	477 (195)	0.073	-	-
MAPR (Palmitate), *µmol ATP $mmol^{-1}$ acetyl-CoA min^{-1}*	488 (169)	447 (86)	429 (169)	0.616	-	-
MAPR (Succinate), *µmol ATP $mmol^{-1}$ acetyl-CoA min^{-1}*	149 (35)	118 (48)	131 (76)	0.521	-	-
Mitochondrial DNA copy number, *relative number*	631 (69)	804 (67)	525 (35)	<0.001	0.041	0.140

HO, Healthy Older group; HY, Healthy Young group. Values are mean (SD). CS, citrate synthase; MAPR, maximal mitochondrial ATP production rates normalised to CS activity for various mitochondrial substrates. Mitochondrial DNA copy numbers expressed relative to nuclear DNA.

During incremental exercise testing at baseline, HY reached a higher workload (p=0.001) and $\dot{V}O_2{}^{PEAK}$ (p<0.001) compared to HO whereas $\dot{V}O_2{}^{PEAK}$ (p=0.026) and peak work rate (p=0.003) were less in COPD relative to HO (Table S3). Measures coinciding with $\dot{V}O_2{}^{PEAK}$ showed that

compared to HO, patients with COPD reached a lower respiratory exchange ratio (RER, p<0.001), minute ventilation ($\dot{V}E$, p=0.020) and heart rate (HR, p=0.071), but utilised a significantly greater proportion of their predicted maximum voluntary ventilation (MVV, p<0.001) (Table S3). Responses to sub-maximal steady-state exercise are shown in Table S4.

Influence of aerobic training and subsequent exercise withdrawal

Habitual physical activity levels in the 1st (week 9) and 4th (week 12) week of exercise withdrawal did not differ from baseline in any group (HO *p=0.961*, HY *p=0.765*, COPD p=0.686), see Table S5 supplementary file). HO and HY participants increased $\dot{V}O_2^{PEAK}$ by 18% (p=0.002) and 21% (p<0.001), respectively, following eight-weeks training (Fig. 1A), but there was no change in $\dot{V}O_2^{PEAK}$ in the COPD group (p=0.603). Following four-weeks of exercise withdrawal, $\dot{V}O_2^{PEAK}$ remained significantly greater than baseline in HO and HY groups (p =0.017 and p=0.004, respectively), whilst $\dot{V}O_2^{PEAK}$ was lower than baseline (p=0.026) and week 8 of exercise training (p=0.033) in the COPD group.

RER during steady-state sub-maximal exercise (at 65% of $\dot{V}O_2^{PEAK}$ at baseline) was reduced in all groups after 8-weeks training (within-group change HO p<0.001, HY p<0.001, COPD p=0.003, Fig. 1B), and remained less than baseline after 4 weeks exercise withdrawal (HO p<0.001, HY *p<0.001,* and COPD p=0.013). HR during steady-state sub-maximal exercise decreased in all groups in the trained state (HO p=0.002, HY *p<0.001,* and COPD p=0.003) and $\dot{V}E$ was reduced in HO and HY (both p<0.05) as shown in Fig. S2.

There was no statistical increase in muscle CS activity in any group with training (HO p=0.120, HY p=0.682, COPD p=0.133), but following four weeks of exercise withdrawal CS activity in the COPD group was less than baseline (p=0.015) and eight weeks of training

(p<0.001), Fig. 2A). Mitochondrial DNA copy number was statistically unchanged from baseline by AET and the return to habitual physical activity in HO (p=0.120) and COPD (p=0.132) volunteers (Fig. 2B). By contrast, there was a robust increase in mitochondrial DNA copy number with AET in the HY group (p<0.001), which declined on return to habitual physical activity (p<0.001), although remaining greater than baseline (p=0.024, Fig. 2B).

Exercise training intervention increased MAPR from baseline in HY volunteers for three substrate combinations (palmitate, p=0.003; glutamate and succinate, p=0.008; glutamate and malate, p=0.011, Fig. 3), and for palmitate in HO volunteers (p0.041, Fig. 3A), but had no robust impact with any substrate combination in COPD (all p>0.05). Withdrawal of AET resulted in the return of MAPR to baseline rates in HY (palmitate, p =0.016; glutamate and succinate, p<0.001; glutamate and malate, p=0.003) and HO volunteers (palmitate, p=0.036), but MAPR was unchanged from baseline in COPD (all substrates p>0.05) (Fig. 3). There was no change in MAPR from baseline for the mitochondrial substrates pyruvate and malate or succinate in any group of volunteers (all p>0.05, data not shown).

Based on changes in muscle mRNA expression from baseline, Fig. 4 illustrates that IPA identified both muscle lipid and carbohydrate metabolism to be altered following 8 weeks of AET and 4 weeks of exercise withdrawal in HO, HY and COPD. The y-axis displays the -log of the p-value for each cellular function, which is a measurement of the likelihood that the association between a set of focus transcripts and a given function is due to random chance with the threshold of significance of the within-group change equivalent to p<0.05. Figure 4 illustrates that based on the collective change in mRNA abundance the consistency of

response of these two cellular functions to exercise training and 4 weeks exercise withdrawal was very similar in HO, HY and COPD.

Fig. 5 highlights the changes in mRNA abundance from baseline for individual genes identified by IPA to compromise the lipid metabolism function shown in Fig. 4 following 8 weeks of training. The number of mRNAs and the magnitude and direction of change of each was similar when comparing HO, HY and COPD groups. The same was true of individual mRNA expression changes from baseline after 4 weeks exercise withdrawal for the lipid metabolism function (Fig. S3). Changes in mRNA abundance following 8 weeks of exercise training (Fig. 6) and 4 weeks exercise withdrawal (Fig. S4) for individual genes deemed to represent carbohydrate metabolism broadly followed the same magnitude and direction of change when comparing HO, HY and COPD groups.

DISCUSSION

The major novel findings of the present study are, firstly, that intrinsic mitochondrial function was not significantly different between HO, HY and COPD at baseline suggesting that mitochondrial function was not impaired in the basal state in patients with COPD or with healthy ageing. Secondly, changes in whole-body and muscle mitochondrial function in response to 8 weeks endurance exercise training at the same relative intensity were robust in HY volunteers, less robust in HO volunteers and largely absent in COPD patients. These diminished responses could be a consequence of the lower absolute muscle loading experienced during training, particularly in COPD where the potential impact of ventilatory limitation to exercise training load exists, but the robust muscle transcriptional response to exercise intervention across all groups speaks against this, and points to post-transcriptional

regulation of muscle adaptation being different during exercise training. Finally, in keeping with the observation in HY volunteers (19) four weeks of exercise training withdrawal returned intrinsic mitochondrial function to that seen in the baseline state in HY and HO volunteers.

It is debated to what extent changes in muscle energy metabolism in COPD reflect muscle level deconditioning and/or a disease-specific COPD mitochondropathy (8, 9, 11-15). Some data indicate that muscle mitochondrial respiration (corrected for total mitochondrial content) in patients with COPD is not impaired compared with older healthy volunteers (11, 13), as has been reported in other chronic disease states, e.g. Type 2 diabetes (32). Conversely, however, there is evidence that low muscle mitochondrial oxidative capacity in COPD cannot be explained by muscle level deconditioning alone and is likely driven by the disease pathophysiology, including a shift away from complex I-driven respiration towards metabolically less-efficient complex II-driven respiration (8), greater mtDNA deletions, increased markers of abnormal fibre specific respiration and suppressed mitochondrial proliferation (e.g. mtDNA copy number) (9, 12). Given mitochondrial oxygen utilisation can be uncoupled from ATP production in pathophysiological states (33), a definitive conclusion as to whether a COPD related mitochondropathy truly exists may be clouded by measurement of mitochondrial respiration as a surrogate of ATP generation, (11, 13) a question addressed by the current study which utilised a sensitive measure of maximal mitochondrial ATP production rates directly. These data in combination with quantification of mitochondrial density (CS), and proliferation (mtDNA copy number) accord with the evidence that intrinsic mitochondrial function in patients is not impaired with ageing (16) or in the presence of COPD (11, 13).

Chronic aerobic exercise training increases several markers of muscle mitochondrial content and function in young (19) and older (16) volunteers, but limited information is available as to whether similar mitochondrial responses to aerobic exercise training occur in patients with COPD, not least because ventilatory limitation in COPD can prevent skeletal muscle from being adequately challenged during whole-body exercise, thereby reducing training adaptation (20). High-intensity restricted muscle group training has been shown to restore mitochondrial function in patients with COPD to that observed in age-matched controls (albeit n=5), although it is unknown whether functional capacity also improved (13). Picard *et al* (11), also pointed to muscle deconditioning, rather than mitochondrial dysfunction, being the driver of lower maximal rates of mitochondrial respiration in patients with COPD. In the present study, exercise training of a large muscle mass was performed at an intensity high enough to increase mitochondrial lipid and carbohydrate oxidation yet within the limits of tolerance for all groups. This exercise regimen produced robust changes in whole-body and muscle mitochondrial function and proliferation in HY volunteers, which were diminished in HO volunteers and absent in COPD patients. We acknowledge that muscle-level loading may have been truncated in the COPD group due to ventilatory limitation restricting whole-body exercise tolerance. Importantly, however, following exercise intervention the muscle transcriptional response of genes directly linked to muscle lipid and carbohydrate use was similar in patients with COPD compared to HO and HY volunteers. Furthermore, a decrease in steady-state RER during sub-maximal exercise across the course of eight weeks training was evident in all groups demonstrating that chronic exercise adaptation in COPD was not limited by fuel mobilisation nor availability during exercise. It would appear rather, that the training regimen employed was not sufficient to elicit adaptation in post-transcriptional regulation of mitochondrial biogenesis or mitochondrial

function, particularly in COPD patients. This may result from ventilatory limitation restricting the magnitude of the muscle level challenge, however it has also been proposed that the plasticity of skeletal muscle adaptive responses to contractile activity is diminished with age and age-related disease, and this extends to mitochondrial proliferation and function, suggesting that a greater contractile stimulus is required to attain a similar phenotype adaptation (34). Emerging sites for such post-transcriptional limitation include muscle translational efficiency (ribosome activity) and translational capacity (ribosome number) (35).

Remarkably $\dot{V}O_2^{PEAK}$ and muscle CS activity in the COPD patients declined below the values recorded at baseline and following exercise training intervention, which was not observed in HY and HO volunteers. There is no obvious explanation for this observation given habitual physical activity levels during the first and fourth week of exercise withdrawal were not significantly different to that measured at baseline. However, it should be acknowledged that triaxial accelerometery, which was used to quantify step count in the current study, cannot quantify the intensity of the activities of daily living and therefore may perhaps explain the apparent deconditioning observed in the COPD group following 4 weeks of exercise withdrawal.

This study, through deliberate selection of sedentary healthy volunteers, better matched for habitual physical activity status of subjects compared to previous publications comparing COPD and health, and furthermore employed a tightly supervised exercise protocol. It is acknowledged that the sample size (although large for a study employing a demanding intervention and detailed metabolic and physiological measures) limits the generalisability of findings to broader populations. The fat-free mass of patients with COPD in this study was

normal, further limiting the generalisability of these findings to patients with low fat-free mass in whom differences in response to aerobic training (particularly at an mRNA level) have previously been observed (36). Finally, a preferential loss of type I oxidative fibres has previously been observed in COPD, leaving a relatively large proportion of type II fibres compared to healthy controls (4, 5). Distinct muscle fibre type responses to exercise training would have added additional insight, however due to limited tissue availability, it was not possible to characterise individual muscle fibre-type responses in this study. A further limitation of the current study is that it does not report muscle responses at a protein level downstream of changes in muscle mRNA abundance.

Our data align with the view that in COPD patients where maximal pulmonary ventilation is constrained, partitioned training strategies such as interval or restricted muscle group training aimed at maximising muscle level training intensity are likely to stimulate greater muscle level adaptation (21, 37), and thereby possibly patient benefit. Furthermore, the findings lend mechanistic support to current clinical rehabilitation guidelines recommending the prescription of high relative whole-body exercise intensities for people with COPD as low relative intensities may be insufficient to provoke robust whole-body and muscle level adaptation (38). It is important to acknowledge in addition, the broader effects of PR such as improvements in confidence, tolerance of breathlessness and mood that may impact on other clinical outcome measures that reflect patient benefit (for example field tests of exercise performance) regardless of relative exercise intensity. Nonetheless, enhancing adaptation at a muscle level has the potential to benefit patients and we suggest novel drug therapies targeting mitochondrial function should take account of the preservation of transcriptional responses to exercise training in the COPD group. Identifying the post-

transcriptional locus of impairment is a critical research question to be addressed in the development of such therapies.

In conclusion, intrinsic mitochondrial function was not impaired with ageing or COPD. However, the magnitude of changes in whole-body responses to exercise, rates of mitochondrial ATP production and mitochondrial proliferation in response to chronic aerobic exercise training were variably blunted with age and further blunted in COPD, possibly as a result of ventilatory limitation. The similarity in muscle mRNA responses directly linked to fat and carbohydrate oxidation across HY, HO and COPD patient groups points to the plasticity of skeletal muscle to exercise induced stress being diminished at the post-transcriptional level in COPD. Our findings are relevant to exercise prescription during pulmonary rehabilitation, suggesting that higher relative work intensities during whole body aerobic training may be needed to stimulate adaptation at a muscle level in COPD and thereby maximise patient benefit.

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Figure legends

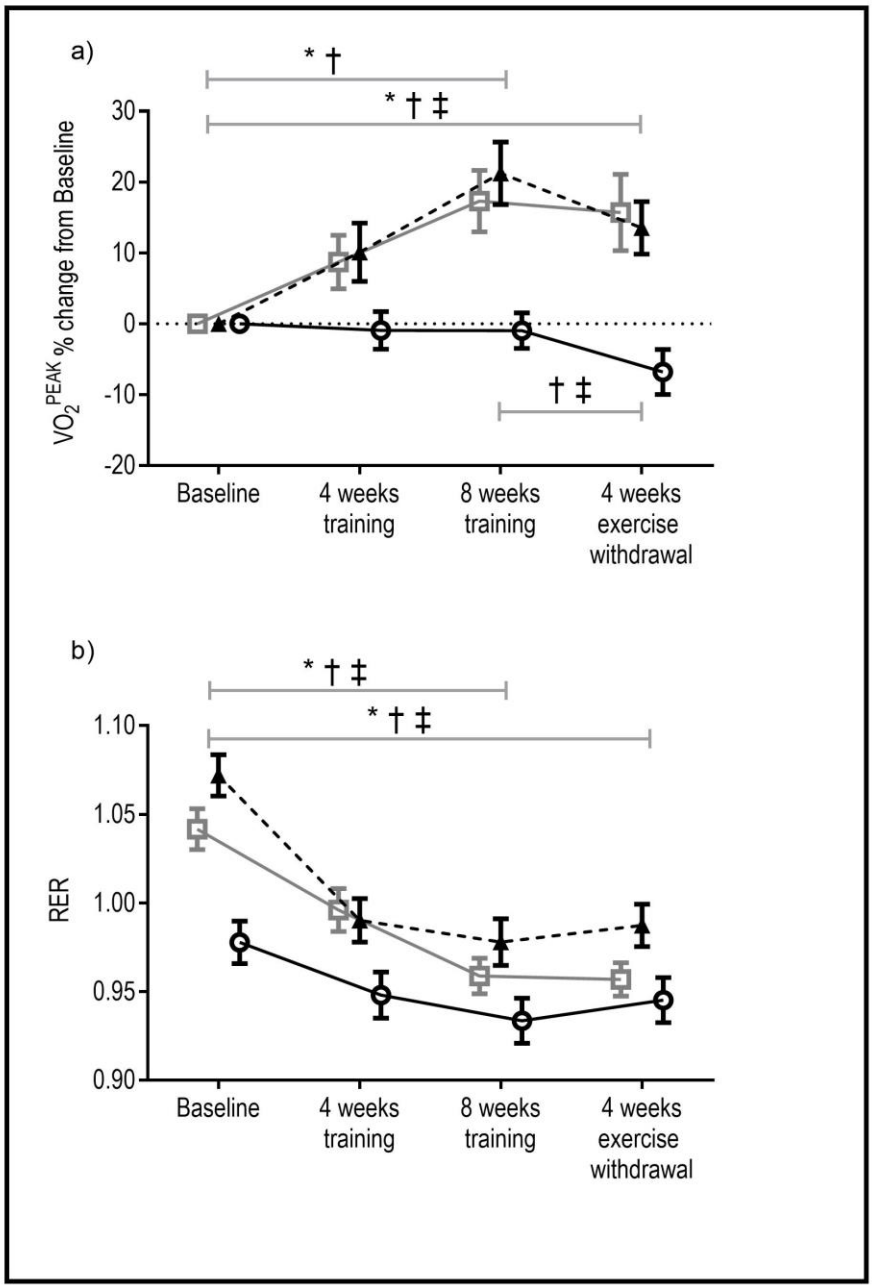


Figure 1. a) Within-group change in $\dot{V}O_2^{PEAK}$ normalised for lean body mass expressed as percent change from baseline after 8 weeks training, and after a subsequent 4 weeks exercise withdrawal where subjects returned to habitual physical activity levels for HO (grey squares), HY (black triangle) and COPD (open circle) groups. b) Respiratory exchange ratio (RER) during steady-state sub-maximal exercise at the same time points as above (a). Within group change $p < 0.05$ for: HO, *; HY, †; COPD, ‡. Values are mean (SEM).

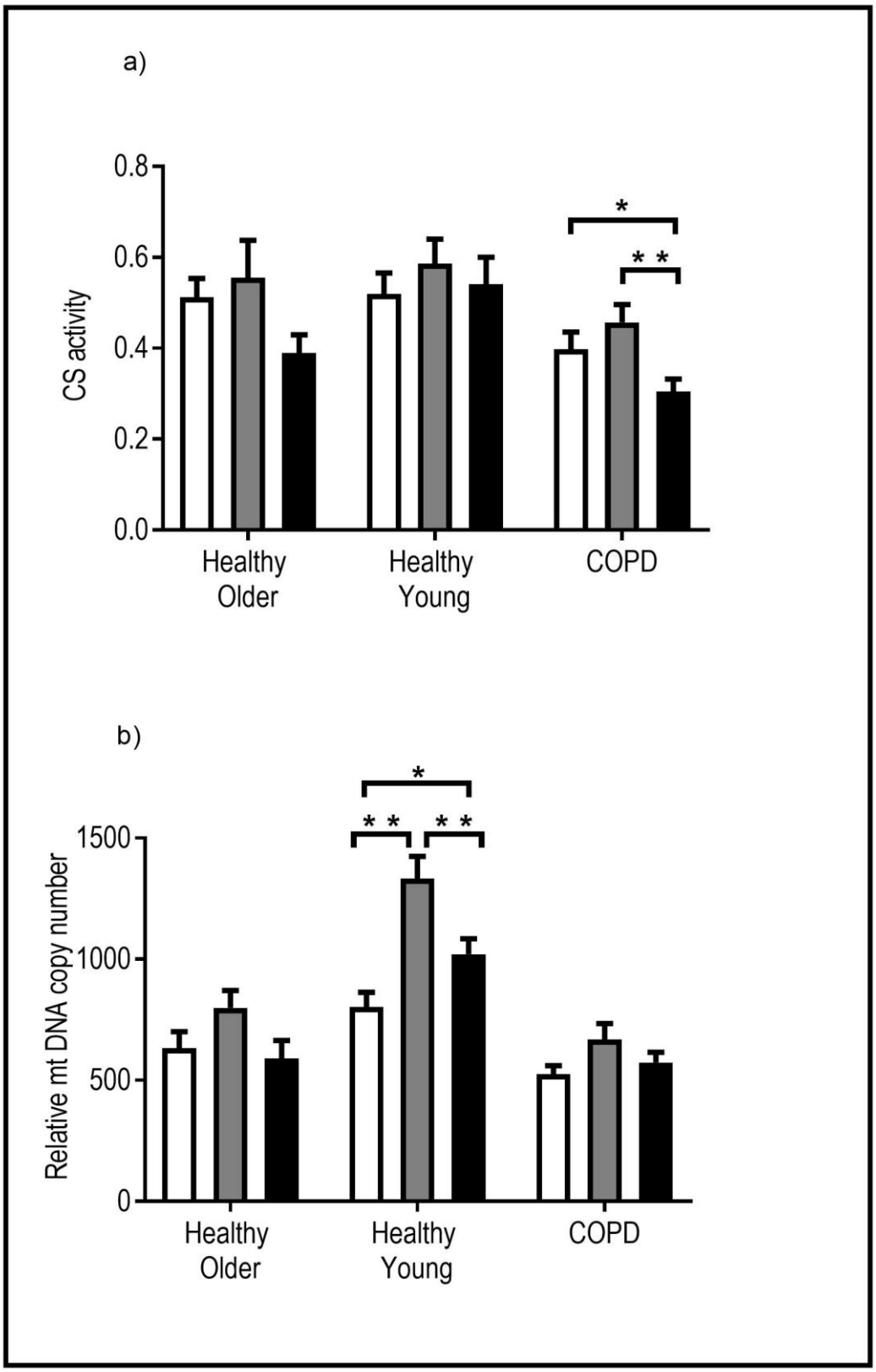

a)
0.8
0.6
0.4
0.2
0.0
CS activity
*
* *
Healthy Older
Healthy Young
COPD
b)
1500
1000
500
0
Relative mt DNA copy number
*
* *
* *
Healthy Older
Healthy Young
COPD

Figure 2. a) Muscle citrate synthase (CS) activity (mmol acetyl CoA min^{-1}.l mitochondrial $suspension^{-1}$); and b) mitochondrial DNA copy number (relative to nuclear DNA copy number) at baseline (open squares), after 8 weeks training (grey squares), and after 4 weeks exercise withdrawal (black squares) where subjects returned to habitual physical activity levels. Values are mean (SEM). *$p < 0.05$, **$p < 0.01$.

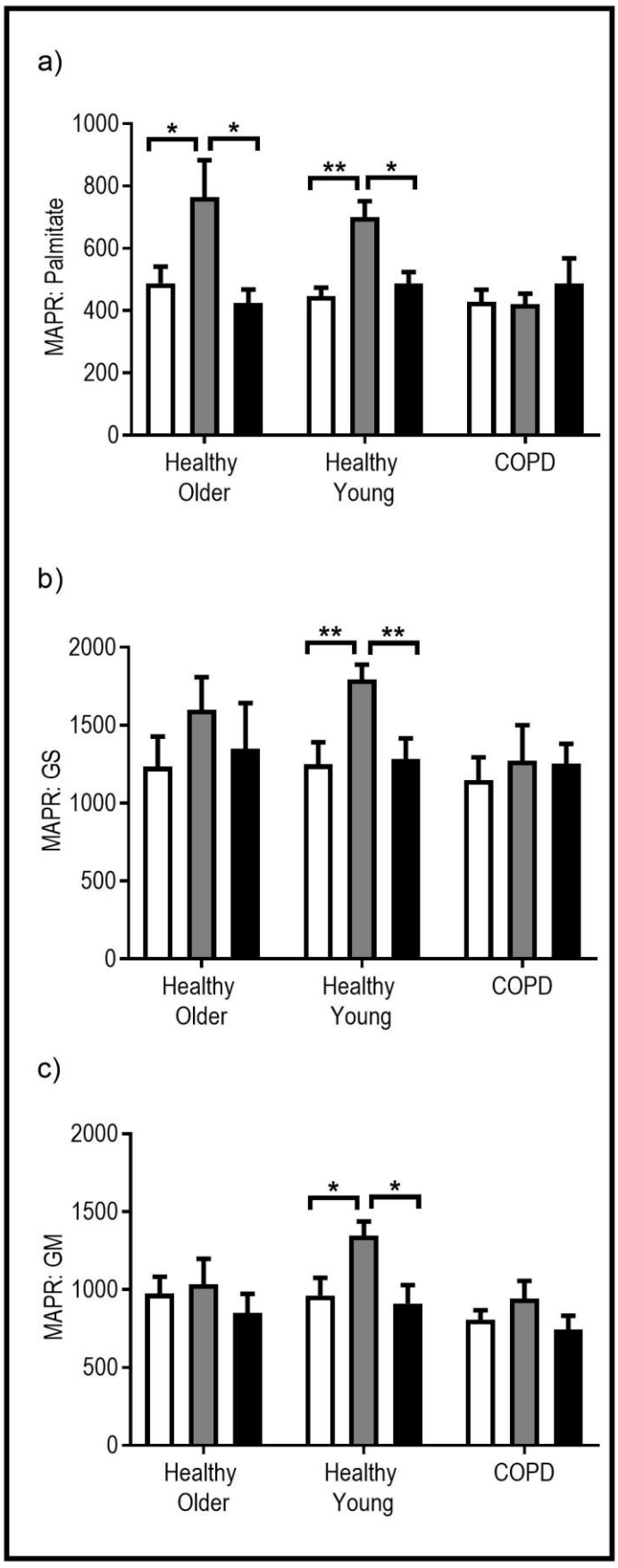

a)
MAPR: Palmitate
1000
800
600
400
200
0
*
*
**
*
Healthy Older
Healthy Young
COPD
b)
MAPR: GS
2000
1500
1000
500
0
**
**
Healthy Older
Healthy Young
COPD
c)
MAPR: GM
2000
1500
1000
500
0
*
*
Healthy Older
Healthy Young
COPD

Figure 3. Muscle intrinsic mitochondrial function assessed by determining maximal mitochondrial ATP production rates (MAPR, µmol ATP $mmol^{-1}$ acetyl-CoA min^{-1}) at baseline (open squares), after 8 weeks training (grey squares), and after 4 weeks exercise withdrawal (black squares) where subjects returned to habitual physical activity levels. a) palmitoyl-L-carnitine (Palmitate); b) glutamate and succinate (GS); c) glutamate and malate (GM). Values shown are mean (SEM). *p<0.05, **p <0.01.

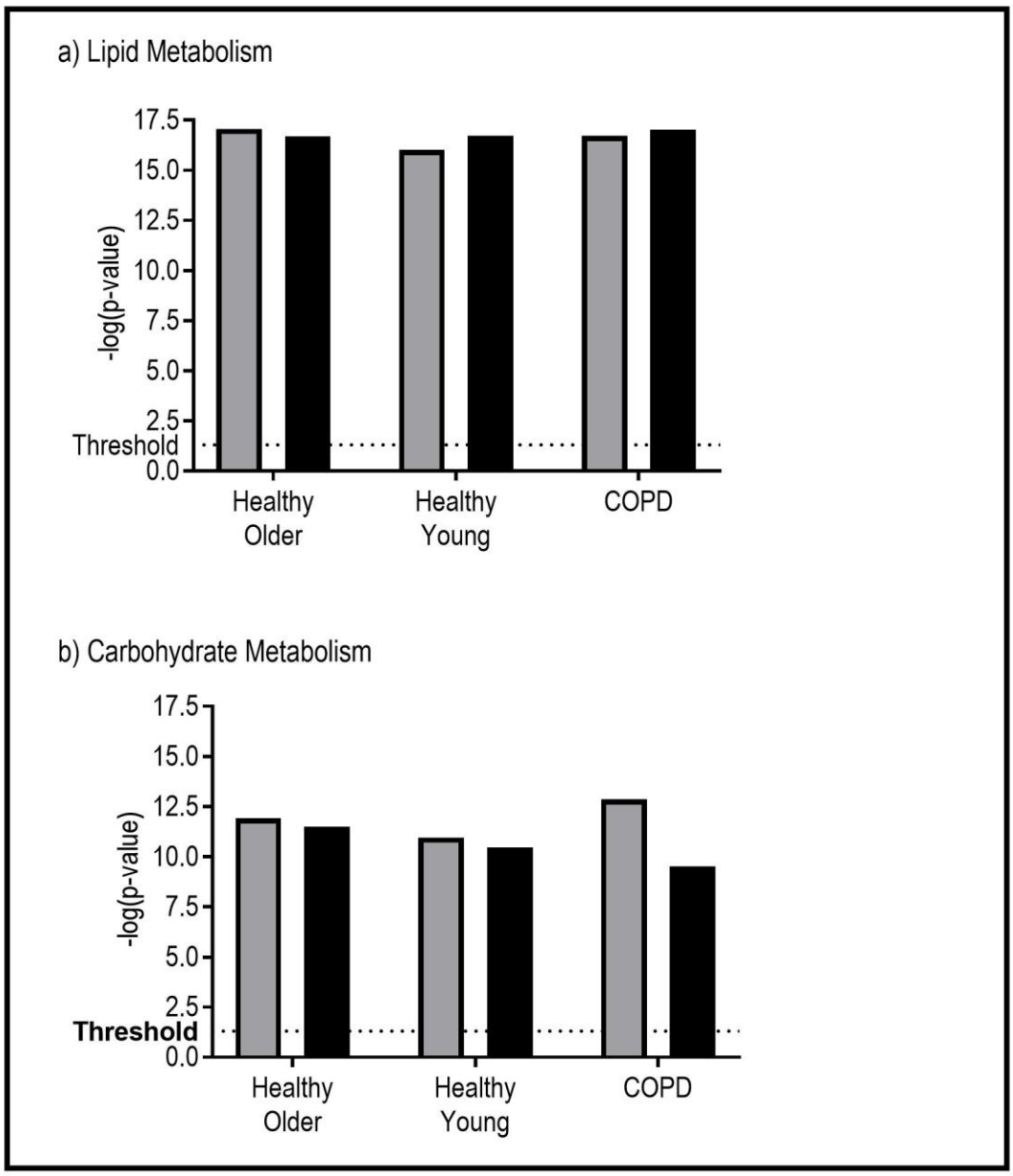


Figure 4. Muscle cellular functions identified by IPA as being altered from baseline after 8 weeks training (grey squares), and after 4 weeks exercise withdrawal (black squares; where subjects returned to habitual physical activity levels) based on mRNA expression data generated using quantitative RT-PCR. Lipid metabolism (a) and carbohydrate metabolism (b) were significantly influenced after both training and exercise withdrawal. The y-axis displays the –log of the p value. The –log of the p value was calculated by Fisher's exact test right-

tailed. Dashed line denotes threshold of statistical significance for within-group change relative to baseline. A -log of the p-value of 1.3 is equivalent to p=0.05).

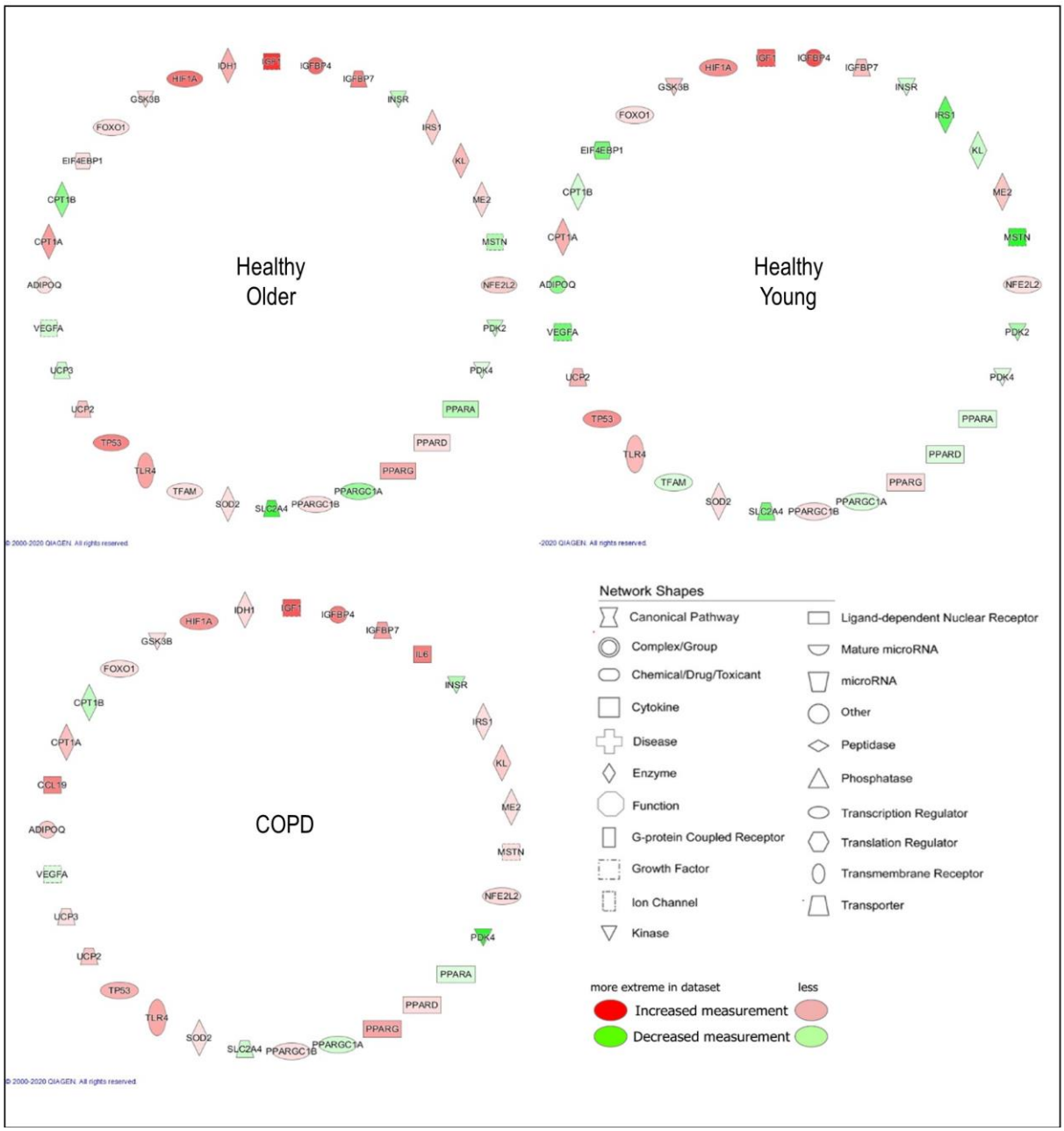


Figure 5. Differentially regulated muscle mRNAs associated with lipid metabolism following 8 weeks training compared to baseline in healthy older, healthy young and COPD groups. Abbreviated gene names are defined in Table S2 of the online supplement.

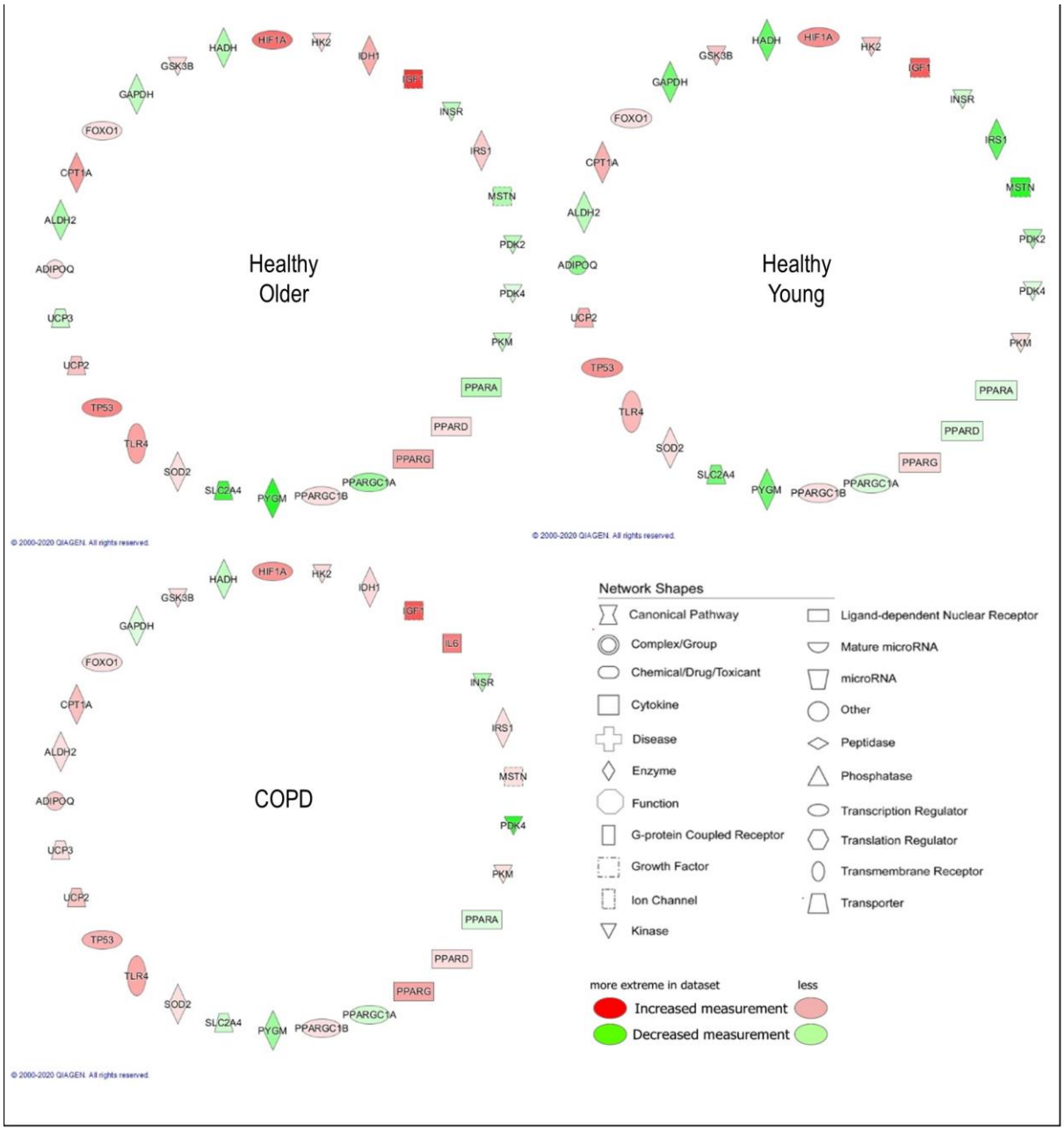


Figure 6. Differentially regulated muscle mRNAs associated with carbohydrate metabolism following 8 weeks training compared to baseline in healthy older, healthy young and COPD groups. Abbreviated gene names are defined in Table S2 of the online supplement.

Online Data Supplement

Whole-body & muscle responses to aerobic exercise training and withdrawal in ageing & COPD

Lorna E Latimer, Dumitru Constantin-Teodosiu, Bhavesh Popat, Despina Constantin, Linzy Houchen-Wolloff, Charlotte E Bolton, Michael C Steiner, Paul L Greenhaff

METHODS

Subjects

Participants for all groups were identified from existing research databases; patients with COPD were recruited from outpatient clinics; and the healthy volunteers were identified via local advertisement. Participants were not engaged in regular exercise training prior to enrollment (<150 min moderate intensity exercise / week and patients had not attended pulmonary rehabilitation in preceding 12 months). Inclusion and exclusion criteria are in Table S1.

Table S1. Participant inclusion / exclusion criteria.

	Inclusion	**Exclusion**
Young Healthy Adults (HY)	Age $\geq$18 & $\leq$35	
	Not engaged in regular exercise programme	Exceeding 150 min / week moderate intensity exercise
	Normal lung function: FEV_1 >80% predicted, FEV_1 / FVC $\geq$70%	Any respiratory diagnosis
Healthy Older Adults (HO)	Age $\geq$60 & $\leq$80	
	Not engaged in regular exercise programme	Exceeding 150 min / week moderate intensity exercise
	Normal lung function: FEV_1 >80% predicted, FEV_1 / FVC $\geq$70%	Any respiratory diagnosis

COPD	Age ≥60 & ≤80	
	Clinical diagnosis of COPD and obstructive spirometry: FEV_1 <80% predicted, FEV_1 / FVC <70%	
	Not engaged in regular exercise programme	Attended PR within last 12 months
	MRC grade ≥3	
	Clinically stable	Exacerbation within last 4 weeks
All Groups	Ability to give informed consent	Any medical condition associated with metabolic disturbance (e.g. type II diabetes), inflammation (e.g. rheumatoid arthritis, inflammatory bowel disease), impaired muscle function or one which affects the ability to perform exercise testing and training (e.g. cardiovascular disease, significant osteoarthritis)
		Receiving systemic corticosteroid medication
		Receiving anticoagulation therapy or condition causing impaired clotting / platelet dysfunction
		Current smoker (ex-smokers < 1 year were acceptable)

Study design

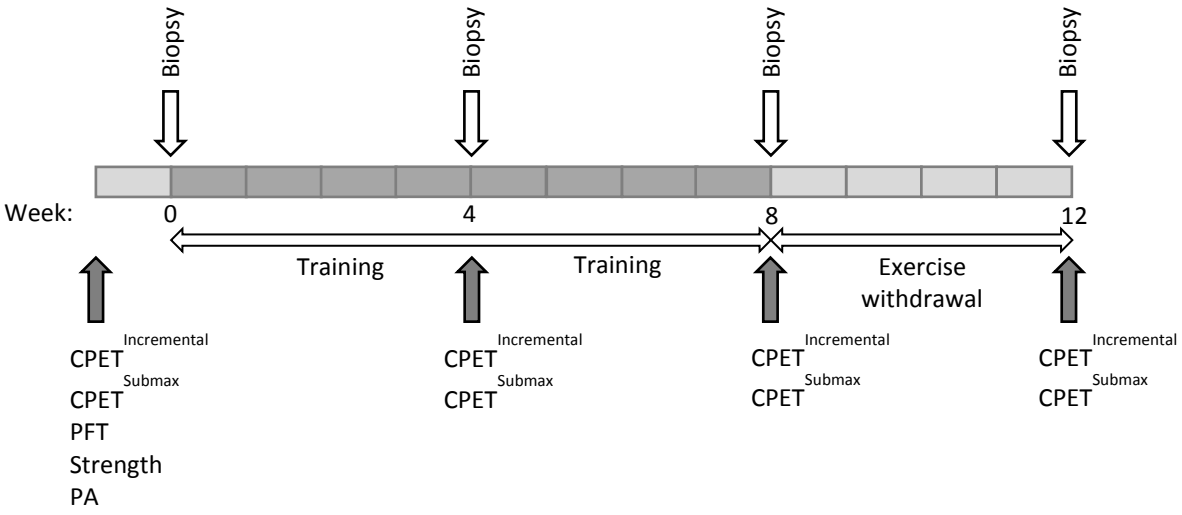


Figure S1. Study protocol followed by all volunteers. Biopsy, microbiopsy of *vastus lateralis*; $CPET^{Incremental}$, symptom-limited incremental cardiopulmonary exercise test; $CPET^{Submax}$, submaximal cardiopulmonary exercise test at workload corresponding to 65% of the workload at $\dot{V}O_2{}^{PEAK}$ in baseline $CPET^{Incremental}$; PFT, pulmonary function test; strength, quadriceps maximal voluntary contraction; PA, physical activity monitoring for 7 days. Training intensity was reset at week four if workload at $\dot{V}O_2{}^{PEAK}$ had increased.

Baseline assessments

Baseline assessments were performed over three visits. At visit 1 measures of anthropometry (height, body mass, body composition by DEXA), pulmonary function and quadriceps strength were performed as was a familiarisation symptom-limited incremental cycling cardiopulmonary exercise test (incremental CPET). At the second visit (minimum 48 hours after visit one) a further incremental CPET (to verify the preceding test) was

performed followed by a submaximal cycling exercise tests (separated by ≥30 minutes resting time). A minimum of 7 days later, at the third visit, a quadriceps muscle biopsy was performed (week 0). Habitual physical activity was assessed over seven days prior to intervention and repeated during the first and last weeks of the exercise withdrawal period.

Anthropometry, pulmonary function, quadriceps strength and physical activity monitoring

Measurements of pulmonary function including lung volumes by plethysmography [S1], body mass index (BMI), body composition (dual energy x-ray absorptiometry, DEXA; Lunar Prodigy, GE Healthcare, Buckinghamshire, United Kingdom), and quadriceps isometric strength at 90° knee and hip flexion (Cybex II Norm: CSMi, Stoughton, USA) were performed at baseline. Habitual physical activity was monitored using a triaxial accelerometer (SenseWear; BodyMedia, Pittsburgh, USA) worn during waking hours on 7 consecutive days before baseline, and during the first (week 9) and final (week 12) week of exercise withdrawal. A minimum of 8 hours of data per day was required to be included in the analysis. The mean step count of the initial 8 hours after waking was calculated for each individual.

Incremental CPET and a submaximal cycling exercise tests

Incremental CPET was performed in accordance with international guidelines [S2] on an electromagnetically braked cycle ergometer (Lode Corival, Groningen, Netherlands) with continuous electrocardiogram, oxygen saturation and blood pressure monitoring. After three min unloading pedalling, workload was increased progressively (ramp protocol) at a rate between 5 W/min and 20 W/min so as to elicit a test of 8 – 12 min duration. All subsequent incremental tests employed the same protocol as the baseline test. Breath by breath measurements of gas exchange ($\dot{V}O_2$, $\dot{V}CO_2$) and ventilation ($\dot{V}E$) were monitored

using a metabolic cart (Ergocard Professional, Medisoft, Sorinnes, Belgium) and reported as fifteen breath rolling averages. Predicted maximum voluntary ventilation (MVV) was calculated as $FEV_{1\ X}$ 37.5 (S3).

Sub-maximal testing was performed at 65% of the workload corresponding to the highest $\dot{V}O_2^{PEAK}$ achieved in either of the baseline tests and was not changed for subsequent tests. Continuous monitoring of cardiorespiratory variables was performed throughout the test and an average reported for the steady-state period. The steady-state period excluded the first three minutes at test workload and isotime measures were calculated for each individual, the duration of which was determined by the shortest test performed by that individual (normally the baseline test). The sub-maximal test was terminated by the investigators after 30 min. The intensity of the sub-maximal test corresponded to the initial training intensity and measures of heart rate, $\dot{V}E$, and respiratory exchange ratio (RER) indicate the physiological stress experienced during training.

Muscle sampling and processing

Muscle biopsy samples were obtained from the *vastus lateralis* muscle of the dominant leg at mid-thigh level using a needle micro-biopsy technique [S4]. Briefly, after the skin was sterilised with Betadine solution, local anaesthetic (lignocaine) was injected subcutaneously and to the depth of the fascia. A small incision (5 mm) was made in the skin and any subcutaneous adipose, through which a 12 g micro-biopsy needle was inserted (Bard Magnum, Arizona, USA). Four passes were performed, each harvesting ~20 mg of tissue. Approximately 40 mg of freshly isolated vastus lateralis muscle tissue was finely diced on a cooled glass plate, and weighed for mitochondrial function and content measurements. The

remaining muscle tissue was immediately dissected free of visible adipose and connective tissue, snap frozen and stored in liquid nitrogen for subsequent DNA and mRNA analyses. The biopsy site was dressed with a butterfly closure, waterproof sterile dressing and a compression bandage to apply light pressure in order to minimise the risk of bleeding or bruising. Subsequent biopsies were performed 2.5 cm from the preceding incision site.

Muscle Mitochondrial measurements

Each sample was homogenised on ice for 3 min in a buffer solution (pH 7.0, KCl 100mM, KH_2PO_4 50 mM, Tris 50 mM, $MgCl_2$ 5mM, EDTA 1 mM, ATP 1.8 mM) using a Teflon pestle homogeniser. The crude homogenate was then centrifuged at 650 g for 3 min at 4^{o}C, and the resultant supernatant was transferred to a test tube and centrifuged at 15,000 g for 3 min at 4^{o}C. The resulting pellet formed contained the mitochondria. Following this, the supernatant was removed and discarded before resuspension of the pellet in 300 µl of the original homogenisation buffer. This was then centrifuged at 15,000 g for 3 min at 4^{o}C. After removal of the supernatant, the pellet was re-suspended in a re-suspension solution (pH 7.0, human serum albumin 0.5 mg/ml, sucrose 240 mM, monopotassium phosphate 15 mM, magnesium acetate tetrahydrate 2mM, EDTA 0.5 mM). The differential centrifugation isolated mitochondrial suspension was then kept on ice immediately prior to measurement of mitochondrial ATP production rates (MAPR).

Mitochondrial ATP production rates (intrinsic mitochondrial function)

Following the method of Wibom et al [S5], 2.5 µl of diluted mitochondrial suspension was added to each well of a luminometer plate. The wells contained 200 µl ATP monitoring reagent (Firefly Luciferase, 357 µM D-luciferine, 14.3 µM L-luciferine, 15 mM BSA, 1 µM Sodium Pyrophosphate Decahydrate, 187.55 mM Sucrose, 18.8 mM monopotassium

phosphate, 2.5 mM magnesium acetate tetrahydrate, 677 µM EDTA (K Salt) pH 7.0), 12.5 µl of 12 mM ADP and 35 µl of the following substrate combinations in duplicate: Glutamate (16.4 mM) and Succinate (15 mM); Glutamate (32.75 mM) and Malate (22 mM); Pyruvate (50 mM) and Malate (22 mM); Palmitoyl-l-carnitine (5 µM) and Malate (1.5 mM); Succinate (2.5 mM); and double-distilled water (ddH_2O). A blank that contained all the above components except the substrate was run in parallel for each sample. Luminescence was continuously recorded for 10 min (BMG LABTECH, Ortenberg, Germany) and an injection of 150 pM ATP standard took place after 4.6 min. The change in luminescence elicited by the ATP standard was used to calculate MAPR. Mitochondrial suspensions were frozen at -80°C for subsequent determination of citrate synthase activity (see below). MAPR values were corrected for maximal citrate synthase activity (routinely used as an index of mitochondrial content / mass) to provide measures of intrinsic mitochondrial function normalised to the mitochondrial content of the suspension.

Citrate synthase activity (mitochondrial content)

Muscle citrate synthase (CS) maximal activity was determined at 37°C on the isolated mitochondrial solution using a kinetic spectrophotometric method to follow the change in absorbance of a 5,5'-dithiobis (2-nitrobenzoic acid)(DNTB) buffered solution as previously described (E6). Briefly, 15 µl mitochondrial suspension was added to 185 µl homogenisation buffer (95% extraction buffer containing 1% triton) and was homogenised using a glass pestle at 200 rpm for 2 min (5). The homogenate was centrifuged at 24,000 g (Eppendorf, Hamburg, Germany) before CS was determined spectrophotometrically in the supernatant [S5]. Acetyl-CoA is formed at a rate determined by the quantity of CS protein present.

Relative mitochondrial DNA copy number Genomic DNA (nDNA) and mitochondrial DNA (mtDNA) were extracted from skeletal muscle Qiagen using a QIAamp® DNA Mini kit according to the manufacturer's instructions. Briefly, the procedure involved initial tissue lysis in a buffer containing proteinase K, incubation for 3 hrs at 56°C to digest the myofibril proteins followed by the spinning of the lysates on silica-membrane-based nucleic acid purification columns and elution of the mtDNA and nDNA with appropriate buffers. Before the addition of buffer AL (Qiagen), 4 µL of free DNase activity RNase A stock solution 7000 U/mL was added to each sample lysate. DNA quality and quantity was assessed by measurement of light at 260, 280 and 230 nm (Nanodrop One Spectrophotometer, Thermoscientific, Waltham, MA, USA). The expression level of hydroxymethylbilane synthase (HMBS; nDNA) and encoded NADH:ubiquinone oxidoreductase core subunit 1 (ND1; mtDNA) was used to evaluate the relative abundance of nuclear and mitochondrial DNA and was quantified using TaqMan probe real-time PCR. The TaqMan probe design for the detection of nDNA levels was based on interrogation of the intron sequence spanning between exons 3-4 of the genomic hydroxymethylbilane synthase (HMBS) gene to avoid any mRNA amplification, if present. The probe design for detection of mtDNA levels was based on interrogation of a stable fragment of the mtDNA loop, namely the mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 1 (ND1). The $2^{-\Delta Ct}$ formula, where $\Delta = Ct_{ND1}-Ct_{HMBS}$, was used to express the relative number of mtDNA copies.

Muscle mRNA expression linked to fuel metabolism

RNA was extracted from ~30 mg snap-frozen muscle as previously described [S7]. First strand cDNA was synthesised from 1 µg of total RNA, using Superscript III reverse transcriptase (Invitrogen Ltd, Paisley, UK) and random primers (Promega, Southampton, UK)

and stored at -80^{o}C until analysis. TaqMan low density arrays were performed using an ABI PRISM 7900HT sequence detection system, and data analysed using SDS 2.1 software (Applied Biosystems, USA). Data were further analysed using RQ Manager software (Applied Biosystems, USA), where the threshold level was normalised across all plates before Ct values were calculated for each gene target and sample. Relative quantification of mRNAs of interest was measured using the $2^{-\Delta\Delta Ct}$ method with hydroxymethylbilane synthase (HMBS) as the endogenous control as it was unaffected by exercise intervention (data not shown). A total of 59 transcripts known to be involved in muscle carbohydrate and lipid metabolism were targeted for analysis in the present study (Table S1). Target selection was led by published data involving high-throughput and targeted RT-PCR approaches from our research group which identified muscle transcripts responsive to exercise intervention [S8], insulin resistance [S9] and changes in fuel metabolism with nutritional [S10] and pharmacological intervention [S11]. Additionally unpublished muscle transcript data from our group from research involving limb immobilisation in healthy volunteers was accessed. To associate altered biological functions to the targeted probe sets, Ct values were uploaded to Ingenuity Pathway Analysis (IPA) software (QIAGEN, Hilden, Germany) for pathway analysis of gene expression data. The overall outcome of IPA (e.g. cellular function) is predicted by calculating a regulation Z-score and an overlap p-value, which are based on: 1, the number of regulated target genes' function; 2, the magnitude of expression change; 3, the direction of expression change; and 4, their concordance with the IPA database, which is constructed from an extensive curated literature database. The overlap p-value was calculated by IPA to identify significantly enriched function pathways from the submitted list of significantly changed genes. These p-values were generated from the right-tailed Fisher's Exact Test, and a significance threshold of $p<0.05$ was used to assess the

statistical significance of the function pathways. In order to control for any enrichment of false positive results when undertaking multiple comparisons (type II errors) IPA utilises Bonferroni's corrected p-value set at $p < 0.05$.

Table S2. mRNA transcripts with functions relating to muscle fuel metabolism that were quantified in this analysis.

Abbreviation	Gene name	ABI ID
ALDH2	Aldehyde dehydrogenase 2 family (mitochondrial)	Hs01007998_m1
G6PD	Glucose-6-phosphate dehydrogenase	Hs00166169_m1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Hs99999905_m1
GLUD1	glutamate dehydrogenase 1	Hs03989560_s1
GYS1	glycogen synthase 1 (muscle)	Hs00157863_m1
HADH	□-hydroxyacyl-CoA dehydrogenase	Hs00193428_m1
HK1	hexokinase 1	Hs00175976_m1
HK2	hexokinase 2	Hs00606086_m1
IDH1	Isocitrate dehydrogenase 1	Hs00271858_m1
PDP2	pyruvate dehyrogenase phosphatase catalytic subunit 2	Hs00380020_m1
PFKM	phosphofructokinase, muscle	Hs00175997_m1
PGM1	phosphoglucomutase 1	Hs01071897_m1
PKM	pyruvate kinase, muscle	Hs00987255_m1
PYGM	phosphorylase, glycogen, muscle	Hs00989942_m1
ACOT1	acyl-CoA thioesterase 1	Hs04195130_s1
ADIPOQ	adiponectin	Hs00605917_m1

APOA1	Apolipoprotein A-I	Hs00163641_m1
APOC1	Apolipoprotein C	Hs00155790_m1
CCL19	chemokine (C-C motif) ligand 19	Hs00171149_m1
CPT1A	carnitine palmitoyltransferase, liver isoform	Hs00912671_m1
CPT1B	carnitine palmitoyltransferase, muscle isoform	Hs00189258_m1
EIF4EBP1	eukaryotic translation initiation factor 4E binding protein 1	Hs00607050_m1
FAXDC2	fatty acid hydroxylase domain containing 2	Hs00260753_m1
FOXO1	Forkhead box protein O1	Hs01054576_m1
HSPA14	heat shock 70kDa protein 14	Hs00212495_m1
HSPD1	heat shock 60kDa protein 1 (chaperonin)	Hs01036747_m1
IDH1	Isocitrate dehydrogenase 1	Hs00271858_m1
IGFBP4	insulin-like growth factor binding protein 4	Hs01057900_m1
IGFBP7	insulin-like growth factor binding protein 7	Hs00266026_m1
IL6	interleukin 6 (interferon, beta 2)	Hs00985639_m1
KL	klotho	Hs00183100_m1
LOXHD1	lipoxygenase homology domains 1	Hs00329848_m1
LPPR2	lipid phosphate phosphatase-related protein type 2	Hs01106565_m1
ME2	malic enzyme 2, NAD(+)-dependent, mitochondrial	Hs00929809_g1
NFE2L2 (NRF2)	nuclear factor, erythroid 2 like 2	Hs00975961_g1
NRF1	nuclear respiratory factor	Hs00192316_m1

	1	
PLA2	Phospholipase A2	Hs00179898_m1
PLD4	phospholipase D family, member 4	Hs00975488_m1
PPARA	peroxisome proliferator-activated receptor alpha	Hs00947536_m1
PPARD	peroxisome proliferator-activated receptor delta	Hs00987011_m1
PPARG	peroxisome proliferator-activated receptor gamma	Hs01115513_m1
PPARGC1A	peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	Hs01016719_m1
PPARGC1B	peroxisome proliferator-activated receptor gamma, coactivator 1 beta	Hs00991677_m1
SOD2	superoxide dismutase, mitochondrial	Hs00167309_m1
TFAM	transcription factor A, mitochondrial	Hs00273372_s1
TLR4	toll-like receptor 4	Hs00152939_m1
TP53 (p53)	tumor protein p53	Hs01034249_m1
UCP2	uncoupling protein 2 in mitochondria	Hs01075227_m1
UCP3	uncoupling protein 3 in mitochondria	Hs01106052_m1
VEGFA	Vascular endothelial growth factor A	Hs00900055_m1
GSK3B	Glycogen synthase kinase-3 beta	Hs01047719_m1
HIF1A	Hypoxia-inducible factor 1-alpha	Hs00153153_m1
IGF1	insulin-like growth factor 1 (somatomedin C)	Hs01547656_m1
INSR	Insulin receptor	Hs00961554_m1
IRS1	Insulin receptor substrate 1	Hs00178563_m1

MSTN	myostatin	Hs00976237_m1
PDK2	pyruvate dehydrogenase kinase, isozyme 2	Hs00176865_m1
PDK4	pyruvate dehydrogenase kinase, isozyme 4	Hs01037712_m1
SLC2A4 (GLUT4)	glucose transporter	Hs00168966_m1

Exercise training intervention

Participant underwent supervised training on an electrically braked cycle ergometer (Lode Corival, Groningen, The Netherlands). Three supervised sessions of 30 min duration were performed per week. Individuals who were unable to compete 30 min continuously were permitted to rest (~5 min) before resuming the session over a total permissible duration of 60 min. Exercise at the prescribed intensity (workload corresponding to 65% $\dot{V}O_2^{PEAK}$) is known to increase muscle lactate accumulation, pyruvate dehydrogenase complex (PDC) activation and flux [S12], and rates of mitochondrial carbohydrate and lipid oxidation from both plasma and muscle sources [S13] well above the resting state, thereby providing a robust stimulus to muscle metabolic adaptation.

Statistical analysis

A power calculation performed on MAPR data (glutamate and succinate) using G-Power software (version 3.1.9.2, Dusseldorf University, Germany) for ANOVA one-way fixed effects given $\alpha = 0.05$, number of groups = 3, power = 0.9 and effect size = 0.6 using the data from Barany et al. [S14] recommended n=9 for the healthy control group, which we rounded up to n=10 for healthy volunteers. Given the inherently variable nature of physiological responses in patients with COPD, this number was increased to n=20 in the COPD group.

Approvals & clinical trial registration

The trail was approved by NHS National Research Ethics Service, West Midlands Committee – Coventry & Warwickshire (reference 13/WM/0075) and registered with the UK Clinical

Research Network (UK CRN) ID: 14080. This trial was registered with www.isrctn.com, reference 10906292.

RESULTS

Baseline

Fourteen HO and 15 HY volunteers consented to participate with 10 from each group completing all study measures. Twenty-seven patients with COPD consented, 20 of whom completed the training intervention and week eight assessments with one drop-out during the exercise withdrawal period leaving 19 COPD patients in the week 12 analysis.

Table S3. Cardiorespiratory measures at $\dot{V}O_2^{PEAK}$ in the baseline incremental test

	Healthy Older (n=10)	**Healthy Younger (n=10)**	**COPD (n=20)**
$\dot{V}O_2^{PEAK}$, *ml.kg lean mass^{-1} min^{-1}*	29.7 (4.0)	44.7 (5.6)**	24.0 (7.4)*
Work load, *W*	113.0 (26.4)	167.0 (43.6)**	71.1 (32.5)**
RER	1.20 (0.09)	1.23 (0.10)	1.05 (0.09)**
$\dot{V}E$, *L min^{-1}*	62.8 (16.4)	82.9 (25.5)*	44.9 (16.6)*
$\dot{V}E$/MVV, *%*	65.4 (15.9)	65.2 (17.0)	103.4 (23.8)**
HR % Predicted Max, *%*	94.4 (10.2)	97.0 (4.8)	85.8 (11.2)*

RER, respiratory exchange ratio; $\dot{V}E$, minute ventilation; MVV, predicted maximum voluntary ventilation; HR, heart rate predicted maximun = 220 – age (years). Values are mean (SD). *, $p < 0.05$ *vs* HO; **, $p < 0.05$ *vs* HO.

Table S4. Baseline physiological responses to steady-state sub-maximal cycling exercise at an intensity corresponding to 65% of the work load achieved at baseline $\dot{V}O_2^{PEAK}$.

	Healthy Older (n=9)	**Healthy Younger (n=10)**	**COPD (n=20)**
Work load, *W*	76 (18)	110 (26)**	46 (21)**
RER	1.04 (0.34)	1.07 (0.37)	0.98 (0.53)**
$\dot{V}E$, *L min^{-1}*	52.2 (11.4)	66.3 (16.9)*	35.7 (11.6)**
$\dot{V}E$/MVV, *%*	53.8 (11.9)	53.4 (15.9)	83.6 (18.8)**
HR % Predicted Max, *%*	86.8 (11.9)	92.6 (3.9)	79.6 (12.1)
Data represent the steady-state period of a cycling exercise test at an individually prescribed work rate (65% of the work rate that corresponded with baseline $\dot{V}O_2^{PEAK}$). RER, respiratory exchange ratio; $\dot{V}E$, minute ventilation; MVV, predicted maximum voluntary ventilation; HR, heart rate predicted maximun = 220 – age (years). Values are mean (SD). *, $p < 0.05$ *vs* HO; **, $p < 0.01$ *vs* HO.			

There were no significant within-group changes over time in physical activity assessed by daily step count (Table S5).

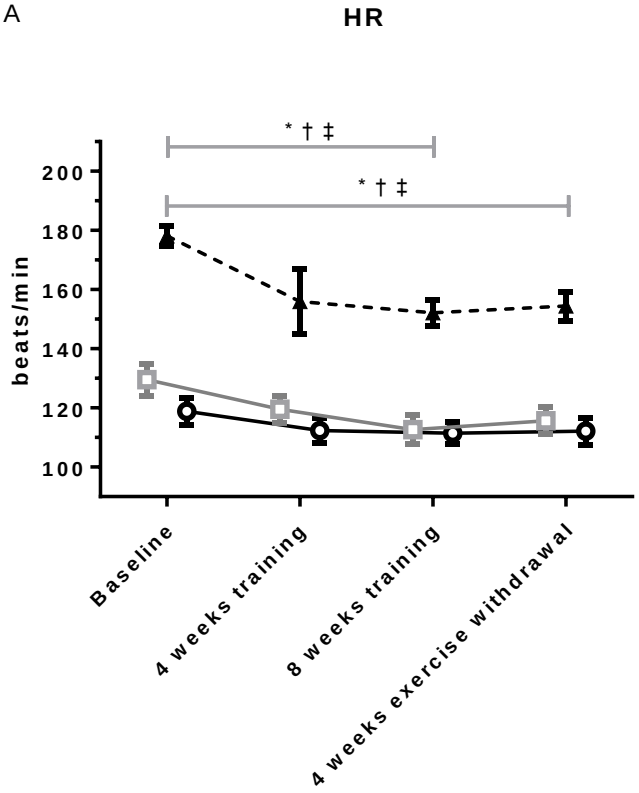
Table S5. Daily step count.

Steps in 8 hours	Baseline	Exercise Withdrawal Week 1	Exercise Withdrawal Week 4	*p* (within group)
Healthy Older	6007 (2088) (n=9)	3976 (579) (n=9)	6012 (2885) (n=9)	NS
Healthy Younger	6180 (3449) (n=9)	6384 (3217) (n=9)	5778 (3212) (n=7)	NS
COPD	4012 (1861) (n=19)	3983 (2077) (n=18)	3772 (2083) (n=18)	NS
Physical activity (daily step count) at baseline (pre-training) and in the 1st and 4th week of				

the exercise withdrawal period. Data are mean (SD).

Heart rate during steady state exercise was reduced from baseline after eight weeks training and after four weeks exercise withdrawal in all groups (Fig. S2 A; all $p < 0.01$) and $\dot{V}E$ was reduced at the same time points in HO ($p < 0.01$) and HY ($p < 0.05$) but was unchanged in COPD (Fig. S2 B).

A


HR
* † ‡
* † ‡
beats/min
200
180
160
140
120
100
Baseline
4 weeks training
8 weeks training
4 weeks exercise withdrawal

B

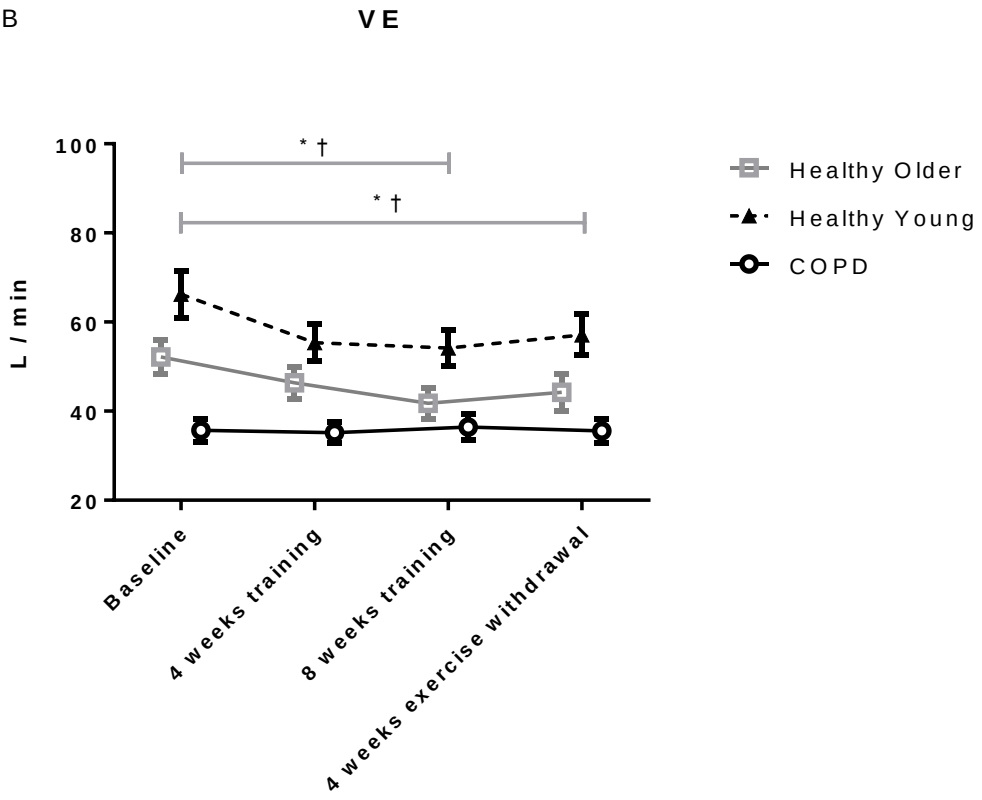

VE
* †
* †
L / min
100
80
60
40
20
Baseline
4 weeks training
8 weeks training
4 weeks exercise withdrawal
Healthy Older
Healthy Young
COPD

Figure S2. Physiological responses to steady-state sub-maximal exercise at a 65% of the work load achieved at $\dot{V}O_2^{PEAK}$ in the baseline test. A, Change in heart rate (HR) after 8 weeks training, and after 4 weeks exercise withdrawal where subjects returned to habitual physical activity levels. B, Respiratory exchange ratio (RER) at the same time points as above (A). Within group change $p < 0.05$ for: HO, *; HY, †; COPD, ‡. Values are mean (SEM).

Lipid Metabolism (After 4 Weeks Exercise Withdrawal)

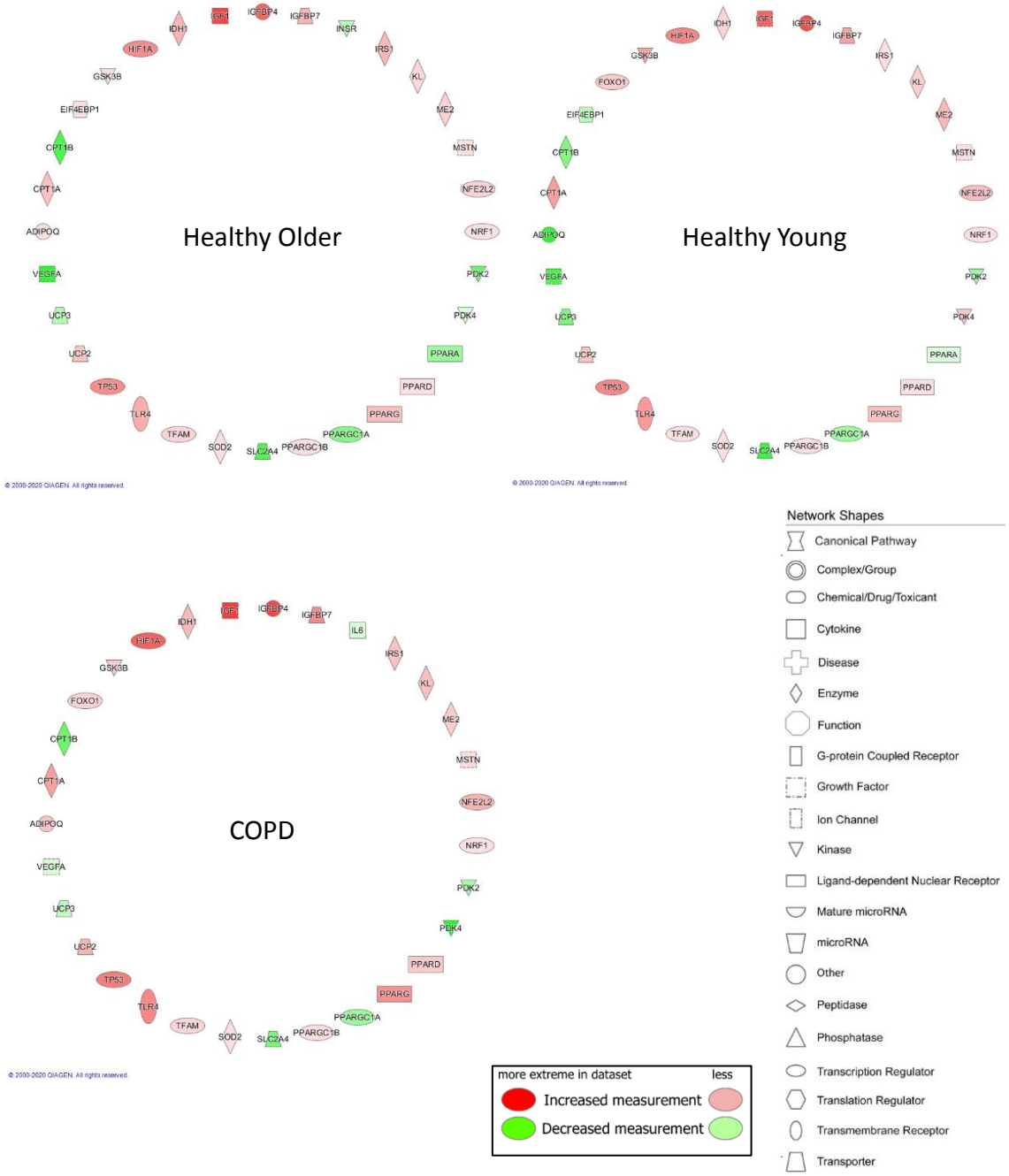


Figure S3. Differentially regulated muscle mRNAs associated with lipid metabolism following 4 weeks exercise withdrawal compared to baseline in healthy older, healthy young and COPD groups. Abbreviated gene names are defined in Table S2.

Carbohydrate Metabolism (After 4 Weeks Exercise Withdrawal)

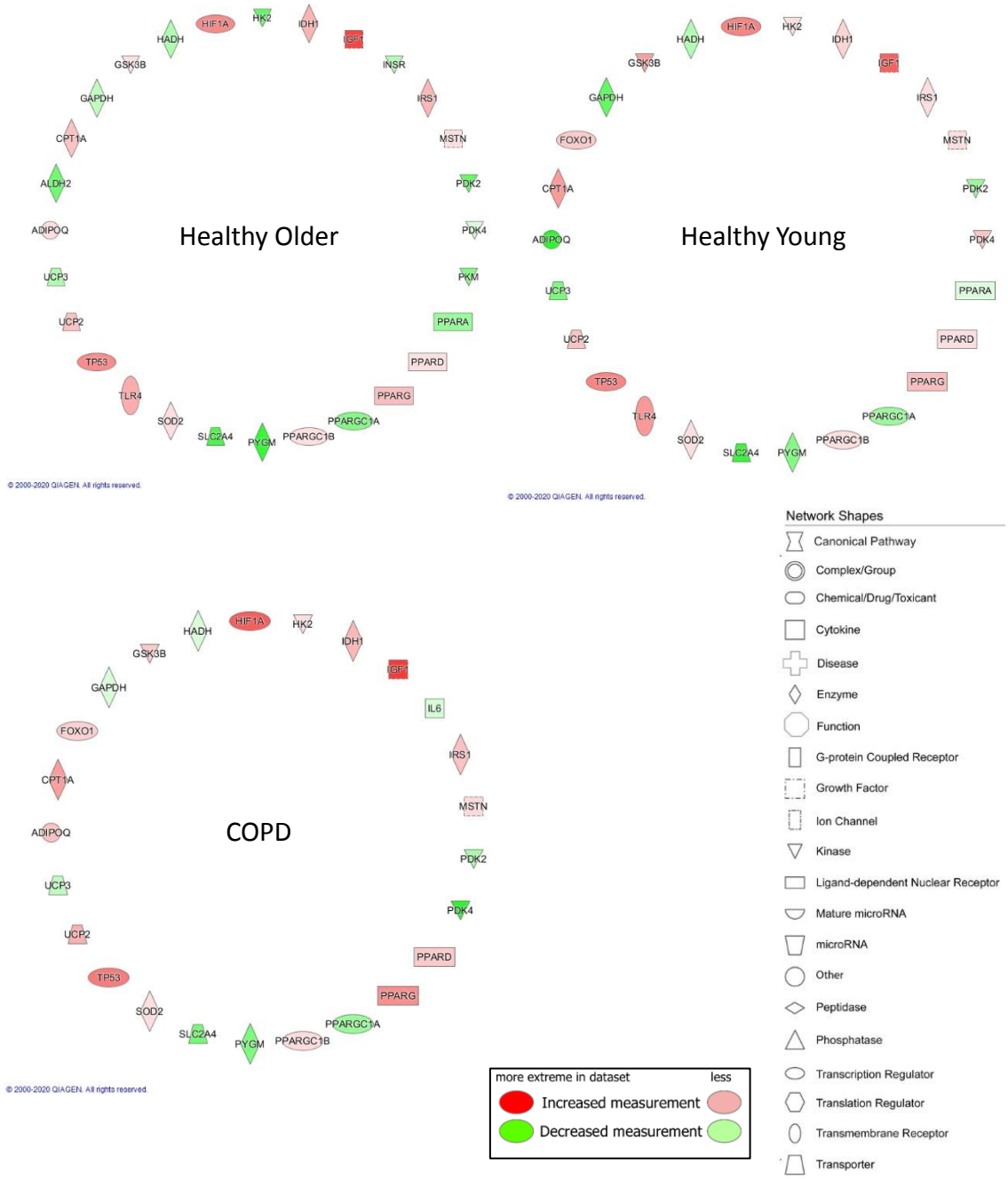


Figure S4. Differentially regulated muscle mRNAs associated with carbohydrate metabolism following 4 weeks exercise withdrawal compared to baseline in healthy older, healthy young and COPD groups. Abbreviated gene names are defined in Table S2.

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