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# High levels of physical activity in later life are associated with enhanced markers of mitochondrial metabolism

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**Abstract**

The age-associated reduction in muscle mass is well characterised, however less is known regarding the mechanisms responsible for the decline in oxidative capacity also observed with advancing age. The purpose of the current study was therefore to compare mitochondrial gene expression and protein content between young and old recreationally active, and older highly active individuals. Muscle biopsies were obtained from the *vastus lateralis* of young males (YG: 22±3 years) and older (OG: 67±2 years) males not previously engaged in formal exercise and older male master cyclists (OT: 65±5 years) who had undertaken cycling exercise for 32±17 yrs. Comparison of gene expression between YG, OG and OT groups revealed greater expression of mitochondrial-related genes, namely electron transport chain (ETC) complexes II, III, IV ($p<.05$) in OT compared to YG and OG. Gene expression of mitofusion (MFN)-1/2, mitochondrial fusion genes, were greater in OT compared to OG ($p<.05$). Similarly, protein content of ETC complexes I, II and IV were significantly greater in OT compared to both YG and OG ($p<.001$). Protein content of peroxisome proliferator-activated receptor gamma, coactivator 1 α (PGC-1α), was greater in OT compared to YG and OG ($p<.001$). Our results suggest that the aging process *per se*, is not associated with a decline in gene expression and protein content of ETC complexes. Mitochondrial-related gene expression and protein content is substantially greater in OT, suggesting that exercise-mediated increases in mitochondrial content can be maintained into later life.

**Keywords:** human, athlete, muscle, mitochondria

Running title: Markers of mitochondrial biology are enhanced in master athletes

**Introduction**

Skeletal muscles of most older individuals are characterized by several adverse morphological changes[1-4], a phenotype directly influenced by physical activity [5-7]. Some groups report an age-associated decline in fibre cross sectional area in sprint-trained older individuals[8] whereas this association is not observed in highly trained master cyclists[6]. This observation therefore questions the extent to which these adverse changes to skeletal muscle health are a function of the aging process or the interaction with age and physical inactivity[8].

Considerable attention has been given to determining the effects of aging on mitochondrial function in skeletal muscle[9]. In addition to being associated with a decline in muscle fatigue resistance, aberrant mitochondrial function is proposed as a major contributor to sarcopenia[10]. In support of this motion, previous research has reported an age-associated decline in mtDNA[11], in addition to a reduction in gene expression of mitochondrial transport chain subunits[12]. The reduction in mitochondrial related gene expression translates to the decline in mitochondrial related protein content observed with aging[11], although this apparent reduction is not observed in middle-aged adults where protein content of electron transport chain (ETC) complex I is greater when compared to young[13]. In addition, aging is associated with an increase in mtDNA mutation-deletions[14], an increase in oxidative damage[12] and altered protein content of factors involved in mitochondrial dynamics[15]. These findings however are not universal, as other groups have reported that aging had no effect on mitochondrial associated gene networks[16], mitochondrial respiration and protein content of ETC subunits[19] and regulators of mitochondrial dynamics[17]. Contrasting results may be due to a number of factors, one of which may be the physical activity status of old individuals included in these studies. For example, Disteffano and colleagues recently demonstrated that cardiorespiratory fitness has a greater influence on mitochondrial respiration than age[17]. Although sedentary aging is associated with a variety of metabolic perturbations, it is suggested that these may be related to the low levels of physical activity rather than to the aging process *per se*[6]. Or

alternatively, physical activity remains beneficial even in advancing age. This latter notion is supported by studies demonstrating that older athletes, when compared to sedentary counterparts, have greater mitochondrial content, function[20], gene expression of ETC subunits[19], and protein content related to mitochondrial dynamics[17]. In older individuals, mitochondrial volume density is correlated with exercise capacity suggesting a role for physical activity to maintain mitochondrial content in this population[21]. Importantly, older individuals retain the ability to positively respond to aerobic exercise training[21,22], placing regular physical exercise as an effective measure for improving mitochondrial content and function throughout the lifespan.

Several studies have sought to characterize the differences in gene and protein expression between either young and sedentary older adults or older sedentary and older athletes. However, few have examined the differences between young and old recreationally active and old highly active master athletes, thereby allowing the combined comparison of age and physical activity levels. Therefore, the purpose of the current study was to compare mitochondrial-related gene expression and protein abundance in the quadriceps muscle between recreationally active young (YG), old individuals who were healthy, but did not undertake regular exercise (OG) and older individuals who had been highly active through cycling exercise for many years (OT). We determined whether genes and proteins related to whole muscle metabolism, mitochondrial biogenesis, dynamics and mitophagy were differentially expressed between groups to further characterize the aging muscle phenotype and the impact of physical activity.

**Methods**

*Ethical Approval.*

Prior to participation written informed consent was obtained from all subjects. Procedures were approved by the National Health Service Wandsworth Research Ethics Committee (reference number 12/LO/0457) and the University of Nottingham Faculty of Medicine and Health Science Research Ethics Committee (B/10/2010), with all procedures conforming to the Declaration of Helsinki. All human tissue collected, stored and analysed was done so in accordance with the Human Tissue Act.

*Participants.*

The OT group is a subset from a large cross-sectional study previously described by Pollock et al. (2015) in which the physiological function of a group of amateur non-elite cyclists aged 55-79 years was evaluated[23]. The inclusion criteria of males was the ability to cycle 100 km in under 6.5h and subjects were required to have undertaken this task twice in the 3 weeks prior to testing[20]. Subjects included in the OG group (67$\pm$2years; 27$\pm$2.8kg/m$^2$; n=8, for subsequent gene expression and protein content analysis) were selected to have similar ages and body mass index (BMI) to those of the OT group (65$\pm$5years; 25.9$\pm$2.1kg/m$^2$; n=7, for subsequent gene expression analysis, n=8 for subsequent protein content analysis). Subjects included in the YG group (21$\pm$3years; 23.9$\pm$2.5 kg/m$^{2;}$ n=6, for subsequent gene expression analysis, n=9 for subsequent protein content analysis) were selected to best match BMI from the older groups. Both YG and OG were recreationally active and performed activities of daily living but none were involved in a formal exercise training programme nor did they participate in more than 2 sessions of purposeful exercise each week. No differences between BMI are observed between groups and no differences between ages are observed between the OT and OG groups. Due to lack of tissue the sample size was smaller for gene expression analysis as stated above.

*Muscle biopsy sampling.*

Following administration of local anaesthetic (1% lidocaine for the YG and OG; 2% lidocaine for the OT) a muscle sample was obtained from the *vastus lateralis* of all participants using the conchotome technique[24] for the YG and OG groups and the Bergstrom needle technique with applied suction for the OT group[6]. All participants in the YG and OG groups were instructed not to exercise for 72h prior to their biopsy which was taken after an overnight fast (~12 h) with water *ad libitum*. Participants in the OT group were asked to maintain habitual levels of physical activity the day prior to their biopsy.

*RNA isolation and Reverse Transcription*

RNA was isolated from 15 – 20 mg of powdered muscle tissue using TRI Reagent/ReliaPrep spin columns. Briefly, all samples were homogenized in 1ml of TRI Reagent (Sigma Aldrich, Gillingham, UK) with the FastPrep 24 5G (MP Biomedicals, Santa Ana, California, USA) at a speed of 6 m/s for 40 seconds. 200ul of chloroform was added to each sample and mixed vigorously for 15 sec then incubated at room temperature for 5 min, then centrifuged at 12000g for 10 min at 4°C. The RNA (aqueous phase) was purified using Reliaprep spin columns (Promega, Madison, Wisconsin, USA) as per manufacturer's instructions. RNA concentrations were determined using the LVis function of the FLUOstar Omega microplate reader. RNA was diluted to 20 μg/μL and reverse transcribed to cDNA using the $RT^2$ First Strand kit (Qiagen, Manchester, UK).

*Quantitative RT-PCR*

Quantitative analysis of 84 genes was completed using custom designed 384-well $RT^2$ PCR Profiler Array (Qiagen) and $RT^2$ SYBR Green Mastermix (Qiagen) on a CFX384 Real-Time PCR Detection System following manufacturer's instructions (Bio-Rad). Briefly, 2.8 ng of cDNA was added to each well. The absence of genomic DNA, the efficiency of reverse-transcription and the efficiency of the PCR assay were assessed on each plate and conformed to the manufacturer's limits in each case. The C(t) values for housekeeper genes beta actin (Refseq# NM_001101), heat shock protein 90 (Refseq#

NM_007355) and beta-2-microglobulin (Refseq# NM_004048) showed no statistical differences between groups. Therefore, the geometric mean C(t) of all three housekeeper genes was used as an internal control[23]. Statistical analysis was carried out on the $\Delta\Delta C(t)$ ($\Delta C(t)$ gene of interest - $\Delta C(t)$ mean of gene of interest of the YG). Data is presented as a fold change from YG as determined using the $2^{-\Delta\Delta C(t)}$ method**26[27]**.

*Immunoblotting*

Approximately 25mg of powdered muscle tissue was homogenized in 300 ul of ice-cold sucrose lysis buffer (50 mM Tris, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM $Na_4P_2O_7$-$10H_2O$, 270 mM sucrose, 1 M Triton-X, 25 mM β-glycerophosphate, 1 μM Trichostatin A, 10 mM Nicatinamide, 1mM 1,4-Dithiothreitol, 1% Phosphatase Inhibitor Cocktail 2; Sigma, 1% Sigma Phosphatase Inhibitor Cocktail 2; Sigma, 4.8% cOmplete Mini Protease Inhibitor Cocktail; Roche) using the FastPrep 24 5G (MP Biomedicals, Santa Ana, California, USA) at a speed of 6 m/s for 40 seconds and repeated 3 times. Samples were then centrifuged at 4°C at a speed of 8000 g for 10 minutes to remove insoluble material. Protein content was determined from the DC protein assay (Bio-Rad, Hercules, California, USA) using FLUOstar Omega at an absorbance of 750nm). Laemmli samples buffer was added and samples were boiled for 5 minutes, equal amounts of protein (20-50 ug) was separated by SDS-PAGE on 8 – 12.5% gels at a constant current of 23 mA per gel. Proteins were transferred to BioTrace NT nitrocellulose membranes (Pall Life Sciences, Pensacola, Florida, USA) via wet transfer at 100 V for one hour on ice. Membranes were stained with Ponceau S (Sigma-Aldrich, Gillingham, UK) and imaged to assure even loading and for future normalization. Membranes were blocked in 3% dry-milk in tris-buffered saline with tween (TBST) for one hour prior to an overnight primary antibody incubation at 4°C. Membranes were washed in TBST three times prior to incubation in appropriate horse radish peroxidase-conjugated secondary antibody at room temperature for one hour. Membranes were then washed in TBST three times prior to antibody detection via enhanced chemiluminescence horseradish peroxidase substrate detection kit (Millipore, Watford, UK). Imaging

and band quantification were undertaken using a G:Box Chemi-XR5 (Syngene, Cambridge, UK). Mean pixel intensity was determined for each band. All bands were normalized to a gel control in addition to the corresponding ponceau image. Statistical analysis was carried out on normalized values for each protein of interest.

*Antibodies*

All primary antibodies were prepared in TBST. Antibodies used were as follows: total OXPHOS human WB antibody cocktail (ab11041; abcam, 1:1000 in unboiled samples), PGC-1Antibody (AB3242; Merck, 1:1000), pyruvate dehydrogenase antibody (PDH; #2784; Cell signalling technology, 1:1000), Citrate synthase (SAB2701077; MitoSciences, 1:1000), Sirtuin3 (SirT3 D22A3; Cell signalling technology, 1:1000). Very long chain acyl CoA dehydrogenase (VLCAD; 1:5000), long chain acyl CoA dehydrogenase (LCAD; 1:5000), medium chain acyl-CoA dehydrogenase (MCAD; 1:5000), were kind gifts from Prof Jerry Vockley, University of Pittsburgh, USA. Appropriate secondary antibodies either anti-rabbit (7074) or anti-mouse (7076) from Cell Signaling Technology were used at a concentration of 1:10000 in TBST.

*Statistical Analysis*

Statistical analysis was performed using Prism 7 (GraphPad Software Incorporated). To assess differences between YG, OG and OT groups a one-way analysis of variance (ANOVA) was completed on individual genes and proteins independently, multiple comparisons were assessed using Tukey's test. The normal distribution of data was verified using the Shapiro-Wilk normality test. Values are presented as mean ± standard deviation.

## Results

### *Mitochondrial related genes expression profile is altered in master athletes*

The mRNA expression of 84 target genes was assessed in YG (n=6), OG (n=8) and OT (n=7) groups, gene expression is presented as a fold change from the YG group (Supplemental Table 1). Of the 84 genes assessed 23 were differentially expressed ($p<.05$) (Supplemental Table 1). Differentially expressed genes of mitochondrial related proteins and transcriptional regulators are presented in Figure 1. Differentially expressed genes related to skeletal muscle structural remodelling are presented in Figure 2. Differentially expressed genes related to substrate metabolism are presented in Figure 3.

### *Mitochondrial related protein content.*

Protein content was assessed in YG (n=9), OG (n=8) and OT (n=8). Protein content of each complex of the electron transport chain was assessed via immunoblotting. There was no difference between YG and OG for any protein assessed. Protein content of complex I, II and IV were 7.1-, 1.9- and 1.3-fold greater in OT compared to OG and 5.6-, 2.1- and 1.2-fold greater compared to YG ($p<.05$) (Fig 4a). There are no differences in protein content of complex III and V across groups ($p>.05$). Protein content of PGC-1α was 2.9-fold greater in OT compared to OG ($p<.05$) and 5.4-fold greater compared to YG ($p<.001$) (Fig 4b). Protein content of CS was 1.9-fold greater in the OT compared to both the OG ($p<.001$) and 1.3-fold greater in the OT compared to the YG ($p<.001$) (Fig 4c). Additionally, protein content of SIRT3 was 1.8-fold greater in OT compared to both OG ($p<.001$) and to YG ($p\leq.001$) (Fig 4d).

### *Protein content related to substrate metabolism.*

Protein content of pyruvate dehydrogenase (PDH), LCAD and MCAD was 3-, 1.8- and 1.6-fold greater, respectively in OT compared to OG and 2.8-, 1.9- and 2.1-fold greater, respectively,

compared to YG ($p<.05$) (Fig 5a,b,c). Protein content of VLCAD was 1.7-fold greater in OT compared to OG ($p<.05$) (Fig 5d).

**Discussion**

Many studies have sought to determine the effect of aging on mitochondrial content and function[11-15,17-19], however few have accounted for the divergent levels of physical activity in the older population. Here we have directly compared young and old recreationally active adults in addition to old highly aerobically trained (master cyclists) adults. The inclusion of these three distinct groups allows the effects of activity to be established and therefore what extent declines in function can be attributed to chronological aging[8]. The primary finding of this study is that highly active older adults, have greater mitochondrial related protein content and gene expression in comparison to recreationally active untrained young and older adults. Importantly, no observable age-associated reduction in mitochondrial related protein content and gene expression (except for CS mRNA expression) was observed in the recreationally active older group in which the negative effects of inactivity are eliminated. In addition, we report no change in OXPHOS, PGC-1α or mitochondrial fusion and fission-related gene expression with age.

Previous work has described a reduction in mtDNA, mitochondrial protein content and ATP production in older adults who had similar physical activity levels to young counterparts (<30 mins, <2 days/week)[11]. However more recent work suggests that mitochondrial related protein content may not be affected by aging[17]. Our results are in line with the latter in which chronological age does not impact mitochondrial related protein content (Fig 4a). A lower expression in OG compared to YG is only observed with respect to CS (Fig 1c) and UQCRC1 (Fig 1d) gene expression. However, this was not observed at the protein level (Fig 4c). Although chronological aging *per se* did not impact mitochondrial related protein content and gene expression in our hands, the impact of high levels of physical activity in advanced age was substantial. We report greater expression of genes related to the electron transport chain (SDHA, SDHB, UQCRC1, UQCRC2 and COX4I2) in the OT compared to

both the YG and OG groups (Fig 1). These results are supported by greater protein content of complex I, II and IV of the electron transport chain in the OT compared to YG and OG groups (Fig 4a). Larsen and co-workers have reported an association between protein content of complex II and V and total mitochondrial content measured via mitochondrial volume by transmission electron microscopy[29]. Although not a direct measure of mitochondrial content, our results are in accordance with previous work demonstrating a greater mitochondrial volume density and content of ETC proteins in old active compared to sedentary adults[20,21]. Given that endurance exercise training is well established as a potent activator of mitochondrial biogenesis[30], the greater mitochondrial related gene expression and protein content in the OT group is likely due to their high physical activity levels and the metabolic demands placed on skeletal muscle.

In addition, we report greater PGC-1α gene expression in the OT compared to the OG group (Fig 1b) and greater PGC-1α protein content in OT compared to both the YG and OG groups (Fig 4b). These results are in partial contrast with previous work reporting a reduction in PGC-1α protein content with aging in both 'high' and 'low' functioning sedentary older adults[28]. As PGC-1α is an important regulatory signalling node for the initiation of mitochondrial biogenesis, a greater expression in the OT group may reflect a greater mitochondrial mass due to greater habitual physical activity levels in this cohort. Thus, our results suggest that mitochondrial biogenesis is primarily affected by activity status rather than aging. PGC-1 α also regulates the gene expression of SIRT3[31], a $NAD^+$-dependent deacetylase highly involved in metabolism[32]. Accordingly, we report greater expression (Fig 1a) and protein content (Fig 4d) of SIRT3, in the OT group compared to both the YG and the OG groups. Again, this observation suggests that aging *per se* is not associated with a reduction in markers of mitochondrial metabolism; rather that exercise training has a positive effect on SIRT3 induction irrespective of age.

Skeletal muscle mitochondrial function is believed, in part, to be regulated by the tumour suppressor p53[35]. Here we demonstrate that gene expression of p53 targets, SESN2 and PMPAIP1,

are increased in the OT group although not affected by aging (Fig 2). ZMAT3 another target of p53, has a greater expression in both OG and OT (Fig 2g,h). Interestingly, no change in p53 expression was observed between groups (p>.05) (Supplemental Table 1). With the design of the current study we are unable to state whether the increase in p53 target genes are beneficial or detrimental in maintaining muscle health.

Information regarding content of proteins involved in mitochondrial dynamics in aging skeletal muscle are equivocal with some reports indicating either no change or a reduction[15,17,28]. Recently, Tezze et al.(2017), have reported greater expression of OPA1, MFN1/2 and DRP1 in "older sportsmen" and young individuals compared to old inactive individuals[15]. Balan et al. (2019) report greater mitochondrial protein content of MFN2 and OPA1 in active young and older adults in comparison to sedentary age-matched subjects however, they do not observe an age-associated reduction in protein content[19]. This is partially confirmed by our findings where expression of MFN1 and MFN2, were greater in OT compared to OG, however, in our hands aging did not result in a reduced expression (Fig 2e,f). This may relate to possible differences in the activity status of the recreational active older participants in the present study and the "sedentary seniors" in the study of Tezze et al. (2017)[15]. Additionally, we did not observe differences in expression of other genes related to mitochondrial dynamics, such as OPA1 and FIS1 (Supplemental Table 1). Our results suggest that aging does not impair the dynamic remodelling of the mitochondrial reticulum but highly active older adults may require enhanced mitochondrial remodelling to support the stresses placed on the skeletal muscle as evidenced by greater gene expression of MFN1/2.

Although we report very few changes associated with chronological aging with respect to protein and gene expression, it is important to note that skeletal muscle of older individuals remains a highly plastic tissue and responds positively to exercise training[37-41]. Although, older individuals' response to resistance exercise training may be attenuated in comparison to young adults, they are able to increase muscle fibre size[41]. In line with this, endurance exercise training in older individuals' results in improved maximal oxygen uptake, mitochondrial enzyme activities, and increase in muscle

fibre size[42-44]. Importantly, a recent analysis of cross-sectional data demonstrates that when older adults commence intense training and competition in later life (>50y), no differences are observed with respect to performance and body composition (fat mass and leg lean mass) compared to adults having trained their entire adult life[22]. Thus, further highlighting the ability of older individuals to positively respond to exercise.

One of the limitations of the present study was the lack of precise quantification of levels of physical activity in the groups that described themselves as being recreationally active. It is likely however, that the OG did not reflect the majority of older people who are known to engage in reduced levels of physical activity[46]. Thus, comparison with the OT group is, if anything, most likely to be an underestimate of the differences in phenotype between lifelong exercisers and most of the older population.

In summary, we observed greater mitochondrial related gene expression and protein content in highly active older individuals compared to young and aged-matched recreationally active individuals. Chronological aging in recreationally active individuals, did not affect mitochondrial related gene and protein expression. Collectively these data suggests that high levels of physical activity, even in advancing age, results in greater mitochondrial-related gene expression and protein content compared to young and age matched untrained individuals. Further, our results highlight that chronological aging does not result in a reduction in mitochondrial content and function and supports the notion that skeletal muscle retains the ability to positively respond to stimuli even in advancing age.

## CONFLICTS OF INTEREST

The authors have no conflicts of interest to disclose.


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**Figure Captions**

**Fig. 1** Mitochondrial related gene expression is greater in highly active older individuals. mRNA of genes related to mitochondrial content in recreationally active young (YG n=6; black bars), old individuals who were healthy, but did not undertake regular exercise (OG n=8; grey bars), older individuals who had been highly active through cycling exercise for many years (OT n=7; open bars). Data are presented as a fold change from YG. (a) sirtuin 3 (SIRT3) (b) peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC-1α) (c) citrate synthase (CS) (d) ubiquinol-cytochrome c reductase core protein I (UQCRC1) (e) ubiquinol-cytochrome c reductase core protein II (UQCRC2) (f) cytochrome c oxidase subunit IV isoform 1 (COX4I2) (g) cytochrome c oxidase assembly homolog 10 (COX10) (h) succinate dehydrogenase complex, subunit A, flavoprotein (SDHA) (i) succinate dehydrogenase complex, subunit B, iron sulfur (SDHB) (j) translocase of inner mitochondrial membrane 8 homolog A (TIMM8A). [a] significantly different from YG, [b] significantly different from OG.

**Fig. 2** Skeletal muscle related gene expression is greater in highly active older individuals. mRNA expression of genes related to skeletal muscle remodelling in recreationally active young (YG n=6; black bars), old individuals who were healthy, but did not undertake regular exercise (OG n=8; grey bars), older individuals who had been highly active through cycling exercise for many years (OT n=7; open bars). Data are presented as a fold change from YG. (a) vascular endothelial growth factor A (VEGFA) (b) vascular endothelial growth factor B (VEGFB) (c) myogenic differentiation 1 (MyoD1) (d) BTG family, member 2 (BTG2) (e) mitofusin 1 (MFN1) (f) mitofusin 2 (MFN2) (g) sestrin 2 (SESN2) (h) phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1) (i) zinc finger, matrin-type 3 (ZMAT3). [a] significantly different from YG ~~OG~~, [b] significantly different from OG.

**Fig. 3** Substrate metabolism related gene expression is greater in highly active older individuals. mRNA expression of genes related to substrate metabolism in recreationally active young (YG n=6; black bars), old individuals who were healthy, but did not undertake regular exercise (OG n=8; grey

bars), older individuals who had been highly active through cycling exercise for many years (OT n=7; open bars). Data are presented as a fold change from YG. (a) isocitrate dehydrogenase 2 (NADP+) (IDH2) (b) malate dehydrogenase 1, NAD (MDH1) (c) pyruvate dehydrogenase kinase, isozyme 4 (PDK4) (d) nuclear receptor subfamily 1, group D, member 2 (NR1D2). [a] significantly different from YG ~~OG~~, [b] significantly different from OG.

**Fig. 4** Mitochondrial related protein content is greater in highly active older individuals. Protein content in recreationally active young (YG n=9; black bars), old individuals who were healthy, but did not undertake regular exercise (OG n=8; grey bars), older individuals who had been highly active through cycling exercise for many years (OT n=8; open bars). (a) Protein content of mitochondrial enzymes (complexes I-V), (b) peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC-1α), (c) citrate synthase (CS) and (d) sirtuin 3 (SIRT3). (e) Representative images of protein content of mitochondrial enzymes (complexes I-V), PGC-1α, CS and SIRT3 in YG, OG and OT. All values were normalized to their respective ponceau stain. Data are presented as mean ± standard deviation. [b] significantly different from OG and YG

**Fig. 5** Substrate metabolism related protein content is greater in highly active older individuals. Protein content in recreationally active young (YG n=9; black bars), old individuals who were healthy, but did not undertake regular exercise (OG n=8; grey bars), older individuals who had been highly active through cycling exercise for many years (OT n=8; open bars). (a) protein content of pyruvate dehydrogenase (PDH), (b) long chain acyl CoA dehydrogenase (LCAD), (c) medium chain acyl-CoA dehydrogenase (MCAD), and (d) very long chain acyl CoA dehydrogenase (VLCAD). (e) Representative images of protein content of PDH, LCAD, MCAD and VLCAD. All values were normalized to their respective ponceau stain. Data are presented as mean ± standard deviation. [a] significantly different from OG, [b] significantly different from OG and YG

**eTable 1. mRNA expression of genes included on the designed RT2 Profiler PCR Array.**

| Name | Abbreviation | Refseq # | YG | OG | OT |
|---|---|---|---|---|---|
| sirtuin 1 | SIRT1 | NM_001142498 | 1.00 | 1.11 | 1.13 |
| sirtuin 3 | SIRT3 | NM_001017524 | 1.00 | 1.04 | 1.53 (c) |
| sirtuin 6 | SIRT6 | NM_001193285 | 1.00 | 1.04 | 1.36 |
| transcription factor A, mitochondrial | TFAM | NM_003201 | 1.00 | 0.85 | 1.05 |
| transcription factor B1, mitochondrial | TFB1M | NM_016020 | 1.00 | 0.96 | 1.22 |
| transcription factor B2, mitochondrial | TFB2M | NM_022366 | 1.00 | 1.15 | 1.19 |
| vascular endothelial growth factor A | VEGFA | NM_001025366 | 1.00 | 1.08 | 2.04 (b) |
| vascular endothelial growth factor B | VEGFB | NM_001243733 | 1.00 | 0.92 | 1.32 (a) |
| vascular endothelial growth factor C | VEGFC | NM_005429 | 1.00 | 1.00 | 1.64 |
| BCL2-associated agonist of cell death | BAD | NM_004322, NM_032989 | 1.00 | 0.81 | 0.99 |
| BCL2-antagonist/killer 1 | BAK1 | NM_001188 | 1.00 | 0.97 | 1.13 |
| BCL2-associated X protein | BAX | NM_004324, NM_138761, NM_138763, NM_138764, NM_138765, NR_027882 | 1.00 | 1.03 | 1.12 |
| BCL2 binding component 3 | BBC3 | NM_001127240 | 1.00 | 1.36 | 0.98 |
| BTG family, member 2 | BTG2 | NM_006763 | 1.00 | 2.27 (d) | 0.73 |
| caspase 9, apoptosis-related cysteine peptidase | CASP9 | NM_001229 | 1.00 | 1.14 | 0.98 |
| Fas cell surface death receptor | FAS | NM_000043 | 1.00 | 0.81 | 0.62 |
| phorbol-12-myristate-13-acetate-induced protein 1 | PMAIP1 | NM_021127 | 1.00 | 1.66 | 3.30 (b) |
| zinc finger, matrin-type 3 | ZMAT3 | NM_152240 | 1.00 | 1.28 (c) | 1.30 (c) |
| breast cancer 1, early onset | BRCA1 | NM_007294 | 1.00 | 1.02 | 1.25 |
| epidermal growth factor receptor | EGFR | NM_005228 | 1.00 | 1.00 | 0.83 |
| growth arrest and DNA-damage-inducible, alpha | GADD45A | NM_001199741 | 1.00 | 0.87 | 1.01 |
| proliferating cell nuclear antigen | PCNA | NM_002592 | 1.00 | 0.99 | 1.05 |
| pituitary tumor-transforming 1 | PTTG1 | NM_004219 | 1.00 | 1.55 | 1.41 |
| retinoblastoma 1 | RB1 | NM_000321 | 1.00 | 0.95 | 0.98 |

| | | | | | |
|---|---|---|---|---|---|
| sestrin 2 | SESN2 | NM_031459 | 1.00 | 1.42 | 2.09 (c) |
| beclin 1, autophagy related | BECN1 | NM_003766 | 1.00 | 0.93 | 1.00 |
| **Name** | **Abbreviation** | **Refseq #** | **YG** | **OG** | **OT** |
| BH3 interacting domain death agonist | BID | NM_001196, NM_001244567, NM_001244569, NM_001244570, NM_001244572, NM_197966, NM_197967 | 1.00 | 1.01 | 1.15 |
| BCL2/adenovirus E1B 19kDa interacting protein 3 | BNIP3 | NM_004052 | 1.00 | 0.80 | 0.91 |
| caspase 3, apoptosis-related cysteine peptidase | CASP3 | NM_004346, NM_032991 | 1.00 | 0.84 | 1.01 |
| caspase 8, apoptosis-related cysteine peptidase | CASP8 | NM_001080124 | 1.00 | 0.97 | 1.15 |
| v-myc avian myelocytomatosis viral oncogene homolog | MYC | NM_002467 | 1.00 | 1.03 | 0.78 |
| cytochrome c oxidase assembly homolog 10 (yeast) | COX10 § | NM_001303 | 1.00 | 0.75 | 1.05 |
| COX18 cytochrome C oxidase assembly factor | COX18 | NM_001033760 | 1.00 | 0.99 | 1.41 |
| fission 1 (mitochondrial outer membrane) homolog (S. cerevisiae) | FIS1 | NM_016068 | 1.00 | 0.86 | 1.03 |
| mitofusin 1 | MFN1 | NM_017927 | 1.00 | 0.88 | 1.12 (a) |
| mitofusin 2 | MFN2 | NM_001127660 | 1.00 | 0.97 | 1.48 (a) |
| optic atrophy 1 (autosomal dominant) | OPA1 | NM_015560 | 1.00 | 0.96 | 1.16 |
| translocase of outer mitochondrial membrane 20 homolog (yeast) | TOMM20 | NM_014765 | 1.00 | 0.89 | 0.92 |
| translocase of outer mitochondrial membrane 22 homolog (yeast) | TOMM22 | NM_020243 | 1.00 | 0.92 | 0.99 |
| translocase of inner mitochondrial membrane 8 homolog A (yeast) | TIMM8A | NM_001145951 | 1.00 | 0.82 | 1.55 (a) |
| translocase of inner mitochondrial membrane 9 homolog (yeast) | TIMM9 | NM_012460 | 1.00 | 0.73 | 0.96 |
| peroxisome proliferator-activated receptor gamma, coactivator 1 alpha | PGC-1α | NM_013261 | 1.00 | 0.73 | 1.42 (a) |
| peroxisome proliferator-activated receptor gamma, coactivator 1 beta | PPARGC1B | NM_001172699, NM_133263 | 1.00 | 0.78 | 1.30 |
| citrate synthase | CS | NM_004077 | 1.00 | 0.77 (d) | 1.28 |

| | | | | | |
|---|---|---|---|---|---|
| hexokinase 2 | HK2 | NM_000189 | 1.00 | 1.53 | 2.50 |
| pyruvate dehydrogenase kinase, isozyme 4 | PDK4 | NM_002612 | 1.00 | 2.88 | 0.43 (a) |
| solute carrier family 2 (facilitated glucose transporter), member 4 | SLC2A4 | NM_001042 | 1.00 | 0.94 | 1.22 |
| **Name** | **Abbreviation** | **Refseq #** | **YG** | **OG** | **OT** |
| myogenic differentiation 1 | MYOD1 | NM_002478 | 1.00 | 0.82 | 1.73 (b) |
| histone deacetylase 3 | HDAC3 | NM_003883 | 1.00 | 0.99 | 0.96 |
| histone deacetylase 5 | HDAC5 | NM_001015053 | 1.00 | 1.22 | 1.19 |
| peroxisome proliferator-activated receptor alpha | PPARA | NM_001001928 | 1.00 | 1.06 | 1.29 |
| peroxisome proliferator-activated receptor delta | PPARD | NM_001171818 | 1.00 | 0.70 | 0.86 |
| NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1, 7.5kDa | NDUFA1 | NM_004541 | 1.00 | 1.10 | 1.10 |
| NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 1, 7kDa | NDUFB1 | NM_004545 | 1.00 | 0.88 | 1.16 |
| NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 1, 6kDa | NDUFC1 | NM_001184986 | 1.00 | 1.03 | 1.12 |
| succinate dehydrogenase complex, subunit A, flavoprotein (Fp) | SDHA | NM_004168 | 1.00 | 0.98 | 1.42 (b) |
| succinate dehydrogenase complex, subunit B, iron sulfur (Ip) | SDHB | NM_003000 | 1.00 | 0.91 | 1.34 (a) |
| cytochrome c-1 | CYC1 | NM_001916 | 1.00 | 0.89 | 1.28 |
| ubiquinol-cytochrome c reductase core protein I | UQCRC1 | NM_003365 | 1.00 | 0.79 (c) | 1.34 (b) |
| ubiquinol-cytochrome c reductase core protein II | UQCRC2 | NM_003366 | 1.00 | 0.87 | 1.28 (a) |
| cytochrome c oxidase subunit IV isoform 1 | COX4I1 | NM_001861 | 1.00 | 0.85 | 1.23 |
| cytochrome c oxidase subunit IV isoform 2 (lung) | COX4I2 | NM_032609 | 1.00 | 1.00 | 1.54 (b) |
| ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle | ATP5A1 | NM_001001935 | 1.00 | 0.86 | 1.18 |
| ATPase, H+ transporting, lysosomal V0 subunit a2 | ATP6V0A2 | NM_012463 | 1.00 | 1.04 | 1.08 |
| isocitrate dehydrogenase 1 (NADP+), soluble | IDH1 | NM_005896 | 1.00 | 0.96 | 1.07 |
| isocitrate dehydrogenase 2 (NADP+), mitochondrial | IDH2 | NM_002168 | 1.00 | 0.90 | 1.34 (a) |
| malate dehydrogenase 1, NAD (soluble) | MDH1§ | NM_001199111 | 1.00 | 1.04 | 1.67 |
| malate dehydrogenase 2, NAD (mitochondrial) | MDH2 | NM_005918 | 1.00 | 0.79 | 0.98 |
| poly (ADP-ribose) polymerase 1 | PARP1 | NM_001618 | 1.00 | 0.96 | 0.97 |

| | | | | | |
|---|---|---|---|---|---|
| poly (ADP-ribose) polymerase 2 | PARP2 | NM_001042618 | 1.00 | 0.86 | 1.05 |
| K(lysine) acetyltransferase 2A | KAT2A | NM_021078 | 1.00 | 1.11 | 1.12 |
| K(lysine) acetyltransferase 2B | KAT2B | NM_003884 | 1.00 | 1.03 | 0.90 |
| **Name** | **Abbreviation** | **Refseq #** | **YG** | **OG** | **OT** |
| nuclear receptor coactivator 1 | NCOA1 | NM_003743 | 1.00 | 1.09 | 1.04 |
| nuclear receptor coactivator 3 | NCOA3 | NM_001174087 | 1.00 | 0.96 | 0.92 |
| nuclear receptor subfamily 1, group D, member 2 | NR1D2 | NM_001145425 | 1.00 | 0.99 | 0.66 (b) |
| estrogen receptor 1 | ESR1 | NM_000125 | 1.00 | 0.71 | 0.82 |
| estrogen receptor 2 (ER beta) | ESR2 | NM_001040275 | 1.00 | 2.28 | 2.54 |
| E1A binding protein p300 | EP300 | NM_001429 | 1.00 | 0.96 | 0.94 |
| CREB binding protein | CREBBP | NM_001079846 | 1.00 | 0.96 | 0.74 |
| cyclin-dependent kinase inhibitor 1A (p21, Cip1) | CDKN1A | NM_000389 | 1.00 | 2.34 | 1.62 |
| tumor protein p53 | TP53 | NM_000546 | 1.00 | 1.03 | 1.12 |

*Note:* YG = young; OG = old; OT = old highly active. Data are expressed as fold change from the YG group. Full names and abbreviations of each gene are included along with the manufacturers Refseq #.

(a) significantly different from OG, (b) significantly different from OG and YG, (c) significantly different from YG, (d) significantly different from YG and OT, §main effect.

Figure 1

FIG 1

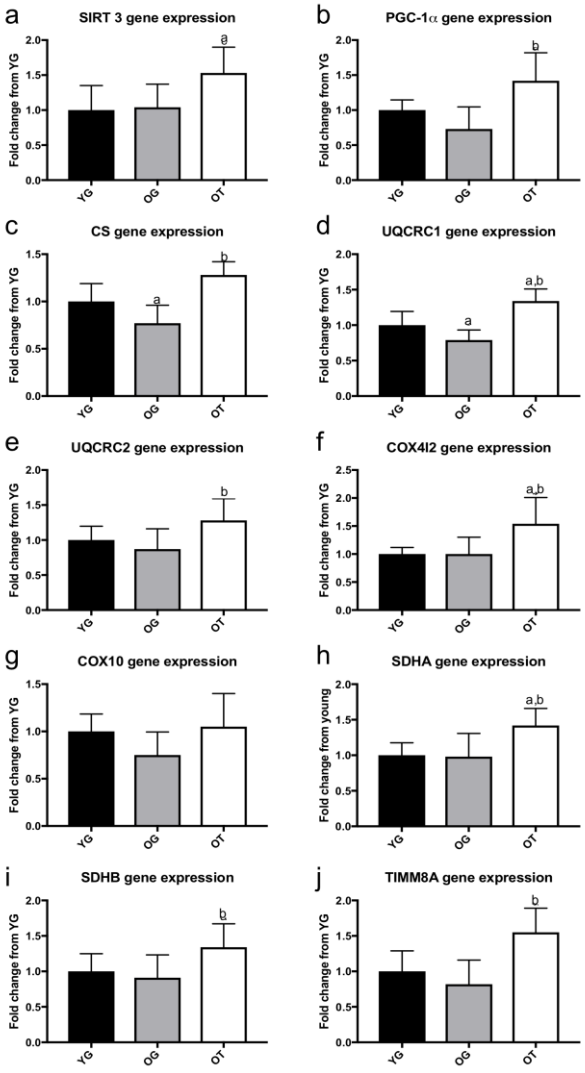

Figure 2

FIG 2

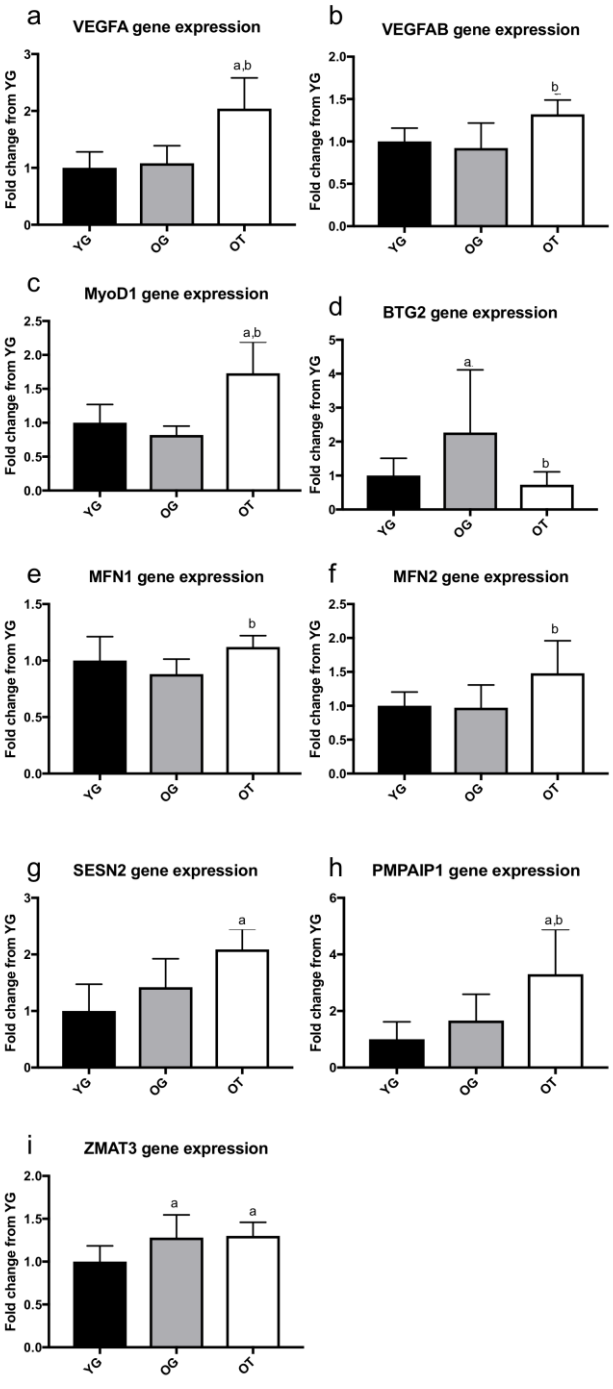

Figure 3

FIG 3

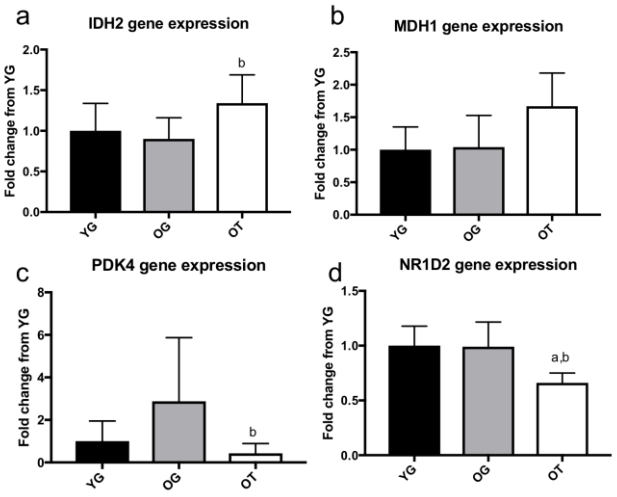

Figure 4

FIG 4

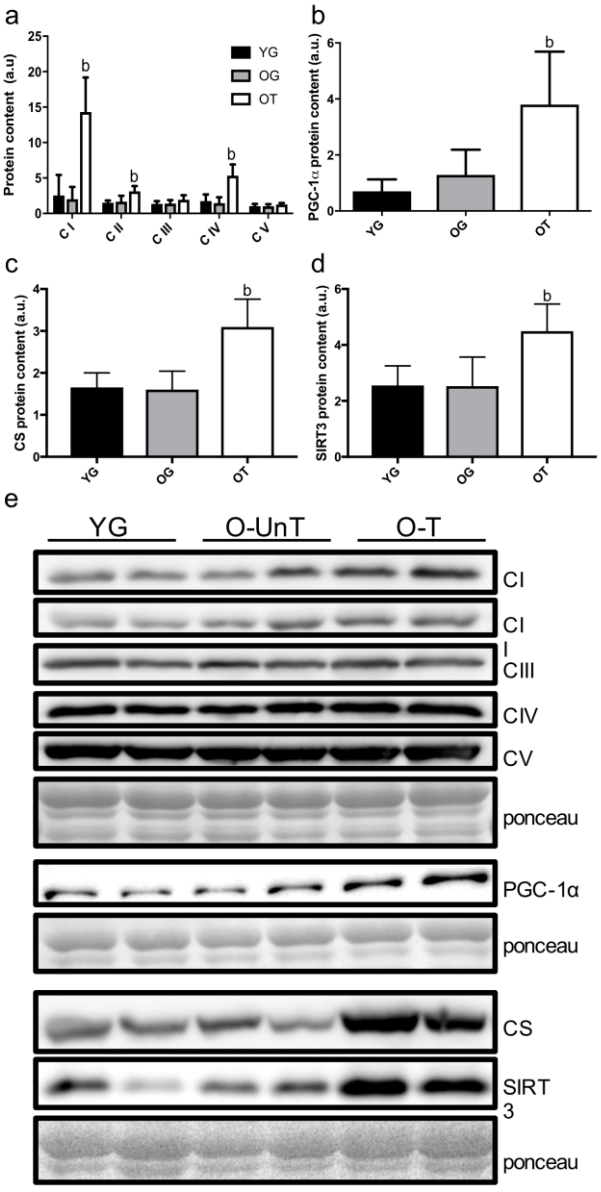

Figure 5

FIG 5