Mitochondria, glycogen, and lipid droplets in skeletal muscle during testosterone treatment and strength training:

a randomized, double-blinded, placebo-controlled trial

Jensen, Richard Christian; Lehman Christensen, Louise; Nielsen, Joachim; Schrøder, Henrik Daa; Kvorning, Thue; Gejl, Kasper Degn; Højlund, Kurt; Glintborg, Dorte; Andersen, Marianne

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Mitochondria, glycogen and lipid droplets in skeletal muscle during testosterone treatment and strength training. A randomized, double blinded, placebo-controlled trial.

Richard Christian Jensen \footnote{Corresponding author at:} \textsuperscript{1,a}, Louise Lehmann Christensen \textsuperscript{1}, Joachim Nielsen \textsuperscript{2}, Henrik Daa Schrøder \textsuperscript{3}, Thue Kvorning \textsuperscript{2}, Kasper Gejl \textsuperscript{2}, Kurt Højlund \textsuperscript{1}, Dorte Glintborg \textsuperscript{1}, Marianne Andersen \textsuperscript{1}

\textsuperscript{1} Department of Endocrinology, Odense University Hospital, Odense C, Denmark.
\textsuperscript{2} Department of Sports Science & Clinical Biomechanics, University of Southern Denmark, Odense M, Denmark.
\textsuperscript{3} Department of Pathology, Odense University Hospital, Odense C, Denmark.

\textbf{Running title:} Intramyocellular mitochondria, glycogen and lipid during testosterone replacement therapy

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\textbf{Key words:} Testosterone replacement therapy, skeletal muscle, metabolism, transmission electron microscopy.

\footnote{Corresponding author at:}

Department of Endocrinology, Odense University Hospital
J.B. Winsløwsvej 17 A, 2, DK-5000 Odense C, Denmark.
Tel: +45 65504917, E-mail: rcjensen@health.sdu.dk
Abstract

Low testosterone levels in ageing men are associated with insulin resistance. Mitochondrial dysfunction, changes in glycogen metabolism, and lipid accumulation are linked to insulin resistance in skeletal muscle. In this randomized, double-blinded, placebo-controlled study, we investigated the effects of six months testosterone replacement therapy (TRT) and strength training (ST) on mitochondrial, glycogen and lipid droplet (LD) content in skeletal muscle of ageing men with subnormal bioavailable testosterone (BioT) levels. Mitochondrial, glycogen, and LD volume fractions in muscle biopsies were estimated by transmission electron microscopy. Insulin sensitivity (insulin-stimulated Rd) and body composition were assessed by euglycaemic-hyperinsulinaemic clamp and dual x-ray absorptiometry, respectively. TRT significantly increased total testosterone levels, BioT and lean body mass (LBM) (p< 0.05), whereas percent body fat decreased (p< 0.05), and insulin sensitivity was unchanged. Baseline mitochondrial volume fraction correlated inversely with percent body fat (ρ = -0.43; p= 0.003). ∆-mitochondrial fraction correlated positively with ∆-total testosterone (ρ = 0.70; p= 0.02), and ∆-glycogen fraction correlated inversely with ∆-LBM (ρ = -0.83; p= 0.002) during six months TRT, but no significant changes were observed in mitochondrial, glycogen and LD volume fractions during TRT and ST. In conclusion, in this exploratory small scale study, the beneficial effects of six months TRT on total testosterone, LBM and percent body fat were not followed by significant changes in fractions of mitochondria, glycogen or lipid in skeletal muscle of ageing men with lowered testosterone levels. Six months ST or combined three months ST+TRT did not change intramyocellular mitochondria, glycogen and LD fractions compared to placebo. However, further studies with a larger sample size are needed.
Abbreviations: BioT: Bioavailable testosterone, BMI: Body mass index, FFA: Free fatty acid, 
LBM: Lean body mass, LD: Lipid droplet, Rd: Rate of glucose disposal, SHBG: Sex hormone 
binding globulin, ST: Strength training, TEM: Transmission electron microscopy, TRT: Testosterone replacement therapy, T2D: type 2 diabetes mellitus, VO2max: Maximal oxygen uptake, 
WC: Waist circumference.
Introduction

Ageing men often have lower bioavailable testosterone (BioT) levels compared to a young reference population (Frost, et al., 2013). The age related decline in testosterone levels is associated with reduced muscle mass (Frost, et al., 2014), increased abdominal adiposity (Glintborg, et al., 2014) and insulin resistance (Grossmann, 2011). Mitochondrial dysfunction was reported in individuals with insulin resistance, as genes and proteins involved in mitochondrial oxidative metabolism (Giebelstein, et al., 2012; Hwang, et al., 2010) and biogenesis (Mootha, et al., 2003; Patti, et al., 2003) were downregulated in skeletal muscle biopsies. This reduction of mitochondrial capacity can result in decreased muscular lipid oxidation and increased lipid accumulation (Lowell, et al., 2005; Petersen, et al., 2003) and subsequently suppress insulin signaling and reduce glycogen synthesis (Lowell, et al., 2005). In accordance, obese subjects with type 2 diabetes mellitus (T2D) had lower intramyocellular volume fraction of glycogen compared to obese normoglycemic participants (Carey, et al., 2003; Levin, et al., 2001), however similar glycogen volume fraction has also been reported (Nielsen, et al., 2010). A cross-sectional study in ageing men reported positive associations between endogenous testosterone levels and insulin sensitivity, aerobic capacity (VO\textsubscript{2max}) and expression of mitochondrial oxidative phosphorylation genes from muscle biopsies (Pitteloud, et al., 2005). Recently, we observed increased lean body mass (LBM) and reduced subcutaneous fat deposits (Frederiksen, et al., 2012) during six months testosterone replacement therapy (TRT) in ageing men, however insulin-stimulated rate of glucose disposal (Rd) was unchanged (Frederiksen, et al., 2012). Moreover, we reported that strength training (ST) for six months increased muscle strength in ageing men with lowered testosterone levels and abdominal obesity, whereas lean body mass (LBM) was unchanged (Kvorning, et al., 2013). To the best of our knowledge, there is no previous
human data on adaptations in mitochondrial, glycogen and lipid droplet (LD) content of skeletal muscle in the ageing male during TRT, placebo and ST, but animal studies reported that testosterone supplementation increased skeletal muscle mitochondrial biogenesis in male mice (Usui, et al., 2014) and glycogenesis in castrated male rats (Ramamani, et al., 1999).

In this study, we investigated effects of TRT, placebo and ST on volume fraction of mitochondria, glycogen and LDs in skeletal muscle biopsies from ageing men with lowered testosterone levels. Furthermore, we examined the associations between intramyocellular volume fraction of mitochondria, glycogen and LDs and body composition, circulating testosterone, insulin-stimulated Rd and triglyceride.

**Materials and methods**

We previously published effects of six months TRT on body composition, substrate metabolism and insulin sensitivity in a single-center, randomized, placebo-controlled, double-blinded study in men aged 60-78 years with lowered bioavailable testosterone (BioT) and waist circumference (WC) > 94 cm (Frederiksen, et al., 2012). The cut-off level for BioT was defined as < 7.3 nmol/L based on observations from a comprehensively characterized reference population of men aged 20–30 years (Nielsen, et al., 2007). Exclusion criteria were haematocrit > 50 %, prostate cancer or prostate specific antigen > 3 ng/dL, previous or on-going malignant disease, severe ischemic heart or respiratory disease, diabetes mellitus, alcohol or drug abuse, abnormal routine blood samples (TSH, ionized calcium, liver and kidney function), treatment with 5-α-reductase inhibitors, morphine or oral glucocorticoids (Frederiksen, et al., 2012). The study was conducted at Odense University Hospital, and screening was started in January 2008 and ended in September 2009. The present study included all data on skeletal muscle volume fraction of mitochondria,
glycogen and LD on all available muscle biopsies at baseline (n=45) and on the effect of TRT, placebo and ST (Fig. 1). Information on intramuscular fractions of mitochondria, glycogen and lipid droplet is not available from dropout participants, as there was no available muscle biopsy from these men.

Ethics

The study was approved by the local ethics committee (Project ID S-20070051), the Danish Medicines Agency (Journal No: 2612-3474), and declared in ClinicalTrials.gov (NCT00700024) and performed in accordance with the 1964 Helsinki Declaration. All participants gave written informed consent at screening visit.

Study protocol (Fig. 2)

Participants were randomly allocated to receive testosterone gel (5 g of gel/50 mg Testim, Ipsen, Paris, France), placebo gel per day or strength training before entry to the groups. After three months of ST, the ST group was further randomized into two groups receiving ST+TRT or ST+placebo (Fig. 2). Randomization list, medicine labelling, randomization and code break envelopes were generated by Ipsen Scandinavia (Krista, Sweden) (Frederiksen, et al., 2012). Sham titrations in both the TRT group and the placebo were externally handled to ensure continued blinding. If BioT was < 7.3 nmol/L after three weeks of intervention, the dose was increased to 10 g gel (100 mg Testim or placebo). Five of 11 participants in the TRT group were increased to 10 g Testim gel, and in the placebo group the dose was increased to 10 g placebo gel in all 13 participants at the three week safety check. Participants were informed not to change their diet,
and refrain from any self-initiated physical exercise training, however were permitted to continue other habitual activities throughout the study period.

**Strength training**

The training group performed 5 minutes of standardized warm up on a stationary bicycle with low resistance (approximately 100 W), followed by a progressive heavy ST program of exercises for the entire body with increased training loads at the start of every other week (Kvorning, et al., 2013). All training sessions were supervised, and participants engaged in a minimum of two out of three weekly training sessions (mean ± SD training adherence 75 ± 8 %) (Kvorning, et al., 2013).

**Testosterone and SHBG**

Testosterone levels were measured in the morning in the fasting state by liquid chromatography tandem mass spectrometry (LC-MS) after ether extraction. For testosterone measurements, the intra-assay coefficient of variation (CV) was less than ±10% for total testosterone > 0.2 nmol/L and less than ±30% in the range between 0.1 and 0.2 nmol/L (Frederiksen, et al., 2012). Sex hormone binding globulin (SHBG) was measured by autoDELFIA assay, and BioT was calculated according to the formulas of Vermeulen (Vermeulen, et al., 1999). The German Society for Clinical Chemistry and Laboratory Medicine (DGKL – Ring trial) undertook the external quality control of the LC-MS measurements.

**Dual X-ray absorptiometry (DXA)**

Total fat mass, central fat mass and lean body mass were measured by DXA using a Hologic
Discovery device (Waltham, MA, USA). The CV was 0.8% for total fat mass and 0.6% for lean
body mass (Frederiksen, *et al*., 2012).

**Euglycaemic hyperinsulinemic clamp**

After an overnight fast an euglycemic-hyperinsulinemic clamp was performed as previously de-
scribed (Frederiksen, *et al*., 2012). In brief, a 120-min basal tracer equilibration period was fol-
lowed by the infusion of insulin at a rate of 40 mU/m$^2$/min for 180 min. Euglycaemia was main-
tained using a variable infusion rate of 20% glucose based on bedside plasma glucose measure-
ments and physiological hyperinsulinemia was obtained at approximately 400 pmol/L during the
insulin-stimulated period. Plasma glucose, serum insulin and plasma free fatty acids (FFA) con-
centrations were measured as previously described (Frederiksen, *et al*., 2012; Hojlund, *et al*.,
2006). Steele’s non-steady-state formulas were used to calculate insulin-stimulated glucose dis-
posal rate (Rd), assuming a glucose distribution volume of 200 mL/kg body weight and a pool
fraction of 0.65 (Hother-Nielsen, *et al*., 1996). A muscle sample was obtained from the *vastus
lateralis muscle* in the resting, basal state using a Bergström needle with suction under local an-
esthesia (10-15 mL lidocaine subcutaneously).

**Transmission electron microscopy (TEM) and skeletal muscle content**

Muscle biopsies were fixed with 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffered (pH
7.3) fixative for 24 hours at 4 °C, and rinsed in 0.1 M sodium cacodylate buffer four times for a
duration of 15 minutes. Following rinsing, the muscle biopsies were postfixed with 1 % osmium
tetroxide in 0.1 M sodium cacodylate buffer for 90 min at 4 °C. Subsequently, muscle biopsies
were washed once in 0.1 M sodium cacodylate buffer and twice in sterile water at 4 °C. The
samples were dehydrated through a graded series of alcohol at 4-20 °C, infiltrated with graded mixtures of propylene oxide and epon at 20 °C, and finally embedded in 100 % epon at 30 °C for 90 min and 60°C for 2 days. Tissue blocks were cut to ultrathin sections using a Leica ultramicrotome separated by 60 nm. The sections were grid contrasted with uranyl acetate (3 % in H2O for 14 min at 60 °C) and lead citrate (for 6 min at room temperature). Sections were photographed in a JEM-1400 plus transmission electron microscope (120 kV) with a Quemesa camera (Olympus Soft Imaging Solutions, Münster, Germany). Ten micrographs per muscle biopsy were photographed in a randomized systematic order for stereological point counting. Micrographs were photographed at a magnification of x5 000. A representative micrograph with mitochondria, glycogen and LD is shown in Fig. 3.

A grid was superimposed over the micrograph in the iTEM computer software (Olympus Soft Imaging Solutions, Münster, Germany) and included test points, defined as two intersecting grid lines. According to standard stereological principles, volume fraction of mitochondria and LD were estimated by counting the test points touching the given structure of interest and dividing them by the total number of test points hitting muscle fibers (Gundersen, et al., 1988; Weibel, 1979). Glycogen volume fraction (Vv) was determined as proposed by Weibel (Weibel, 1980), where the effect of section thickness was taken into account: 

\[
V_v = A_A - t \left\{ \left( \frac{1}{\pi} \right) B_A - N_A \left[ \frac{(t+H)}{t} \right] \right\},
\]

where \( A_A \) is the glycogen area fraction, \( t \) is the section thickness (60 nm), \( B_A \) is the boundary length density, \( N_A \) is the number of particles per area, and \( H \) is the average glycogen profile diameter. Glycogen particles were assumed to be spherical (Melendez-Hevia, et al., 1993). The average glycogen profile diameter was measured using iTEM software. This was done blinded by the first author. Volume fractions of mitochondria and glycogen were assessed in grid sizes with squares of 500x500 nm (corresponding to 0.25 \( \mu \)m\(^2\), and density test point of
330 /micrograph), and LDs were evaluated in grid sizes with squares of 125x125 nm (corresponding to 0.016 µm², and density test point of 5 460 /micrograph), hence ensuring the principle of Cavalieri (Broskey, et al., 2013).

Assessment of cardiorespiratory fitness

VO₂max was initially determined by an incremental test to exhaustion, but due to general discomfort the maximal test was replaced by the Astrand submaximal cycle test (Astrand, et al., 1954) (Online: n= 3, Aastrand: n= 18). Prior to both tests, subjects performed a 6-min. standardized warm-up at 60 W. The initial workload in the incremental online test was 60-120 W for 3-min., followed by 3-min. with additional 30 W. Thereafter, power output was increased by 30 W every minute until exhaustion. VO₂ was calculated continuously throughout the test by using a mixing chamber system (Oxycon Pro, Erich JÄGER GmbH, Hoechberg, Germany), and VO₂max was defined as the highest mean VO₂ over 30 sec. In the submaximal test, VO₂max was determined from the Astrand nomogram and age-corrected (Astrand, et al., 1954).

Statistical analyses

Sample size was determined by the effect of TRT on lean body mass (Frederiksen, et al., 2012). Data were not normally distributed. Data are presented as median and interquartile range (IQR). Differences at baseline, between and within groups were analyzed with Mann Whitney U-test for unpaired data and Wilcoxon’s signed-rank test for paired data. Delta (Δ) values were calculated as post-treatment minus pre-treatment level. The effect of TRT and placebo were analyzed by comparing Δ-values of skeletal muscle mitochondrial, glycogen and lipid content, Δ biochemical variables, and Δ-body composition using Mann-Whitney U-test as described by Altman (Altman,
2010). Spearman’s non-parametric ρ correlation coefficient was performed estimating bivariate associations between intramyocellular mitochondrial, glycogen and lipid volume fraction and biochemical variables, and body composition at baseline and after intervention. Statistics were performed using STATA version 14.2 (StataCorp, College Station, Texas, USA), p-values < 0.05 were considered significant.

Results

Changes in clinical and metabolic characteristics during TRT and placebo

In consistence with the entire study population (Frederiksen, et al., 2012), we found no significant difference in biochemical assessments, body composition, insulin-stimulated Rd (Table 1) or aerobic power (VO_{2max}) (data not shown) between TRT, placebo or ST at baseline. In line with previous papers (Frederiksen, et al., 2012; Petersson, et al., 2014), we also found that TRT significantly increased total testosterone, BioT and LBM, and decreased percent body fat, but had no effect on insulin sensitivity measured as insulin-stimulated Rd or the ability of insulin to suppress FFA in this subgroup of individuals with attainable muscle biopsies (Supplemental table 1).

Skeletal muscle composition at baseline and during TRT, placebo and ST (Table 2)

Mitochondrial and glycogen volume fraction were comparable between TRT and placebo before treatment, but the volume fraction of LDs was higher in the TRT-group compared to placebo (p<0.05). No placebo-controlled change was found in skeletal muscle volume fraction of mitochondria, glycogen and LDs in response to three and six months TRT, three and six months ST, or three months combined ST+TRT.
**Baseline associations (Table 3)**

In all available muscle biopsies at baseline (n=45), mitochondrial fraction was inversely associated with percent body fat and circulating fasting FFA levels and tended to correlate positively with insulin-stimulated Rd ($p = 0.06$). Glycogen fraction was positively associated with circulating triglyceride levels and tended to correlate inversely with insulin-stimulated Rd ($p = 0.09$). LD fraction was positively associated with circulating triglyceride levels and inversely with SHBG levels.

**Associations of changes during TRT and placebo**

**3 months**

In the TRT group (n=11), $\Delta$-mitochondrial fraction was positively associated with total testosterone, and $\Delta$-glycogen fraction was negatively associated with $\Delta$-insulin-stimulated Rd (Supplemental table 2).

No significant associations were found during placebo (n=13), except $\Delta$-glycogen fraction was negatively associated with insulin sensitivity (Supplemental table 3).

**6 months**

In the TRT group (n=11), we found that $\Delta$-mitochondrial fraction was positively associated with $\Delta$-total testosterone, $\Delta$-insulin-stimulated Rd, and correlated inversely with $\Delta$-insulin-stimulated FFA levels (Table 4). Moreover, the TRT-induced increment in $\Delta$-LBM correlated inversely with $\Delta$-glycogen fraction. $\Delta$-LD fraction was negatively associated with $\Delta$-insulin-stimulated-FFA (Table 4).
In the placebo group (n=13), Δ-glycogen fraction also correlated inversely with Δ-LBM, and Δ-LD fraction was negatively associated with Δ-insulin-stimulated Rd (Supplemental table 5).

**Associations of changes during ST**

In the ST group (n=14), Δ-mitochondrial fraction was positively associated with Δ-LBM and inversely with Δ-fat percentage, and Δ-glycogen fraction was positively associated with Δ-insulin sensitivity Rd during three months (Supplemental table 4).

After randomization of ST group into ST+TRT (n=7) and ST+placebo (n=7), the groups were too small for assessing associations after six months.

**Discussion**

In this RCT, we investigated the effects of six months TRT, placebo and ST on volume fractions of mitochondria, glycogen and LD in skeletal muscle biopsies from ageing men with lowered testosterone levels. **TRT significantly increased total testosterone levels** and LBM, whereas percent body fat decreased, and insulin-stimulated Rd was unchanged. **We found that baseline mitochondrial fraction was significantly inversely correlated with percent body fat. Δ-mitochondrial fraction was positively associated with both Δ-total testosterone, and Δ-glycogen fraction correlated inversely with Δ-LBM during six months TRT, but no significant changes in mitochondrial, glycogen and LD fractions were detected during TRT**.

Our observation of an inverse baseline association between mitochondrial fraction and percent body fat was in accordance with a recent study by Bharadwaj *et al.* (Bharadwaj, *et al.*, 2015), where mitochondrial content correlated negatively with percent body fat in ageing men. However, Bharadwaj *et al.* used citrate synthase activity as a biochemical marker of mitochondrial con-
tent instead of the gold standard method, TEM (Larsen, et al., 2012). **We found that changes in mitochondrial fraction were positively associated with changes in total testosterone during TRT, but skeletal muscle mitochondrial fraction was not significantly affected. Our results were compatible with previous data from the same study cohort, where TRT resulted in unchanged levels of mRNA and proteins involved in mitochondrial biogenesis (Petersson, et al., 2014).**

Despite clinical relevant increase in testosterone levels, we observed no significant change in glycogen fraction during TRT. In contrast, an experimental rat study observed that testosterone deficiency was associated with decreased glycogen storage and synthesis in skeletal muscle at baseline, and glycogen stores were normalized in response to 30 days high dose testosterone supplementation (Ramamani, et al., 1999). The discrepancy between study observations may be a result of non-physiological testosterone levels in an experimental animal study population (Ramamani, et al., 1999).

We cannot fully explain the lack of improvement in glycogen fraction and insulin-stimulated Rd during TRT (Frederiksen, et al., 2012), as we saw an advantageous increase in LBM (Frederiksen, et al., 2012) and decreased total fat mass (Frederiksen, et al., 2012). However, visceral adipose tissue deposit was unchanged during TRT, and serum adiponectin levels and subcutaneous fat deposits were reduced (Frederiksen, et al., 2012). Myostatin may add to our understanding of unchanged glycogen fractions, as myostatin levels are significantly elevated during TRT (Dalbo, et al., 2017; Dubois, et al., 2014), and myostatin represents a potential key mediator of insulin resistance (Cleasby, et al., 2014). Unfortunately, there is currently no reliable myostatin assay, and this hypothesis remains to be tested.

We observed a higher fraction of lipid droplets at baseline in the TRT group compared to the placebo group, which may reflect differences in physical activity, however, mitochondrial frac-
tion, and VO2\text{max} at baseline were comparable between the groups, indicating similar aerobic capacity. The unchanged LD fraction during TRT in the present study was consistent with our previously published data, showing unchanged muscle mRNA levels of genes involved in lipid metabolism during TRT (Petersson, et al., 2014). Unchanged lipid fraction during TRT in the present study could be explained by the previously reported decrease in serum adiponectin during TRT (Frederiksen, et al., 2012). Decreased adiponectin may have counterbalanced the potential beneficial direct effect of increasing testosterone levels on lipid metabolism in skeletal muscle, as recombinant adiponectin has been reported to induce muscle fatty acid oxidation (Yoon, et al., 2006).

Sedentary lifestyle is increasing in the western world, and there is a growing interest in effects of ST. We demonstrated that intramuscular mitochondria, glycogen and LD fractions did not change during six months ST or three months combined ST and TRT. Our unchanged mitochondrial content during ST was in line with previous papers investigating ST effects after three months in ageing non-obese men (Flack, et al., 2016) and after 14 weeks in ageing adults (Parise, et al., 2005), however, Flack et al. and Parise et al. used the citrate synthase activity as a biochemical marker for mitochondrial content. In contrast, aerobic training increased mitochondrial volume fraction after 10 weeks in ageing obese men with and without T2D (Nielsen, et al., 2010) and after 16 weeks in healthy elderly adults (Broskey, et al., 2014). Aerobic training combined with ST may be an effective means of ameliorating an age-related decrease in mitochondrial content, as this combination may increase mitochondrial biogenesis (Wang, et al., 2011). Interestingly, we reported a positive association between changes in glycogen fraction and changes in insulin-stimulated Rd during three months of ST. This finding suggested that strength training may contribute to improvements in insulin-mediated glucose uptake and storage capacity in age-
ing men with lowered testosterone levels, these data are in agreement with studies in healthy older adults (Prior, et al., 2015) and insulin resistant men (Dela, et al., 2014). Despite this association, it was not reflected by increased glycogen content in our study and may be due to dropout.

Strengths and limitations may apply to the present study. The first author performed the stereological method in a blinded manner based on a recently validated methodological article by Broskey et al. (Broskey, et al., 2013). Furthermore, we used three independent modalities to assess lipid metabolism: Muscle LD fraction, circulating triglycerides and FFA. We acknowledge that eligible men were included only based on a single measurement of BioT, however, all men in the placebo group had BioT < 7.3 nmol/l at reassessment at three weeks. We have previously published that reference intervals for total testosterone were similar between healthy young and ageing men, in contrast, FreeT and BioT assessments were lower in ageing men explained by increased SHBG levels (Frost, et al., 2013). Despite that the Vermeulen formula relevantly includes SHBG as a parameter in the estimation of BioT (Vermeulen, et al., 1999), calculated BioT is not as exact as the actual measurement of BioT. The free hormone hypothesis is challenged, and it is suggested to take the variable binding affinity of testosterone to SHBG into account through a multi-step dynamic allosteric model (Goldman, et al., 2017; Handelsman, 2017; Ly, et al., 2010). This study was restricted to the inclusion of ageing men with lowered testosterone levels and without any known metabolic disease, and we cannot exclude more prominent results in e.g. men with T2D. In the original study design, sample size was based on the effects of TRT on LBM (Frederiksen, et al., 2012). We acknowledge that our study includes a subgroup of the original cohort that will limit the power to detect the effects of TRT on intramuscular mitochondrial, glycogen and LD fractions due to underpowering including low sample size and high dropout rate.
Conclusion

TRT was associated with increased **total testosterone**, LBM and decreased percent body fat, whereas we found no significant changes in intramuscular mitochondrial, glycogen and LD fractions in ageing men with lowered testosterone levels. Intramyocellular mitochondria, glycogen and LD fractions were not changed in response to six months ST or combined three months ST+TRT compared to placebo. **However, the study was conducted with a novel and an exploratory approach, so further studies with a larger sample size are needed.**

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Disclosures

The authors declare that they have no conflict of interest.

Author contributions

RCJ: Writing of the manuscript, literature search, data collection, data analysis, data interpretation, tables, and figures. LLC: Study design, data collection, writing of the manuscript and data
interpretation. JN, HDS, KH: Writing of the manuscript, data interpretation, and data analyses.

TK: Study design, data collection, writing of the manuscript and data interpretation. KG: Data collection, writing of the manuscript and data interpretation. DG: Supervision, writing of the manuscript, data interpretation, and data analyses. MA: Supervision, study design, writing of the manuscript, literature search, data collection, data interpretation, and data analyses.
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FIGURE LEGENDS

Figure 1 Consort flow chart of included individuals. ‘Baseline’ represents total individuals with muscle biopsies included in the baseline bivariate association. NA: Individuals without muscle biopsies after baseline visit and not included in the longitudinal analysis.

Figure 2 Study design

Fig. 3 Representative transmission electron micrograph. Ultrastructure of skeletal muscle fibers (original magnification x5 000; scale bar = 2 µm). Glycogen granules (G), mitochondrion (M), lipid droplet (L)
Mitochondria, glycogen and lipid droplets in skeletal muscle during testosterone treatment and strength training. A randomized, double blinded, placebo-controlled trial.

Richard Christian Jensen 1,a, Louise Lehmann Christensen 1, Joachim Nielsen 2, Henrik Daa Schrøder 3, Thue Kvorning 2, Kasper Gejl 2, Kurt Højlund 1, Dorte Glintborg 1, Marianne Andersen 1

1 Department of Endocrinology, Odense University Hospital, Odense C, Denmark.
2 Department of Sports Science & Clinical Biomechanics, University of Southern Denmark, Odense M, Denmark.
3 Department of Pathology, Odense University Hospital, Odense C, Denmark.

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a Corresponding author at:
Department of Endocrinology, Odense University Hospital
J.B. Winsløwsvej 17 A, 2, DK-5000 Odense C, Denmark.
Tel: +45 65504917, E-mail: rcjensen@health.sdu.dk
Abstract

Low testosterone levels in ageing men are associated with insulin resistance. Mitochondrial dysfunction, changes in glycogen metabolism, and lipid accumulation are linked to insulin resistance in skeletal muscle. In this randomized, double-blinded, placebo-controlled study, we investigated the effects of six months testosterone replacement therapy (TRT) and strength training (ST) on mitochondrial, glycogen and lipid droplet (LD) content in skeletal muscle of ageing men with subnormal bioavailable testosterone (BioT) levels. Mitochondrial, glycogen, and LD volume fractions in muscle biopsies were estimated by transmission electron microscopy. Insulin sensitivity (insulin-stimulated Rd) and body composition were assessed by euglycaemic-hyperinsulinaemic clamp and dual x-ray absorptiometry, respectively. TRT significantly increased total testosterone levels, BioT and lean body mass (LBM) (p< 0.05), whereas percent body fat decreased (p< 0.05), and insulin sensitivity was unchanged. Baseline mitochondrial volume fraction correlated inversely with percent body fat (ρ = -0.43; p = 0.003). ∆-mitochondrial fraction correlated positively with ∆-total testosterone (ρ = 0.70; p = 0.02), and ∆-glycogen fraction correlated inversely with ∆-LBM (ρ = -0.83; p = 0.002) during six months TRT, but no significant changes were observed in mitochondrial, glycogen and LD volume fractions during TRT and ST. In conclusion, in this exploratory small scale study, the beneficial effects of six months TRT on total testosterone, LBM and percent body fat were not followed by significant changes in fractions of mitochondria, glycogen or lipid in skeletal muscle of ageing men with lowered testosterone levels. Six months ST or combined three months ST+TRT did not change intramyocellular mitochondria, glycogen and LD fractions compared to placebo. However, further studies with a larger sample size are needed.
**Abbreviations**: BioT: Bioavailable testosterone, BMI: Body mass index, FFA: Free fatty acid, LBM: Lean body mass, LD: Lipid droplet, Rd: Rate of glucose disposal, SHBG: Sex hormone binding globulin, ST: Strength training, TEM: Transmission electron microscopy, TRT: Testosterone replacement therapy, T2D: type 2 diabetes mellitus, VO\(_{2\text{max}}\): Maximal oxygen uptake, WC: Waist circumference.
Introduction

Ageing men often have lower bioavailable testosterone (BioT) levels compared to a young reference population (Frost, et al., 2013). The age related decline in testosterone levels is associated with reduced muscle mass (Frost, et al., 2014), increased abdominal adiposity (Glintborg, et al., 2014) and insulin resistance (Grossmann, 2011). Mitochondrial dysfunction was reported in individuals with insulin resistance, as genes and proteins involved in mitochondrial oxidative metabolism (Giebelstein, et al., 2012; Hwang, et al., 2010) and biogenesis (Mootha, et al., 2003; Patti, et al., 2003) were downregulated in skeletal muscle biopsies. This reduction of mitochondrial capacity can result in decreased muscular lipid oxidation and increased lipid accumulation (Lowell, et al., 2005; Petersen, et al., 2003) and subsequently suppress insulin signaling and reduce glycogen synthesis (Lowell, et al., 2005). In accordance, obese subjects with type 2 diabetes mellitus (T2D) had lower intramyocellular volume fraction of glycogen compared to obese normoglycemic participants (Carey, et al., 2003; Levin, et al., 2001), however similar glycogen volume fraction has also been reported (Nielsen, et al., 2010). A cross-sectional study in ageing men reported positive associations between endogenous testosterone levels and insulin sensitivity, aerobic capacity (VO$_{2\text{max}}$) and expression of mitochondrial oxidative phosphorylation genes from muscle biopsies (Pitteloud, et al., 2005). Recently, we observed increased lean body mass (LBM) and reduced subcutaneous fat deposits (Frederiksen, et al., 2012) during six months testosterone replacement therapy (TRT) in ageing men, however insulin-stimulated rate of glucose disposal (Rd) was unchanged (Frederiksen, et al., 2012). Moreover, we reported that strength training (ST) for six months increased muscle strength in ageing men with lowered testosterone levels and abdominal obesity, whereas lean body mass (LBM) was unchanged (Kvorning, et al., 2013). To the best of our knowledge, there is no previous
human data on adaptations in mitochondrial, glycogen and lipid droplet (LD) content of skeletal muscle in the ageing male during TRT, placebo and ST, but animal studies reported that testosterone supplementation increased skeletal muscle mitochondrial biogenesis in male mice (Usui, et al., 2014) and glycogenesis in castrated male rats (Ramamani, et al., 1999).

In this study, we investigated effects of TRT, placebo and ST on volume fraction of mitochondria, glycogen and LDs in skeletal muscle biopsies from ageing men with lowered testosterone levels. Furthermore, we examined the associations between intramyocellular volume fraction of mitochondria, glycogen and LDs and body composition, circulating testosterone, insulin-stimulated Rd and triglyceride.

**Materials and methods**

We previously published effects of six months TRT on body composition, substrate metabolism and insulin sensitivity in a single-center, randomized, placebo-controlled, double-blinded study in men aged 60-78 years with lowered bioavailable testosterone (BioT) and waist circumference (WC) >94 cm (Frederiksen, et al., 2012). The cut-off level for BioT was defined as < 7.3 nmol/L based on observations from a comprehensively characterized reference population of men aged 20–30 years (Nielsen, et al., 2007). Exclusion criteria were haematocrit > 50 %, prostate cancer or prostate specific antigen > 3 ng/dL, previous or on-going malignant disease, severe ischemic heart or respiratory disease, diabetes mellitus, alcohol or drug abuse, abnormal routine blood samples (TSH, ionized calcium, liver and kidney function), treatment with 5-α-reductase inhibitors, morphine or oral glucocorticoids (Frederiksen, et al., 2012). The study was conducted at Odense University Hospital, and screening was started in January 2008 and ended in September 2009. The present study included all data on skeletal muscle volume fraction of mitochondria,
glycogen and LD on all available muscle biopsies at baseline (n=45) and on the effect of TRT, placebo and ST (Fig. 1). Information on intramuscular fractions of mitochondria, glycogen and lipid droplet is not available from dropout participants, as there was no available muscle biopsy from these men.

**Ethics**

The study was approved by the local ethics committee (Project ID S-20070051), the Danish Medicines Agency (Journal No: 2612-3474), and declared in ClinicalTrials.gov (NCT00700024) and performed in accordance with the 1964 Helsinki Declaration. All participants gave written informed consent at screening visit.

**Study protocol (Fig. 2)**

Participants were randomly allocated to receive testosterone gel (5 g of gel/50 mg Testim, Ipsen, Paris, France), placebo gel per day or strength training before entry to the groups. After three months of ST, the ST group was further randomized into two groups receiving ST+TRT or ST+placebo (Fig. 2). Randomization list, medicine labelling, randomization and code break envelopes were generated by Ipsen Scandinavia (Krista, Sweden) (Frederiksen, *et al*., 2012). Sham titrations in both the TRT group and the placebo were externally handled to ensure continued blinding. If BioT was < 7.3 nmol/L after three weeks of intervention, the dose was increased to 10 g gel (100 mg Testim or placebo). Five of 11 participants in the TRT group were increased to 10 g Testim gel, and in the placebo group the dose was increased to 10 g placebo gel in all 13 participants at the three week safety check. Participants were informed not to change their diet,
and refrain from any self-initiated physical exercise training, however were permitted to continue other habitual activities throughout the study period.

**Strength training**

The training group performed 5 minutes of standardized warm up on a stationary bicycle with low resistance (approximately 100 W), followed by a progressive heavy ST program of exercises for the entire body with increased training loads at the start of every other week (Kvorning, et al., 2013). All training sessions were supervised, and participants engaged in a minimum of two out of three weekly training sessions (mean ± SD training adherence 75 ± 8%) (Kvorning, et al., 2013).

**Testosterone and SHBG**

Testosterone levels were measured in the morning in the fasting state by liquid chromatography tandem mass spectrometry (LC-MS) after ether extraction. For testosterone measurements, the intra-assay coefficient of variation (CV) was less than ±10% for total testosterone > 0.2 nmol/L and less than ±30% in the range between 0.1 and 0.2 nmol/L (Frederiksen, et al., 2012). Sex hormone binding globulin (SHBG) was measured by autoDELFIA assay, and BioT was calculated according to the formulas of Vermeulen (Vermeulen, et al., 1999). The German Society for Clinical Chemistry and Laboratory Medicine (DGKL – Ring trial) undertook the external quality control of the LC-MS measurements.

**Dual X-ray absorptiometry (DXA)**

Total fat mass, central fat mass and lean body mass were measured by DXA using a Hologic
Discovery device (Waltham, MA, USA). The CV was 0.8% for total fat mass and 0.6% for lean body mass (Frederiksen, et al., 2012).

**Euglycaemic hyperinsulinemic clamp**

After an overnight fast an euglycemic-hyperinsulinemic clamp was performed as previously described (Frederiksen, et al., 2012). In brief, a 120-min basal tracer equilibration period was followed by the infusion of insulin at a rate of 40 mU/m²/min for 180 min. Euglycaemia was maintained using a variable infusion rate of 20% glucose based on bedside plasma glucose measurements and physiological hyperinsulinemia was obtained at approximately 400 pmol/L during the insulin-stimulated period. Plasma glucose, serum insulin and plasma free fatty acids (FFA) concentrations were measured as previously described (Frederiksen, et al., 2012; Hojlund, et al., 2006). Steele’s non-steady-state formulas were used to calculate insulin-stimulated glucose disposal rate (Rd), assuming a glucose distribution volume of 200 mL/kg body weight and a pool fraction of 0.65 (Hother-Nielsen, et al., 1996). A muscle sample was obtained from the vastus lateralis muscle in the resting, basal state using a Bergström needle with suction under local anesthesia (10-15 mL lidocaine subcutaneously).

**Transmission electron microscopy (TEM) and skeletal muscle content**

Muscle biopsies were fixed with 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffered (pH 7.3) fixative for 24 hours at 4 °C, and rinsed in 0.1 M sodium cacodylate buffer four times for a duration of 15 minutes. Following rinsing, the muscle biopsies were postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 90 min at 4 °C. Subsequently, muscle biopsies were washed once in 0.1 M sodium cacodylate buffer and twice in sterile water at 4 °C. The
samples were dehydrated through a graded series of alcohol at 4-20 °C, infiltrated with graded mixtures of propylene oxide and epon at 20 °C, and finally embedded in 100 % epon at 30 °C for 90 min and 60°C for 2 days. Tissue blocks were cut to ultrathin sections using a Leica ultramicrotome separated by 60 nm. The sections were grid contrasted with uranyl acetate (3 % in H₂O for 14 min at 60 °C) and lead citrate (for 6 min at room temperature). Sections were photographed in a JEM-1400 plus transmission electron microscope (120 kV) with a Quemesa camera (Olympus Soft Imaging Solutions, Münster, Germany). Ten micrographs per muscle biopsy were photographed in a randomized systematic order for stereological point counting. Micrographs were photographed at a magnification of x5 000. A representative micrograph with mitochondria, glycogen and LD is shown in Fig. 3.

A grid was superimposed over the micrograph in the iTEM computer software (Olympus Soft Imaging Solutions, Münster, Germany) and included test points, defined as two intersecting grid lines. According to standard stereological principles, volume fraction of mitochondria and LD were estimated by counting the test points touching the given structure of interest and dividing them by the total number of test points hitting muscle fibers (Gundersen, et al., 1988; Weibel, 1979). Glycogen volume fraction (Vₐ) was determined as proposed by Weibel (Weibel, 1980), here the effect of section thickness was taken into account:

$$Vₐ = Aₐ - t \left\{ \frac{1}{\pi} Bₐ - Nₐ \left[ \frac{(t+H)}{(t+H)} \right] \right\}$$

where $Aₐ$ is the glycogen area fraction, $t$ is the section thickness (60 nm), $Bₐ$ is the boundary length density, $Nₐ$ is the number of particles per area, and $H$ is the average glycogen profile diameter. Glycogen particles were assumed to be spherical (Melendez-Hevia, et al., 1993). The average glycogen profile diameter was measured using iTEM software. This was done blinded by the first author. Volume fractions of mitochondria and glycogen were assessed in grid sizes with squares of 500x500 nm (corresponding to 0.25 µm², and density test point of
330/micrograph), and LDs were evaluated in grid sizes with squares of 125x125 nm (corresponding to 0.016 µm², and density test point of 5 460/micrograph), hence ensuring the principle of Cavalieri (Broskey, et al., 2013).

**Assessment of cardiorespiratory fitness**

VO$_{2\text{max}}$ was initially determined by an incremental test to exhaustion, but due to general discomfort the maximal test was replaced by the Astrand submaximal cycle test (Astrand, et al., 1954) (Online: n= 3, Aastrand: n= 18). Prior to both tests, subjects performed a 6-min. standardized warm-up at 60 W. The initial workload in the incremental online test was 60-120 W for 3-min., followed by 3-min. with additional 30 W. Thereafter, power output was increased by 30 W every minute until exhaustion. VO$_2$ was calculated continuously throughout the test by using a mixing chamber system (Oxycon Pro, Erich JAEGER GmbH, Hoechberg, Germany), and VO$_{2\text{max}}$ was defined as the highest mean VO$_2$ over 30 sec. In the submaximal test, VO$_{2\text{max}}$ was determined from the Astrand nomogram and age-corrected (Astrand, et al., 1954).

**Statistical analyses**

Sample size was determined by the effect of TRT on lean body mass (Frederiksen, et al., 2012). Data were not normally distributed. Data are presented as median and interquartile range (IQR). Differences at baseline, between and within groups were analyzed with Mann Whitney U-test for unpaired data and Wilcoxon’s signed-rank test for paired data. Delta (Δ) values were calculated as post-treatment minus pre-treatment level. The effect of TRT and placebo were analyzed by comparing Δ-values of skeletal muscle mitochondrial, glycogen and lipid content, Δ-biochemical variables, and Δ-body composition using Mann-Whitney U-test as described by Altman (Altman,
Spearman’s non-parametric $\rho$ correlation coefficient was performed estimating bivariate associations between intramyocellular mitochondrial, glycogen and lipid volume fraction and biochemical variables, and body composition at baseline and after intervention. Statistics were performed using STATA version 14.2 (StataCorp, College Station, Texas, USA), p-values < 0.05 were considered significant.

**Results**

**Changes in clinical and metabolic characteristics during TRT and placebo**

In consistence with the entire study population (Frederiksen, *et al.*, 2012), we found no significant difference in biochemical assessments, body composition, insulin-stimulated Rd (Table 1) or aerobic power (VO$_{2\text{max}}$) (data not shown) between TRT, placebo or ST at baseline. In line with previous papers (Frederiksen, *et al.*, 2012; Petersson, *et al.*, 2014), we also found that TRT significantly increased total testosterone, BioT and LBM, and decreased percent body fat, but had no effect on insulin sensitivity measured as insulin-stimulated Rd or the ability of insulin to suppress FFA in this subgroup of individuals with attainable muscle biopsies (Supplemental table 1).

**Skeletal muscle composition at baseline and during TRT, placebo and ST (Table 2)**

Mitochondrial and glycogen volume fraction were comparable between TRT and placebo before treatment, but the volume fraction of LDs was higher in the TRT-group compared to placebo (p<0.05). No placebo-controlled change was found in skeletal muscle volume fraction of mitochondria, glycogen and LDs in response to three and six months TRT, three and six months ST, or three months combined ST+TRT.
Baseline associations (Table 3)

In all available muscle biopsies at baseline (n=45), mitochondrial fraction was inversely associated with percent body fat and circulating fasting FFA levels and tended to correlate positively with insulin-stimulated Rd ($p = 0.06$). Glycogen fraction was positively associated with circulating triglyceride levels and tended to correlate inversely with insulin-stimulated Rd ($p = 0.09$). LD fraction was positively associated with circulating triglyceride levels and inversely with SHBG levels.

Associations of changes during TRT and placebo

3 months

In the TRT group (n=11), $\Delta$-mitochondrial fraction was positively associated with total testosterone, and $\Delta$-glycogen fraction was negatively associated with $\Delta$-insulin-stimulated Rd (Supplemental table 2).

No significant associations were found during placebo (n=13), except $\Delta$-glycogen fraction was negatively associated with insulin sensitivity (Supplemental table 3).

6 months

In the TRT group (n=11), we found that $\Delta$-mitochondrial fraction was positively associated with $\Delta$-total testosterone, $\Delta$-insulin-stimulated Rd, and correlated inversely with $\Delta$-insulin-stimulated FFA levels (Table 4). Moreover, the TRT-induced increment in $\Delta$-LBM correlated inversely with $\Delta$-glycogen fraction. $\Delta$-LD fraction was negatively associated with $\Delta$-insulin-stimulated-FFA (Table 4).
In the placebo group (n=13), Δ-glycogen fraction also correlated inversely with Δ-LBM, and Δ-LD fraction was negatively associated with Δ-insulin-stimulated Rd (Supplemental table 5).

**Associations of changes during ST**

In the ST group (n=14), Δ-mitochondrial fraction was positively associated with Δ-LBM and inversely with Δ-fat percentage, and Δ-glycogen fraction was positively associated with Δ-insulin sensitivity Rd during three months (Supplemental table 4).

After randomization of ST group into ST+TRT (n=7) and ST+placebo (n=7), the groups were too small for assessing associations after six months.

**Discussion**

In this RCT, we investigated the effects of six months TRT, placebo and ST on volume fractions of mitochondria, glycogen and LD in skeletal muscle biopsies from ageing men with lowered testosterone levels. TRT significantly increased total testosterone levels and LBM, whereas percent body fat decreased, and insulin-stimulated Rd was unchanged. We found that baseline mitochondrial fraction was significantly inversely correlated with percent body fat. Δ-mitochondrial fraction was positively associated with both Δ-total testosterone, and Δ-glycogen fraction correlated inversely with Δ-LBM during six months TRT, but no significant changes in mitochondrial, glycogen and LD fractions were detected during TRT.

Our observation of an inverse baseline association between mitochondrial fraction and percent body fat was in accordance with a recent study by Bharadwaj et al. (Bharadwaj, et al., 2015), where mitochondrial content correlated negatively with percent body fat in ageing men. However, Bharadwaj et al. used citrate synthase activity as a biochemical marker of mitochondrial con-
tent instead of the gold standard method, TEM (Larsen, et al., 2012). We found that changes in mitochondrial fraction were positively associated with changes in total testosterone during TRT, but skeletal muscle mitochondrial fraction was not significantly affected. Our results were compatible with previous data from the same study cohort, where TRT resulted in unchanged levels of mRNA and proteins involved in mitochondrial biogenesis (Petersson, et al., 2014).

Despite clinical relevant increase in testosterone levels, we observed no significant change in glycogen fraction during TRT. In contrast, an experimental rat study observed that testosterone deficiency was associated with decreased glycogen storage and synthesis in skeletal muscle at baseline, and glycogen stores were normalized in response to 30 days high dose testosterone supplementation (Ramamani, et al., 1999). The discrepancy between study observations may be a result of non-physiological testosterone levels in an experimental animal study population (Ramamani, et al., 1999).

We cannot fully explain the lack of improvement in glycogen fraction and insulin-stimulated Rd during TRT (Frederiksen, et al., 2012), as we saw an advantageous increase in LBM (Frederiksen, et al., 2012) and decreased total fat mass (Frederiksen, et al., 2012). However, visceral adipose tissue deposit was unchanged during TRT, and serum adiponectin levels and subcutaneous fat deposits were reduced (Frederiksen, et al., 2012). Myostatin may add to our understanding of unchanged glycogen fractions, as myostatin levels are significantly elevated during TRT (Dalbo, et al., 2017; Dubois, et al., 2014), and myostatin represents a potential key mediator of insulin resistance (Cleasby, et al., 2014). Unfortunately, there is currently no reliable myostatin assay, and this hypothesis remains to be tested.

We observed a higher fraction of lipid droplets at baseline in the TRT group compared to the placebo group, which may reflect differences in physical activity, however, mitochondrial frac-
tion, and VO$_{2 \text{max}}$ at baseline were comparable between the groups, indicating similar aerobic capacity. The unchanged LD fraction during TRT in the present study was consistent with our previously published data, showing unchanged muscle mRNA levels of genes involved in lipid metabolism during TRT (Petersson, et al., 2014). Unchanged lipid fraction during TRT in the present study could be explained by the previously reported decrease in serum adiponectin during TRT (Frederiksen, et al., 2012). Decreased adiponectin may have counterbalanced the potential beneficial direct effect of increasing testosterone levels on lipid metabolism in skeletal muscle, as recombinant adiponectin has been reported to induce muscle fatty acid oxidation (Yoon, et al., 2006).

Sedentary lifestyle is increasing in the western world, and there is a growing interest in effects of ST. We demonstrated that intramuscular mitochondria, glycogen and LD fractions did not change during six months ST or three months combined ST and TRT. Our unchanged mitochondrial content during ST was in line with previous papers investigating ST effects after three months in ageing non-obese men (Flack, et al., 2016) and after 14 weeks in ageing adults (Parise, et al., 2005), however, Flack et al. and Parise et al. used the citrate synthase activity as a biochemical marker for mitochondrial content. In contrast, aerobic training increased mitochondrial volume fraction after 10 weeks in ageing obese men with and without T2D (Nielsen, et al., 2010) and after 16 weeks in healthy elderly adults (Broskey, et al., 2014). Aerobic training combined with ST may be an effective means of ameliorating an age-related decrease in mitochondrial content, as this combination may increase mitochondrial biogenesis (Wang, et al., 2011). Interestingly, we reported a positive association between changes in glycogen fraction and changes in insulin-stimulated Rd during three months of ST. This finding suggested that strength training may contribute to improvements in insulin-mediated glucose uptake and storage capacity in age-
ing men with lowered testosterone levels, these data are in agreement with studies in healthy older adults (Prior, et al., 2015) and insulin resistant men (Dela, et al., 2014). Despite this association, it was not reflected by increased glycogen content in our study and may be due to dropout.

Strengths and limitations may apply to the present study. The first author performed the stereological method in a blinded manner based on a recently validated methodological article by Broskey et al. (Broskey, et al., 2013). Furthermore, we used three independent modalities to assess lipid metabolism: Muscle LD fraction, circulating triglycerides and FFA. We acknowledge that eligible men were included only based on a single measurement of BioT, however, all men in the placebo group had BioT < 7.3 nmol/l at reassessment at three weeks. We have previously published that reference intervals for total testosterone were similar between healthy young and ageing men, in contrast, FreeT and BioT assessments were lower in ageing men explained by increased SHBG levels (Frost, et al., 2013). Despite that the Vermeulen formula relevantly includes SHBG as a parameter in the estimation of BioT (Vermeulen, et al., 1999), calculated BioT is not as exact as the actual measurement of BioT. The free hormone hypothesis is challenged, and it is suggested to take the variable binding affinity of testosterone to SHBG into account through a multi-step dynamic allosteric model (Goldman, et al., 2017; Handelsman, 2017; Ly, et al., 2010). This study was restricted to the inclusion of ageing men with lowered testosterone levels and without any known metabolic disease, and we cannot exclude more prominent results in e.g. men with T2D. In the original study design, sample size was based on the effects of TRT on LBM (Frederiksen, et al., 2012). We acknowledge that our study includes a subgroup of the original cohort that will limit the power to detect the effects of TRT on intramuscular mitochondrial, glycogen and LD fractions due to underpowering including low sample size and high dropout rate.
**Conclusion**

TRT was associated with increased total testosterone, LBM and decreased percent body fat, whereas we found no significant changes in intramuscular mitochondrial, glycogen and LD fractions in ageing men with lowered testosterone levels. Intramyocellular mitochondria, glycogen and LD fractions were not changed in response to six months ST or combined three months ST+TRT compared to placebo. However, the study was conducted with a novel and an exploratory approach, so further studies with a larger sample size are needed.

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**Disclosures**

The authors declare that they have no conflict of interest.

**Author contributions**

RCJ: Writing of the manuscript, literature search, data collection, data analysis, data interpretation, tables, and figures. LLC: Study design, data collection, writing of the manuscript and data
interpretation. JN, HDS, KH: Writing of the manuscript, data interpretation, and data analyses. TK: Study design, data collection, writing of the manuscript and data interpretation. KG: Data collection, writing of the manuscript and data interpretation. DG: Supervision, writing of the manuscript, data interpretation, and data analyses. MA: Supervision, study design, writing of the manuscript, literature search, data collection, data interpretation, and data analyses.
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FIGURE LEGENDS

**Figure 1** Consort flow chart of included individuals. ‘Baseline’ represents total individuals with muscle biopsies included in the baseline bivariate association. NA: Individuals without muscle biopsies after baseline visit and not included in the longitudinal analysis.

**Figure 2** Study design

**Fig. 3** Representative transmission electron micrograph. Ultrastructure of skeletal muscle fibers (original magnification x5000; scale bar = 2 µm). Glycogen granules (G), mitochondrion (M), lipid droplet (L)
Figure 1 Consort flow chart of included individuals.

'Baseline' represents total individuals with muscle biopsies included in the baseline bivariate association. NA: Individuals without muscle biopsies after baseline visit and not included in the longitudinal analysis.
Figure 2 Study design

Randomization

Placebo

Testosterone

Strength training

Strength training + Placebo

Strength training + Testosterone

Month 0 3 6
Figure 3 Representative transmission electron micrograph

Ultrastructure of skeletal muscle fibers (original magnification x5 000; scale bar = 2 µm).

Mitochondrion (M), glycogen granules (G), lipid droplet (L).
Table 1 Baseline data

<table>
<thead>
<tr>
<th></th>
<th>Placebo (n=13)</th>
<th>Testosterone (n=11)</th>
<th>Strength training (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>68 (65; 72)</td>
<td>69 (65; 72)</td>
<td>68.5 (63.0; 73.0)</td>
</tr>
<tr>
<td>Total testosterone (nmol/L)</td>
<td>12.3 (8.9; 16.1)</td>
<td>10.0 (9.2; 14.4)</td>
<td>93.1 (88.0; 101.7)</td>
</tr>
<tr>
<td>SHBG (nmol/L)</td>
<td>43 (39; 56)</td>
<td>35 (26; 45)</td>
<td>29.9 (28.2; 33.2)</td>
</tr>
<tr>
<td>BioT (nmol/L)</td>
<td>4.6 (3.4; 6.3)</td>
<td>5.0 (4.1; 5.4)</td>
<td>14.4 (12.1; 17.6)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.4 (26.5; 33.2)</td>
<td>29.8 (28.1; 33.4)</td>
<td>0.3 (0.2; 0.3)</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>100 (98; 113)</td>
<td>109 (105; 115)</td>
<td>5.4 (4.9; 6.2)</td>
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<tr>
<td>LBM (kg)</td>
<td>60.9 (58.8; 69.0)</td>
<td>60.0 (56.8; 71.2)</td>
<td>65.5 (60.0; 72.2)</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>26.2 (21.5; 28.3)</td>
<td>28.2 (26.1; 31.1)</td>
<td>108.0 (105.0; 115.0)</td>
</tr>
<tr>
<td>Triglyceride (mmol/L)</td>
<td>1.4 (0.9; 1.6)</td>
<td>1.5 (1.4; 1.7)</td>
<td>26.2 (24.2; 28.8)</td>
</tr>
<tr>
<td>FFA fasting (mmol/L)</td>
<td>0.49 (0.37; 0.57)</td>
<td>0.52 (0.44; 0.61)</td>
<td>28.0 (24.6; 31.5)</td>
</tr>
<tr>
<td>FFA clamp (mmol/L)</td>
<td>0.06 (0.05; 0.07)</td>
<td>0.06 (0.04; 0.07)</td>
<td>15.0 (13.7; 17.5)</td>
</tr>
<tr>
<td>Rd clamp (mg/min/m²)</td>
<td>190 (153; 350)</td>
<td>184 (135; 242)</td>
<td>1.2 (1.0; 1.6)</td>
</tr>
</tbody>
</table>

Data presented as median (interquartile range). SHBG, sexual hormone binding globulin; BioT, bioavailable testosterone; BMI, body mass index; WC, waist circumference; LBM, lean body mass; FFA, free fatty acid; Rd, insulin-stimulated rate of glucose disposal.
Table 2 Muscle characteristics during placebo, testosterone replacement therapy (TRT) and strength training (ST)

<table>
<thead>
<tr>
<th>Months</th>
<th>Placebo</th>
<th>TRT</th>
<th>ST and placebo</th>
<th>ST and TRT</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Median (IQR)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total mitochondria, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t = 0</td>
<td>4.5 (3.9; 5.7)</td>
<td>5.2 (3.8; 7.4)</td>
<td>5.1 (4.2; 5.8)</td>
<td></td>
</tr>
<tr>
<td>t = 3</td>
<td>4.9 (3.6; 6.1)</td>
<td>4.9 (4.3; 6.1)</td>
<td>5.8 (4.6; 6.3)</td>
<td></td>
</tr>
<tr>
<td>t = 6</td>
<td>5.2 (4.5; 6.6)</td>
<td>5.4 (4.9; 6.5)</td>
<td>5.5 (4.0; 8.0)</td>
<td>5.8 (5.5; 7.4)</td>
</tr>
<tr>
<td>Total glycogen, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t = 0</td>
<td>3.6 (3.0; 4.0)</td>
<td>3.8 (3.3; 4.5)</td>
<td>3.7 (3.0; 4.3)</td>
<td></td>
</tr>
<tr>
<td>t = 3</td>
<td>3.8 (3.1; 4.5)</td>
<td>4.1 (3.2; 4.4)</td>
<td>4.3 (3.5; 4.8)</td>
<td></td>
</tr>
<tr>
<td>t = 6</td>
<td>4.3 (3.6; 4.5)</td>
<td>4.4 (3.7; 4.9)</td>
<td>4.0 (3.3; 5.1)</td>
<td>4.8 (4.3; 5.2)</td>
</tr>
<tr>
<td>Total lipid droplets, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t = 0</td>
<td>0.30 (0.26; 0.34)</td>
<td>0.61 (0.44; 0.94)*</td>
<td>0.39 (0.28; 0.60)</td>
<td></td>
</tr>
<tr>
<td>t = 3</td>
<td>0.42 (0.22; 0.58)</td>
<td>0.52 (0.27; 0.56)</td>
<td>0.47 (0.32; 0.80)</td>
<td></td>
</tr>
<tr>
<td>t = 6</td>
<td>0.40 (0.22; 0.51)</td>
<td>0.53 (0.41; 0.59)</td>
<td>0.64 (0.40; 0.93)</td>
<td>0.42 (0.37; 0.88)</td>
</tr>
</tbody>
</table>

Data presented as median (interquartile range (IQR)).

*P<0.05 TRT vs. placebo at baseline.
Table 3: Bivariate associations at baseline between muscle characteristics and testosterone, body composition and circulating triglyceride, and glucose metabolism (n= 45)

|                          | Mitochondria | Glycogen | Lipid droplets |
|--------------------------|--------------|----------|----------------|----------------|
|                          | ρ            | P-value  | ρ              | P-value        | ρ              | P-value |
| Total Testosterone (nmol/L) | -0.02        | 0.91     | 0.06           | 0.68           | -0.24          | 0.11    |
| SHBG (nmol/L)             | 0.20         | 0.18     | -0.14          | 0.36           | -0.30          | 0.04    |
| LBM (kg)                  | 0.14         | 0.35     | 0.11           | 0.49           | 0.10           | 0.50    |
| Fat (%)                   | -0.43        | 0.003    | -0.05          | 0.74           | 0.02           | 0.88    |
| Triglyceride (mmol/L)     | 0.15         | 0.34     | 0.35           | 0.02           | 0.51           | <0.001  |
| FFA basal (mmol/L)        | -0.33        | 0.02     | -0.13          | 0.38           | -0.11          | 0.50    |
| FFA clamp (mmol/L)        | -0.17        | 0.27     | -0.08          | 0.62           | 0.24           | 0.12    |
| Rd clamp (mg/min/m²)      | 0.29         | 0.06     | -0.25          | 0.09           | 0.01           | 0.95    |

Spearman’s ρ correlation coefficient, significant results in bold (p< 0.05).

SHBG: Sex hormone binding globulin, LBM: Lean body mass, FFA: Free fatty acid, Rd: Rate of insulin-stimulated glucose disposal.
Table 4 Bivariate associations between changes (Δ) in muscle characteristics and testosterone assessments, body composition, circulating triglyceride and glucose metabolism during 6 months testosterone replacement therapy (n= 11)

<table>
<thead>
<tr>
<th>Δ Mitochondria</th>
<th>Δ Glycogen</th>
<th>Δ Lipid droplets</th>
</tr>
</thead>
<tbody>
<tr>
<td>ρ</td>
<td>P-value</td>
<td>ρ</td>
</tr>
<tr>
<td>Δ Total Testosterone (nmol/L)</td>
<td>0.70</td>
<td>0.02</td>
</tr>
<tr>
<td>Δ SHBG (nmol/L)</td>
<td>-0.12</td>
<td>0.73</td>
</tr>
<tr>
<td>Δ LBM (kg)</td>
<td>-0.23</td>
<td>0.50</td>
</tr>
<tr>
<td>Δ Fat (%)</td>
<td>-0.10</td>
<td>0.77</td>
</tr>
<tr>
<td>Δ Triglyceride (mmol/L)</td>
<td>0.15</td>
<td>0.65</td>
</tr>
<tr>
<td>Δ FFA basal (mmol/L)</td>
<td>-0.28</td>
<td>0.40</td>
</tr>
<tr>
<td>Δ FFA clamp (mmol/L)</td>
<td>-0.70</td>
<td>0.02</td>
</tr>
<tr>
<td>Δ Rd clamp (mg/min/m²)</td>
<td>0.74</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Spearman’s ρ correlation coefficient, significant results in bold (p< 0.05).

SHBG: Sex hormone binding globulin, LBM: lean body mass, FFA: free fatty acid, Rd: rate of insulin-stimulated glucose disposal.
### Supplemental table 1 Effects of testosterone replacement therapy (TRT) or placebo on body composition, testosterone, metabolic parameters and content of glycogen, mitochondria and lipid in skeletal muscle

<table>
<thead>
<tr>
<th></th>
<th>Placebo (n= 13)</th>
<th>TRT (n= 11)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>68 (65; 72)</td>
<td>69 (65; 72)</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Total testosterone (nmol/L)</strong></td>
<td>12.3 (8.9; 16.1)</td>
<td>10.0 (9.2; 14.4)</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>SHBG (nmol/L)</strong></td>
<td>43 (39; 50)</td>
<td>35 (26; 45)</td>
<td>0.40</td>
</tr>
<tr>
<td><strong>BioT (nmol/L)</strong></td>
<td>4.6 (3.4; 6.3)</td>
<td>5.0 (4.1; 5.4)</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>28.4 (26.5; 33.2)</td>
<td>29.8 (28.1; 33.4)</td>
<td>0.08</td>
</tr>
<tr>
<td><strong>WC (cm)</strong></td>
<td>100 (98; 113)</td>
<td>109 (105; 115)</td>
<td>0.70</td>
</tr>
<tr>
<td><strong>LBM (kg)</strong></td>
<td>60.9 (58.8; 69.0)</td>
<td>60.0 (56.8; 71.2)</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>Fat (%)</strong></td>
<td>26.2 (21.5; 28.3)</td>
<td>28.2 (26.1; 31.1)</td>
<td>0.006</td>
</tr>
<tr>
<td><strong>Triglyceride (mmol/L)</strong></td>
<td>1.4 (0.9; 1.6)</td>
<td>1.5 (1.4; 1.7)</td>
<td>0.58</td>
</tr>
<tr>
<td><strong>FFA fasting (mmol/L)</strong></td>
<td>0.49 (0.37; 0.57)</td>
<td>0.52 (0.44; 0.61)</td>
<td>0.98</td>
</tr>
<tr>
<td><strong>FFA clamp (mmol/L)</strong></td>
<td>0.06 (0.05; 0.07)</td>
<td>0.06 (0.04; 0.07)</td>
<td>0.07</td>
</tr>
<tr>
<td><strong>Rd clamp (mg/min/m²)</strong></td>
<td>190 (153; 350)</td>
<td>184 (135; 242)</td>
<td>0.51</td>
</tr>
</tbody>
</table>

Data presented as median (interquartile range).

* P<0.05 TRT vs. placebo at baseline.  * P<0.05 between groups (delta (Δ) values).

P-value refers to Δ during TRT vs. Δ during placebo.

SHBG: Sex hormone binding globulin, BioT: Bioavailable testosterone, BMI: Body mass index, WC: Waist circumference, LBM: Lean body mass, FFA: Free fatty acid, Rd: Rate of insulin-stimulated glucose disposal
**Supplementary Table 2** Associations after 3 months testosterone replacement therapy between changes (Δ) in muscle characteristics and testosterone assessments, body composition and circulating triglyceride

<table>
<thead>
<tr>
<th></th>
<th>Δ Mitochondria</th>
<th>Δ Glycogen</th>
<th>Δ Lipid droplets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ρ P-value</td>
<td>ρ P-value</td>
<td>ρ P-value</td>
</tr>
<tr>
<td>Δ Total Testosterone (nmol/L)</td>
<td>0.66 0.04</td>
<td>0.28 0.43</td>
<td>0.67 0.03</td>
</tr>
<tr>
<td>Δ SHBG (nmol/L)</td>
<td>-0.07 0.84</td>
<td>-0.47 0.17</td>
<td>0.08 0.83</td>
</tr>
<tr>
<td>Δ LBM (kg)</td>
<td>-0.31 0.39</td>
<td>-0.07 0.85</td>
<td>-0.32 0.37</td>
</tr>
<tr>
<td>Δ Total body fat (kg)</td>
<td>-0.30 0.41</td>
<td>-0.33 0.35</td>
<td>-0.03 0.93</td>
</tr>
<tr>
<td>Δ Fat (%)</td>
<td>-0.16 0.65</td>
<td>-0.18 0.63</td>
<td>0.12 0.75</td>
</tr>
<tr>
<td>Δ Triglyceride (mmol/L)</td>
<td>0.35 0.33</td>
<td>0.26 0.47</td>
<td>0.08 0.83</td>
</tr>
<tr>
<td>Δ FFA basal (mmol/L)</td>
<td>-0.65 0.04</td>
<td>0.14 0.70</td>
<td>-0.44 0.20</td>
</tr>
<tr>
<td>Δ FFA clamp (mmol/L)</td>
<td>0.40 0.26</td>
<td>0.87 &lt;0.01</td>
<td>0.18 0.61</td>
</tr>
<tr>
<td>Δ Rd clamp (mg/min/m²)</td>
<td>-0.13 0.73</td>
<td>-0.81 &lt;0.01</td>
<td>0.07 0.86</td>
</tr>
</tbody>
</table>

Spearman's rank correlation. Significant results in bold (p< 0.05). SHBG, sexual hormone binding globulin; LBM, lean body mass; FFA, free fatty acid; Rd, insulin-stimulated rate of glucose disposal.
**Supplementary Table 3** Associations after 3 months placebo between changes (Δ) in muscle characteristics and testosterone assessments, body composition and circulating triglyceride

<table>
<thead>
<tr>
<th></th>
<th>Δ Mitochondria</th>
<th></th>
<th>Δ Glycogen</th>
<th></th>
<th>Δ Lipid</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ρ</td>
<td>P-value</td>
<td>ρ</td>
<td>P-value</td>
<td>ρ</td>
<td>P-value</td>
</tr>
<tr>
<td>Δ Total Testosterone (nmol/l)</td>
<td>0.20</td>
<td>0.52</td>
<td>0.03</td>
<td>0.93</td>
<td>-0.10</td>
<td>0.75</td>
</tr>
<tr>
<td>Δ SHBG (nmol/L)</td>
<td>0.33</td>
<td>0.27</td>
<td>-0.26</td>
<td>0.40</td>
<td>-0.34</td>
<td>0.26</td>
</tr>
<tr>
<td>Δ LBM (kg)</td>
<td>0.14</td>
<td>0.64</td>
<td>-0.20</td>
<td>0.51</td>
<td>0.05</td>
<td>0.87</td>
</tr>
<tr>
<td>Δ Total body fat (kg)</td>
<td>-0.40</td>
<td>0.18</td>
<td>0.00</td>
<td>1.00</td>
<td>0.26</td>
<td>0.38</td>
</tr>
<tr>
<td>Δ Fat (%)</td>
<td>-0.38</td>
<td>0.20</td>
<td>0.25</td>
<td>0.41</td>
<td>0.20</td>
<td>0.52</td>
</tr>
<tr>
<td>Δ Triglyceride (mmol/l)</td>
<td>-0.01</td>
<td>0.99</td>
<td>0.04</td>
<td>0.89</td>
<td>0.12</td>
<td>0.71</td>
</tr>
<tr>
<td>Δ FFA basal (mmol/L)</td>
<td>0.26</td>
<td>0.38</td>
<td>-0.03</td>
<td>0.93</td>
<td>-0.32</td>
<td>0.28</td>
</tr>
<tr>
<td>Δ FFA clamp (mmol/L)</td>
<td>-0.29</td>
<td>0.33</td>
<td>0.36</td>
<td>0.23</td>
<td>-0.10</td>
<td>0.74</td>
</tr>
<tr>
<td>Δ Rd clamp (mg/min/m²)</td>
<td>0.35</td>
<td>0.25</td>
<td>-0.74</td>
<td>0.00</td>
<td>0.06</td>
<td>0.85</td>
</tr>
</tbody>
</table>

Spearman’s rank correlation. Significant results in bold (p< 0.05). SHBG, sexual hormone binding globulin; LBM, lean body mass; FFA, free fatty acid; Rd, insulin-stimulated rate of glucose disposal.
**Supplementary Table 4** Associations after 3 months strength training between changes (Δ) in muscle characteristics and testosterone assessments, body composition and circulating triglyceride

<table>
<thead>
<tr>
<th></th>
<th>∆ Mitochondria</th>
<th></th>
<th>∆ Glycogen</th>
<th></th>
<th>∆ Lipid droplets</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ρ</td>
<td>P-value</td>
<td>ρ</td>
<td>P-value</td>
<td>ρ</td>
<td>P-value</td>
</tr>
<tr>
<td>∆ Total Testosterone (nmol/L)</td>
<td>-0.25</td>
<td>0.42</td>
<td>0.26</td>
<td>0.38</td>
<td>0.30</td>
<td>0.32</td>
</tr>
<tr>
<td>∆ SHBG (nmol/L)</td>
<td>-0.19</td>
<td>0.54</td>
<td>0.38</td>
<td>0.19</td>
<td>0.02</td>
<td>0.94</td>
</tr>
<tr>
<td>∆ LBM (kg)</td>
<td>0.79</td>
<td>&lt;0.01</td>
<td>0.08</td>
<td>0.79</td>
<td>-0.18</td>
<td>0.55</td>
</tr>
<tr>
<td>∆ Total body fat (kg)</td>
<td>-0.36</td>
<td>0.22</td>
<td>-0.15</td>
<td>0.63</td>
<td>0.19</td>
<td>0.53</td>
</tr>
<tr>
<td>∆ Fat (%)</td>
<td>-0.60</td>
<td>0.03</td>
<td>-0.06</td>
<td>0.84</td>
<td>0.19</td>
<td>0.54</td>
</tr>
<tr>
<td>∆ Triglyceride (mmol/L)</td>
<td>-0.05</td>
<td>0.87</td>
<td>0.55</td>
<td>0.06</td>
<td>-0.21</td>
<td>0.49</td>
</tr>
<tr>
<td>∆ FFA basal (mmol/L)</td>
<td>0.11</td>
<td>0.73</td>
<td>-0.28</td>
<td>0.35</td>
<td>0.09</td>
<td>0.78</td>
</tr>
<tr>
<td>∆ FFA clamp (mmol/L)</td>
<td>-0.26</td>
<td>0.39</td>
<td>-0.06</td>
<td>0.86</td>
<td>0.24</td>
<td>0.42</td>
</tr>
<tr>
<td>∆ Rd clamp (mg/min/m²)</td>
<td>0.32</td>
<td>0.28</td>
<td><strong>0.65</strong></td>
<td><strong>0.02</strong></td>
<td>-0.37</td>
<td>0.21</td>
</tr>
</tbody>
</table>

Spearman's rank correlation. Significant results in bold (p< 0.05). SHBG, sexual hormone binding globulin; LBM, lean body mass; FFA, free fatty acid; Rd, insulin-stimulated rate of glucose disposal.
**Supplementary Table 5** Associations after 6 months placebo between changes (Δ) in muscle characteristics and testosterone assessments, body composition and circulating triglyceride

<table>
<thead>
<tr>
<th></th>
<th>Δ Mitochondria</th>
<th></th>
<th>Δ Glycogen</th>
<th></th>
<th>Δ Lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ρ</td>
<td>P-value</td>
<td>ρ</td>
<td>P-value</td>
<td>ρ</td>
</tr>
<tr>
<td>Δ Total Testosterone (nmol/l)</td>
<td>0.06</td>
<td>0.85</td>
<td>0.18</td>
<td>0.57</td>
<td>0.42</td>
</tr>
<tr>
<td>Δ SHBG (nmol/L)</td>
<td>0.00</td>
<td>0.99</td>
<td>-0.13</td>
<td>0.67</td>
<td>0.23</td>
</tr>
<tr>
<td>Δ LBM (kg)</td>
<td>-0.28</td>
<td>0.36</td>
<td>-0.65</td>
<td>0.02</td>
<td>-0.50</td>
</tr>
<tr>
<td>Δ Total body fat (kg)</td>
<td>-0.25</td>
<td>0.42</td>
<td>-0.53</td>
<td>0.06</td>
<td>-0.17</td>
</tr>
<tr>
<td>Δ Fat (%)</td>
<td>-0.08</td>
<td>0.79</td>
<td>-0.52</td>
<td>0.07</td>
<td>-0.09</td>
</tr>
<tr>
<td>Δ Triglyceride (mmol/l)</td>
<td>-0.47</td>
<td>0.11</td>
<td>-0.19</td>
<td>0.53</td>
<td>0.28</td>
</tr>
<tr>
<td>Δ FFA basal (mmol/L)</td>
<td>0.06</td>
<td>0.84</td>
<td>0.15</td>
<td>0.63</td>
<td>0.07</td>
</tr>
<tr>
<td>Δ FFA clamp (mmol/L)</td>
<td>-0.09</td>
<td>0.77</td>
<td>-0.24</td>
<td>0.44</td>
<td>0.29</td>
</tr>
<tr>
<td>Δ Rd clamp (mg/min/m²)</td>
<td>-0.09</td>
<td>0.78</td>
<td>-0.32</td>
<td>0.28</td>
<td>-0.64</td>
</tr>
</tbody>
</table>

Spearman’s rank correlation. Significant results in bold (p< 0.05). SHBG, sexual hormone binding globulin; LBM, lean body mass; FFA, free fatty acid; Rd, insulin-stimulated rate of glucose disposal.
**Table 1:** Sensitivity analysis on main effect, Lean Body Mass, at an individual level

<table>
<thead>
<tr>
<th>ID</th>
<th>LBM baseline (kg)</th>
<th>LBM 6 months (kg)</th>
<th>∆LBM (kg)</th>
<th>Placebo</th>
<th>Testosterone replacement therapy</th>
</tr>
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<tbody>
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<td>3</td>
<td>57.7</td>
<td>56.7</td>
<td>-1.0</td>
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<td></td>
</tr>
<tr>
<td>6</td>
<td>61.8</td>
<td>61.8</td>
<td>-0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>70.4</td>
<td>67.03</td>
<td>-3.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>60.8</td>
<td>60.6</td>
<td>-0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>60.9</td>
<td>60.0</td>
<td>-0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>56.8</td>
<td>56.2</td>
<td>-0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>57.3</td>
<td>56.5</td>
<td>-0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>76.5</td>
<td>76.5</td>
<td>-0.003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>72.7</td>
<td>74.4</td>
<td>1.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>67.6</td>
<td>67.1</td>
<td>-0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>60.6</td>
<td>60.8</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>56.5</td>
<td>57.7</td>
<td>1.2</td>
<td></td>
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<tr>
<td>51</td>
<td>81.1</td>
<td>80.6</td>
<td>-0.5</td>
<td></td>
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<tr>
<td>55</td>
<td>69.0</td>
<td>70.7</td>
<td>1.7</td>
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<td>57</td>
<td>75.0</td>
<td>76.0</td>
<td>1.0</td>
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<tr>
<td>61</td>
<td>71.1</td>
<td>71.1</td>
<td>-0.04</td>
<td></td>
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</tr>
<tr>
<td>65</td>
<td>78.8</td>
<td>79.2</td>
<td>0.4</td>
<td></td>
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</tr>
<tr>
<td>68</td>
<td>58.8</td>
<td>59.2</td>
<td>0.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Individuals with no muscle biopsies (not included in this study) are shaded.

**Table 2:** Sensitivity analysis on main effect, Lean Body Mass (LBM) according to samples

<table>
<thead>
<tr>
<th>LBM (kg)</th>
<th>Placebo</th>
<th>TRT</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All, (n)</td>
<td>18</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>-0.05 (-0.6; 0.4)</td>
<td>1.4 (0.03; 2.7)*</td>
<td>0.01</td>
</tr>
<tr>
<td>Individuals with muscle biopsies (n)</td>
<td>13</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>-0.04 (-0.5; 0.4)</td>
<td>2.0 (0.8; 3.0)*</td>
<td>0.01</td>
</tr>
</tbody>
</table>

P-value: differences between the TRT and placebo group tested with non-parametric unpaired Wilcoxon rank sum test