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ARTICLE

Molecular Biology

Mitochondrial DNA copy number associates with insulin sensitivity and aerobic capacity, and differs between sedentary, overweight middle-aged males with and without type 2 diabetes

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Abstract

Background/objectives Increased risk of type 2 diabetes mellitus (T2DM) is linked to impaired muscle mitochondrial function and reduced mitochondrial DNA copy number ($mtDNA^{num}$). However, studies have failed to control for habitual physical activity levels, which directly influences both mtDNA copy number and insulin sensitivity. We, therefore, examined whether physical conditioning status (maximal oxygen uptake, $\dot{V}O_{2max}$) was associated with skeletal muscle mitochondrial volume and $mtDNA^{num}$, and was predictive of T2DM in overweight, middle-aged men.

Methods Whole-body physiological (ISI—insulin sensitivity index, HOMA-IR, $\dot{V}O_{2max}$) and muscle biochemical/molecular (vastus lateralis; $mtDNA^{num}$, mitochondrial and glycolytic enzymes activity, lipid content and markers of lipid peroxidation) measurements were performed in three groups of overweight, middle-aged male volunteers ($n = 10$ per group): sedentary T2DM (ST2DM); sedentary control (SC) and non-sedentary control (NSC), who differed in aerobic capacity (ST2DM < SC < NSC).

Results $mtDNA^{num}$ was greater in NSC versus SC and ST2DM ($P < 0.001$; $P < 0.001$), and less in ST2DM versus SC ($P < 0.01$). Across all groups, $mtDNA^{num}$ positively correlated with ISI ($P < 0.001$; $r = 0.688$) and $\dot{V}O_{2max}$ (normalised to free fat mass; $r = 0.684$, $P < 0.001$), and negatively correlated to HOMA-IR ($r = -0.544$, $P < 0.01$). The activity of mitochondrial enzymes (GluDH, CS and β-HAD) was greater in NSC than ST2DM ($P < 0.01$, $P < 0.001$ and $P < 0.05$) and SC ($P < 0.05$, $P < 0.01$ and $P < 0.05$), but similar between ST2DM and SC. Intramuscular-free fatty acids, triglycerides and malondialdehyde contents were similar between ST2DM and SC.

Conclusions Body composition and indices of muscle mitochondrial volume/function were similar between SC and ST2DM. However, $mtDNA^{num}$ differed and was positively associated with ISI, HOMA-IR and $\dot{V}O_{2max}$ across all groups. Collectively, the findings support the contention that habitual physical activity is a key component of T2DM development, possibly by influencing $mtDNA^{num}$.

Introduction

Excess caloric intake and lack of physical activity are primary causes of increasing obesity prevalence worldwide. Being overweight or obese is commonly associated with elevated circulating-free fatty acid concentrations and increased risk of metabolic inflexibility (defined as the inability of skeletal muscle to switch from fat to carbohydrate oxidation in response to increased circulating glucose and insulin concentrations), a central feature of insulin resistance (IR) and type 2 diabetes mellitus (T2DM). However, not all obese people develop T2DM, and not all T2DM patients are obese. Alternatively, increased risk of T2DM development has been linked to reduced muscle

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mitochondrial function (defects in intrinsic mitochondrial ATP production) and reduced mitochondrial DNA copy number ($mtDNA^{num}$) [1–5]. However, these observations have not been consistent across studies, and some would argue that declines in mitochondrial function are normalised when differences in physical activity levels ($\dot{V}O_2$), mitochondrial content and insulin action are considered [6–8]. It is also noteworthy that mitochondrial respiration (with or without normalisation for mitochondrial content) does not change following gastric-bypass induced improvements in insulin sensitivity [9]. Therefore, whether reduced intrinsic mitochondrial function is causative in the induction of IR/T2DM, or contributes to increased susceptibility to T2DM, or arises as a consequence of existing IR remains an openly debated topic.

Mitochondria are the site of cellular oxidative phosphorylation processes in which fat, carbohydrates and amino acids are oxidatively decarboxylated to produce reducing equivalents, which are subsequently used to generate ATP. Also, mitochondrial function is an integral part of glucose-stimulated insulin secretion in pancreatic beta-cells [10]. The dynamic equilibrium between mtDNA synthesis and degradation determines the $mtDNA^{num}$, which is relatively stable under normal physiological conditions. However, changes in $mtDNA^{num}$ are associated with pathological changes in tissues and organs. Human mtDNA resides in hundreds to thousands of copies in each cell and encodes for 13 structural proteins, which are subunits of the oxidative phosphorylation electron transport chain, in addition to 2 ribosomal RNAs (rRNA) and 22 transfer RNAs (tRNA). However, mtDNA replication, transcription, translation and repair is controlled by proteins encoded by nuclear DNA (nDNA) [11]. Qualitative changes in the mtDNA sequence induced by the mitochondrial reactive oxygen species (ROS), such as mutations and deletions, have been implicated in the pathogenesis of T2DM [12]. However, this can only account for a small proportion of patients with T2DM [13].

Nevertheless, it was demonstrated that the content of mtDNA decreased in patients with T2DM [14–17] and that reduced mtDNA levels precede the development of diabetes [14], although not consistently [7, 8]. A confounding factor that may have contributed to these conflicting observations is age since an age-related decline in $mtDNA^{num}$ was previously identified in isolated human islets [15, 16] and rodent skeletal muscle [18]. Similarly, regular physical activity by amplifying the signal for mitochondrial biogenesis can increase mitochondrial content and function in young and older volunteers [19], and also increases insulin sensitivity (IS) [20], while deconditioning has the opposite effects [21]. Overall, therefore, it would be useful if one could control for those factors known to contribute to variation in the mtDNA copy number, such as age and aerobic training status (maximal oxygen uptake), to provide more informative insight of the role of $mtDNA^{num}$ in T2DM risk.

The present study therefore aimed to identify whether indices of whole-body insulin sensitivity index (ISI) and IR were associated with (1) maximal oxygen uptake, (2) indices of skeletal muscle intrinsic mitochondrial function, (3) measures of skeletal muscle mitochondrial volume or (4) skeletal muscle $mtDNA^{num}$ in a cohort of middle-aged male volunteers clustered into sedentary T2DM (ST2DM), sedentary control (SC) and non-sedentary control (NSC) sub-cohorts.

Materials and methods

Study participants

This study was part of a previous project from which skeletal muscle fatty acid transporter protein expression has been reported [22]. A total of 10 male ST2DM patients, 10 normoglycaemic SC male volunteers and 10 normoglycaemic NSC male volunteers provided informed consent to participate in this study, which was approved by the Maastricht University Medical Ethics Committee. All volunteers were overweight (BMI > 25, Table 1). The ST2DM and SC volunteers were matched for age, BMI and whole-body fat mass (FM) (Table 1), and none was or had been engaged in a physical activity training programme.

In contrast, the NSC volunteers reported cycling 3–4 times each week for more than 45 min. The inclusion of ST2DM patients was based entirely on their medical condition, and confirmation was verified with an oral glucose tolerance test. Patient medication included metformin, amaryl, lipitor, glucophage, avandia, tolbutamide, daonil and statins. The average duration of clinical T2DM up to the start of the study was 7.5 ± 1.1 years. Anthropometric, physiological and biochemical parameters for each group of volunteers are shown in Table 1.

Study protocol

Subjects reported to the laboratory after an overnight fast. Following 30 min of supine rest, a vastus lateralis muscle biopsy sample was obtained from each volunteer under local anaesthesia (lignocaine 2%) using a Bergstrom biopsy needle. The muscle biopsy specimens were snap-frozen in liquid nitrogen and stored at −80 °C until analyses were undertaken at a later date.

Following a further 30 min of supine rest, a catheter (Baxter BV, Utrecht, the Netherlands) was inserted into an antecubital vein, and a blood sample was drawn ($t = 0$ min), after which 75 g of glucose (dissolved in 250 mL water) was ingested, and a further blood sample was collected at $t =$

Table 1 Anthropometric, physiological and biochemical characteristics of participants

	Type 2 diabetes $n = 10$	Sedentary control $n = 10$	Non-sedentary control $n = 10$
Age (years)	58.9 ± 1.7	60.0 ± 2.1	57.4 ± 0.9
BMI (kg/m^2)	28.9 ± 1.2	27.5 ± 0.5	25.5 ± 0.7*
Whole-body fat (%)	28.8 ± 1.8	29.2 ± 1.3	17.2 ± 1.2*,#
Whole-body FFM (kg)	64.0 ± 1.9	61.3 ± 1.5	63.6 ± 1.0
Fasting plasma glucose (mmol/L)	9.0 ± 0.4	5.5 ± 0.2*	5.7 ± 0.1*
Plasma glucose$_{120\,min}$ (mmol/L)	16.8 ± 1.0	5.3 ± 0.49*	5.3 ± 0.4*
Fasting serum insulin$_{0\,min}$ (mI U/L)	8.8 ± 0.9	7.9 ± 1.6	5.1 ± 0.6*
Serum insulin$_{120\,min}$ (mI U/L)	45.2 ± 7.8	48.4 ± 8.0	29.4 ± 6.3
Insulin sensitivity index (ISI) mL^2/kg/μI U/min	46.8 ± 1.8	83.6 ± 5.8*	103.0 ± 8.6**
HOMA-IR	3.63 ± 0.35	1.82 ± 0.37***	1.30 ± 0.15***
HbA1c (%)	7.30 ± 0.3	5.83 ± 0.2*	5.78 ± 0.1*
$\dot{V}O_{2max}$ (L/min)	2.90 ± 0.20	3.19 ± 0.19	3.80 ± 0.12**,#
$\dot{V}O_{2max}$ (mL/min/kg FFM)	45.0 ± 2.3	52.0 ± 2.7*	59.8 ± 1.6***,#
Wmax	205 ± 16	206 ± 18	300 ± 9**,#

BMI body mass index, *FFM* free fat mass

*,**,***Significantly different from Type 2 diabetes group ($P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively)

#Significantly different from the sedentary control group ($P < 0.05$)

120 min. Plasma glucose concentrations (Table 1) were measured (Yellow Spring glucose analyser) to assess glucose intolerance and type 2 diabetes according to the American Diabetes Association guidelines (www.diabetes.org) while serum, which was stored for 2 years at −80 °C, was used to assess insulin concentration (ELISA kit; Mercodia, Uppsala, Sweden). A small blood specimen collected at 0 time was used to measure the glycosylated haemoglobin HbA1c using an A1CNow$^+$ device (Medisave, UK).

Maximal power output (Wmax) and maximal oxygen uptake ($\dot{V}O_{2max}$) were determined on an electronically braked cycle ergometer (Lode Excalibur, Groningen, the Netherlands) during an incremental exhaustive exercise test undertaken 1 week before muscle biopsy sampling (Table 1). Oxygen uptake ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) were measured continuously (Oxycon; Mijnhart, Breda, the Netherlands). Body composition was assessed using the hydrostatic weighing method in the morning after an overnight fast. Simultaneously, residual lung volume was measured by the helium-dilution technique using a spirometer (Volugraph 2000; Mijnhart, Bunnik, the Netherlands). Body weight was measured with a digital balance. Body fat percentage was calculated using Siri's equation [23]. Fat-free mass (FFM) was calculated by subtracting FM from total body weight (Table 1).

Insulin sensitivity index ($ISI_{0,120}$)

Insulin sensitivity index ($ISI_{0,120}$) was calculated using serum insulin and plasma glucose concentrations in a fasted state (0 min) and 120 min post-oral glucose ingestion according to Gutt et al. [24]. The $ISI_{0,120}$ index (mL^2/kg/μI U/min^{-1}) was defined as:

$$ISI_{0,120} = \frac{75,000 + (G_0 - G_{120}) \times 0.19 \times m}{120 \times G_{mean} \times \log(I_{mean})},$$

where 75,000 represents the oral glucose load in mg, G_0 represents fasting plasma glucose concentration (mg/dL), G_{120} represents plasma glucose concentration at 120 min (mg/dL), 0.19 represents glucose space in L/kg body weight, m represents body mass (kg), 120 represents duration of the test (min), I_{mean} represents mean serum insulin concentration during the test (mIU/L) and G_{mean} represents mean plasma glucose concentration during the test (mmol/L).

HOMA-IR

The homeostatic model assessment (HOMA), which is a method used to quantify insulin resistance, was calculated as:

$$\frac{\text{Plasma glucose concentration (mmol/L)} \times \text{serum insulin concentration (mIU/L)}}{22.5}$$

where 22.5 is a normalising factor representing the product of normal fasting plasma glucose concentration of 4.5 mmol/L and normal fasting plasma insulin concentration of 5 μI U/mL.

Muscle lipid peroxidation

Muscle malondialdehyde (MDA) content was determined as an indicator of lipid peroxidation products based on the method of Erdelmeier et al. [25]. Briefly, frozen muscle tissue was homogenised in 5 mmol/L butylated hydroxytoluene in 20 mmol/L phosphate buffer pH 7.4, followed by centrifugation at 3000 × *g* at 4 °C. Clear muscle lysate was acidic hydrolysed at 60 °C for 80 min followed by mixing with N-methyl-2-phenylindol in 3:1 (v/v) acetonitrile: methanol, incubation at 45 °C for 60 min and finally centrifuged at 15,000 × *g* for 10 min to clarify. Absorbance was measured spectrophotometrically at 586 nm. The concentration of MDA (μmol/L/mg protein) was calculated using 1,1,3,3-tetramethoxypropane as a standard.

Muscle-free fatty acid and triglyceride content

Frozen muscle aliquots were homogenised in a Potter glass homogeniser for 3 min with 200 μL buffer (10 mmol/L Tris/HCl, pH 7.0 containing 154 mmol/L NaCl, 1 mmol/L EDTA and 1% Triton X-100)/mg wet weight. The muscle lysates were then centrifuged at 24,000 × *g* for 10 min. The pellets were discarded, and each supernatant was split into two aliquots. In the first aliquot, levels of unbound-free fatty acids (NEFA) only were measured using a WAKO NEFA assay kit, while in the second aliquot, through alkaline hydrolysis, the pools of free NEFA and NEFA released from triglycerides hydrolysis were determined. A 10-point standard curve generated from 1 mmol/L stock oleic acid solution was run in parallel. The triglycerides content was calculated by subtracting the free NEFA from the pooled NEFA values.

Muscle enzymes activities

The activities of muscle mitochondrial glutamate dehydrogenase (GluDH), citrate synthase (CS), and β-hydroacyl-CoA dehydrogenase (HAD) and cytosolic Gly3P dehydrogenase (Gly3PDH) were measured as described previously [21]. Briefly, following the lysis of frozen muscle pieces (~5 mg wet weight) in buffer containing K_2HPO_4 and Triton X-100 using a Potter Elvehjem homogeniser the enzyme activities were measured spectrophotometrically in the presence of suitable cofactors, activators and buffers specific for each enzyme.

Relative mtDNA copy number

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

Statistical analysis

Data in text, tables and figures are expressed as mean ± SEM, with $n = 10$ in each experimental group. Between-group differences were determined using one-factor analysis of variance (ANOVA). A Scheffe's post hoc test was applied in the case of a significant F-ratio to locate between-group differences. Significance was set at the 0.05 level of confidence. The strength of the linear correlations between the investigated variables (r) was obtained using Pearson's correlation. Correlations were deemed to be significant at 0.05 and 0.01 levels (two-tailed). The contribution of the variation in mitochondrial enzyme activity, mtDNA, FFM and $\dot{V}O_{2max}$/FFM to overall variation in ISI and HOMA-IR was determined using a linear regression model (IBM SPSS Statistics 24 package). Sample size calculation was calculated using G-Power software (version 3.1.9.2, Dusseldorf University, Germany) for ANOVA one-way fixed effects given $\alpha = 0.05$, number of groups = 3, power = 0.8 and effect size = 0.6.

Results

Participant anthropometric, physiological and biochemical characteristics

The anthropometric, physiological and biochemical characteristics of the ST2DM, SC and NSC groups are shown in Table 1. Subjects did not differ in age and whole-body FFM. All volunteers were overweight (BMI > 25), and body mass index in ST2DM was significantly greater than in NSC. Whole-body fat (%) in NSC was significantly less than in ST2DM and SC and similar between ST2DM and SC groups.

Fasting plasma glucose concentration in ST2DM was significantly greater than NSC and SC. Post-feeding (120 min) plasma glucose concentration in ST2DM was

Table 2 Muscle content of lipids and their oxidation product (malondialdehyde) in sedentary control volunteers ($n = 10$) and sedentary T2DM patients ($n = 10$)

Muscle metabolites	Sedentary control	Sedentary T2DM patients
Malondialdehyde (μmol/mg protein)	30.1 ± 4.2	32.4 ± 5.4
Intramuscular-free fatty acids (mmol/kg dry matter)	11.66 ± 0.68	15.21 ± 2.92
Intramuscular triglycerides (mmol/kg dry matter)	1.10 ± 0.04	0.85 ± 0.13

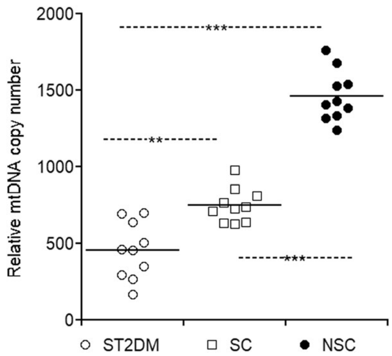

Fig. 1 Relative mtDNA copy number in vastus lateralis muscle of sedentary Type 2 diabetes mellitus (ST2DM), sedentary control (SC) and non-sedentary control (NSC) volunteers. Data represent mean ± SEM and individual values. Significant difference between groups depicted as: $^{**}P < 0.01$; $^{***}P < 0.001$

significantly greater than SC and NSC. Fasting serum insulin concentration in ST2DM was significantly greater than NSC. Post-feeding (120 min) serum insulin concentration was not different across groups, although it tended to be less in NSC than in SC and ST2DM. The ISI in SC and NSC was significantly greater than in ST2DM, and HOMA-IR in SC and NSC was significantly less than in ST2DM. Percentage glycated haemoglobin (HbA1c) in ST2DM was significantly greater than in NSC and SC. Maximal oxygen uptake in ST2DM was no different from SC, but when normalised to free FM (mL/min/kg FFA), $\dot{V}O_{2max}$ in SC was significantly greater than in ST2DM. Irrespective of the reference base, $\dot{V}O_{2max}$ in NSC was significantly greater than in ST2DM and SC (Table 1). Maximal power output in NSC was significantly greater than SC and ST2DM.

Muscle biopsy analyses

The muscle content of free fatty acids, triglycerides, determined as indices of muscle lipid availability and the muscle content of MDA, determined as an index of lipid peroxidation are presented in Table 2. Due to a scarcity of muscle tissue in some of the subjects in the NSC group, muscle metabolites could not be determined in all volunteers, and therefore, this group was omitted. Nevertheless, no difference was observed between ST2DM and SC for any parameter.

The individual mtDNA copy number and the mean relative mtDNA copy number displayed in Fig. 1 illustrate that on average NSC had a significantly greater number of mtDNA copies than SC ($P < 0.001$) and ST2DM ($P < 0.001$; 1461 ± 52, 749 ± 34 and 454 ± 58, respectively). The SC mtDNA copy number was also greater than ST2DM ($P < 0.01$).

The maximal activity of three mitochondrial enzymes (GluDH, CS and HAD) was determined as indices of mitochondrial volume and function (CS). The activity of all was greater in NSC compared with ST2DM and SC, but no differences were seen between ST2DM and SC (Fig. 2a). The maximal activity of the Gly3PDH enzyme was used as a marker of capacity for glycolytic energy production. There was no significant difference between SC and ST2DM volunteers, but Gly3PDH activity was less in NSC compared with both groups (both $P < 0.001$; Fig. 2b).

Pearson correlations between the relative mtDNA copy number and ISI, HOMA-IR, $\dot{V}O_{2max}$ normalised to FFM and mitochondrial enzyme activities across groups are presented in Table 3. Across all individuals, $mtDNA^{num}$ highly associated with ISI, HOMA-IR, mitochondrial enzyme activities (GlutDH and CS) and $\dot{V}O_{2max}$/FFM.

Discussion

The present study demonstrates that in a cohort of overweight, middle-aged male volunteers, $mtDNA^{num}$ is ordered as NSC > SC > ST2DM. Furthermore, across all individuals, $mtDNA^{num}$ was highly correlated with ISI, HOMA-IR, mitochondrial volume markers (GlutDH and CS activities) and $\dot{V}O_{2max}$ normalised to FFM, while the associations between indices of mitochondrial volume and ISI and HOMA-IR were less robust. These observations, together with the knowledge that there was no difference in body composition or muscle lipids between SC and ST2DM volunteers, leads to the conclusion that $mtDNA^{num}$ is a sensitive index of insulin sensitivity, which most likely reflects mitochondrial mass and supports the notion that regular exercise exerts a protective role against the development of IR and T2DM.

In the present study, a significant difference in $mtDNA^{num}$ was observed between SC and ST2DM groups, despite no between-group differences in muscle CS activity. Frequently, CS activity shows concordance with proteins entirely coded by mtDNA, such as complexes I, II and IV

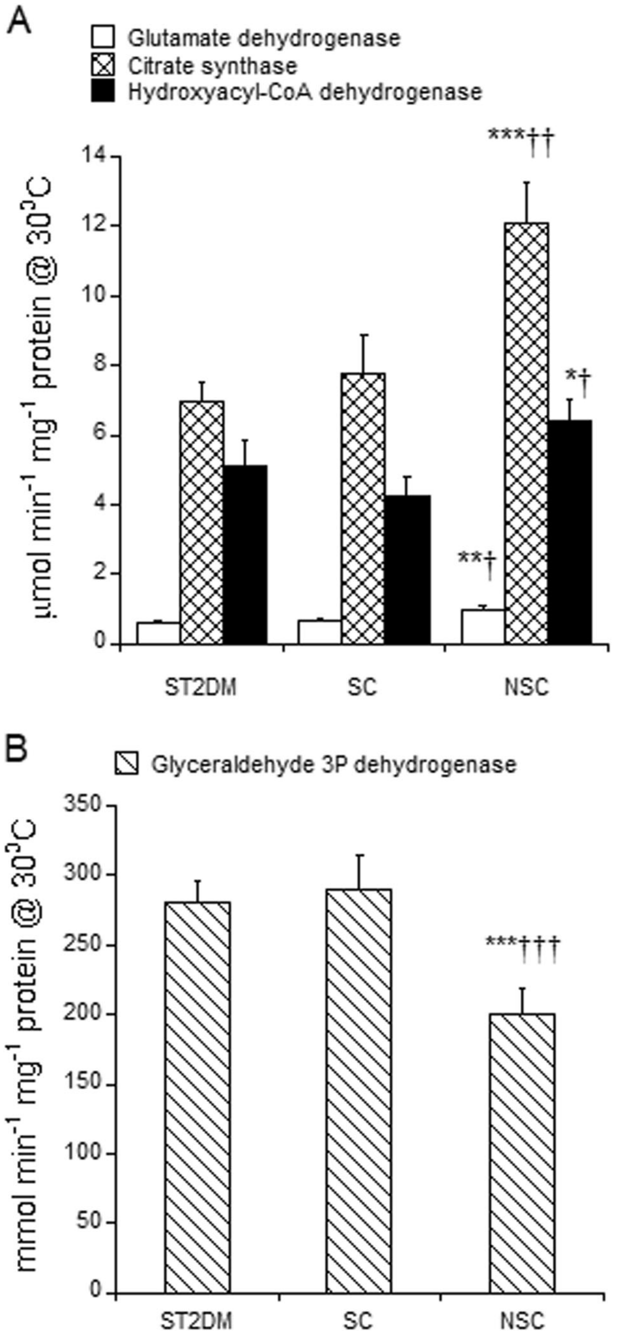

Fig. 2 Mitochondrial volume markers (**a** glutamate dehydrogenase, citrate synthase and β-hydroxyacyl-CoA dehydrogenase activity) and glycolytic capacity index (**b** glyceraldehyde-3P-dehydrogenase) in vastus lateralis muscle of sedentary Type 2 diabetes mellitus (ST2DM), sedentary control (SC) and non-sedentary control (NSC) volunteers. Data represent mean ± SEM and individual values. *,**,***Significantly different from ST2DM; $P < 0.05$, $P < 0.01$, and $P < 0.010$, respectively. †,††,†††Significantly different from SC; $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively

activity in both young and older subjects [26, 27], and it is often used as an index of muscle 'mitochondrial content/volume'. However, it has to be recognised that CS protein is coded by nDNA, rather than by mtDNA, indicating that the discordance between CS activity and mtDNA in the present study may not be entirely unexpected. In line with the CS findings, other mitochondrial enzyme activity measurements (GlutDH and HAD), body composition measurements (% whole-body fat and FFM), muscle levels of free fatty acids, triglycerides and MDA (a marker of lipid peroxidation; all of which have been reported to be causative of muscle IR), were similar in the SC and ST2DM groups. However, the $\dot{V}O_{2max}$ normalised to FFM in SC was significantly greater than in the T2DM group, presumably reflecting greater habitual physical activity levels in SC compared with ST2DM. In line with this contention, Table 3 illustrates that $mtDNA^{num}$ was found to associate strongly with indices of insulin sensitivity (ISI and HOMA-IR) and $\dot{V}O_{2max}$ normalised to FFM across all volunteers, and far better than the other muscle level measurements made.

It is worth commenting that the whole-body ISI did not associate with $\dot{V}O_{2max}$ across all study volunteers. This finding may be accounted for by the presence of additional factors that contribute to the biological variation of ISI and $\dot{V}O_{2max}$. Indeed, our linear regression model showed that 68% of the variation of ISI was accounted for by the variation of the mtDNA, GlutDH and FFM (all muscle related), while the variation of $\dot{V}O_{2max}$ was more most likely to be accounted for by adaptations of the cardiovascular and pulmonary systems in addition to those adaptations occurring at the muscle level.

Declines in $mtDNA^{num}$ and mitochondrial function have been linked to human ageing [28] and thereby age-related reductions in physical function [29]. This age-related decline in $mtDNA^{num}$ does not appear to be gender-related as $mtDNA^{num}$ in women and men was found to be almost the same [30]. In contrast, $mtDNA^{num}$ appears to be preserved in heart muscle with ageing, presumably due to its continuous contraction state [18]. In keeping with these observations, human $mtDNA^{num}$ also appears to be tissue-specific. Thus, values are reported to be greatest in muscle tissue, followed by blood vessels, and lowest in leucocytes (in both T2DM and control subjects) [31]. Although oxidative stress stimulates mitochondrial biogenesis, it also induces a greater degree of apoptosis in T2DM, resulting in a decrease in muscle tissue $mtDNA^{num}$ [32].

In conclusion, we report here the existence of a significant difference in ISI, HOMA-IR and $mtDNA^{num}$ across SC and T2DM volunteers, despite indices in mitochondrial volume and function, and body composition and muscle-free fatty acids, triglycerides and MDA being similar between sedentary and T2DM volunteers. Moreover, we found that $mtDNA^{num}$ strongly correlates with indices of insulin sensitivity and $\dot{V}O_{2max}$, which most likely reflect mitochondrial mass and supports the evidence that non-sedentary behaviour in the form of regular exercise exerts a protective role against the development of IR and T2DM.

Table 3 Pearson correlations between muscle $mtDNA^{num}$, whole-body insulin sensitivity index (ISI) and insulin resistance (HOMA-IR), and several muscle mitochondrial capacity indices (glutamate dehydrogenase—GlutDH, citrate synthase—CS, and β-hydroxyacyl-CoA dehydrogenase activity—HAD) and $\dot{V}O_{2max}$ normalised to free fat mass (FFM), in three groups of late middle-aged males clustered according to aerobic capacity and the presence of T2DM

	mtDNA	ISI	HOMA-IR	GlutDH	CS	HAD	$\dot{V}O_{2max/FFM}$
mtDNA							
Pearson correlation	–	0.688	−0.542	0.603	0.604	0.382	0.684
Sig (two-tailed)	–	0.001	0.002	0.001	0.001	0.037	0.001
ISI							
Pearson correlation	0.688	–	−0.662	0.488	0.328	0.187	0.178
Sig (two-tailed)	0.001	–	−0.001	0.006	0.077	0.321	0.348
HOMA-IR							
Pearson correlation	−0.542	−0.662	–	−0.424	−0.325	−0.112	−0.229
Sig (two-tailed)	0.002	0.001	–	0.020	0.080	0.554	0.224
GlutDH							
Pearson correlation	0.603	0.488	−0.424	–	0.715	0.605	0.217
Sig (two-tailed)	0.001	0.006	0.020	–	0.001	0.001	0.249
CS							
Pearson correlation	0.604	0.328	−0.325	0.715	–	0.654	0.161
Sig (two-tailed)	0.001	0.077	0.080	0.001	–	0.001	0.394
HAD							
Pearson correlation	0.382	0.187	−0.112	0.605	0.654	–	0.350
Sig (two-tailed)	0.037	0.321	0.554	0.001	0.001	–	0.105
$\dot{V}O_{2max/FFM}$							
Pearson correlation	0.684	0.278	−0.229	0.217	0.161	0.350	–
Sig (two-tailed)	0.001	0.240	0.224	0.249	0.394	0.105	–

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Author contributions DC-T—conducting experiments, collection, analysis, and interpretation of data, figures, literature search, writing of the manuscript and final approval. DC—data collection, analysis and interpretation, writing of the manuscript and final approval. MMP—study design, conducting experiments, and manuscript final approval. LBV—study design, conducting experiments and manuscript final approval. LvL—study design, data interpretation and manuscript final approval. PLG—data interpretation, writing of the manuscript and final approval.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Patients and healthy volunteers consent obtained.

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