

Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects

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Key points

- Several biochemical measures of mitochondrial components are used as biomarkers of mitochondrial content and muscle oxidative capacity. However, no studies have validated these surrogates against a morphological measure of mitochondrial content in human subjects.
- The most commonly used markers (citrate synthase activity, cardiolipin content, mitochondrial DNA content (mtDNA), complex I–V protein, and complex I–IV activity) were correlated with a measure of mitochondrial content (transmission electron microscopy) and muscle oxidative capacity (respiration in permeabilized fibres).
- Cardiolipin content followed by citrate synthase activity and complex I activity were the biomarkers showing the strongest association with mitochondrial content.
- mtDNA was found to be a poor biomarker of mitochondrial content.
- Complex IV activity was closely associated with mitochondrial oxidative phosphorylation capacity.

Abstract Skeletal muscle mitochondrial content varies extensively between human subjects. Biochemical measures of mitochondrial proteins, enzyme activities and lipids are often used as markers of mitochondrial content and muscle oxidative capacity (OXPHOS). The purpose of this study was to determine how closely associated these commonly used biochemical measures are to muscle mitochondrial content and OXPHOS. Sixteen young healthy male subjects were recruited for this study. Subjects completed a graded exercise test to determine maximal oxygen uptake ($\dot{V}_{O_2\text{peak}}$) and muscle biopsies were obtained from the vastus lateralis. Mitochondrial content was determined using transmission electron microscopy imaging and OXPHOS was determined as the maximal coupled respiration in permeabilized fibres. Biomarkers of interest were citrate synthase (CS) activity, cardiolipin content, mitochondrial DNA content (mtDNA), complex I–V protein content, and complex I–IV activity. Spearman correlation coefficient tests and Lin's concordance tests were applied to assess the absolute and relative association between the markers and mitochondrial content or OXPHOS. Subjects had a large range of $\dot{V}_{O_2\text{peak}}$ (range 29.9–71.6 ml min⁻¹ kg⁻¹) and mitochondrial content (4–15% of cell volume). Cardiolipin content showed the strongest association with mitochondrial content followed by CS and complex I activities. mtDNA was not related to mitochondrial content. Complex IV activity showed the strongest association with muscle oxidative capacity followed by complex II activity. We conclude

that cardiolipin content, and CS and complex I activities are the biomarkers that exhibit the strongest association with mitochondrial content, while complex IV activity is strongly associated with OXPHOS capacity in human skeletal muscle.

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Abbreviations CS, citrate synthase; dw, dry weight; IMF, intramyofibrillar; MHC, myocin heavy chain; mtDNA, mitochondrial DNA; OXPHOS, oxidative capacity; SS, subsarcolemmal; TEM, transmission electron microscopy; ww, wet weight.

Introduction

A common experimental approach in bioenergetic research is the determination of mitochondrial content. Mitochondrial volume or content is an important quantitative indicator of oxidative capacity and is often used to normalize global measures of muscle bioenergetic capacity. Even though it is well established that the mitochondrial population exists in a three-dimensional network (Ogata & Yamasaki, 1997), two-dimensional imaging using transmission electron microscopy (TEM) is still regarded as the golden standard for measuring mitochondrial fractional area (mitochondrial content). Since the TEM technique is time consuming and may not be available for many laboratories, biochemical measures of mitochondrial proteins, lipids, enzyme activities and DNA have often been used as surrogate measures of mitochondrial content (biomarkers). However, it is not known which of these commonly used markers of mitochondrial content has the strongest association with a morphological measure of the actual mitochondrial content.

During the past two decades there has been an increased interest in determining the intrinsic functional properties of mitochondria. Studies on isolated mitochondria or permeabilized muscle fibres have shown that acute exercise (Fernström *et al.* 2004), high fat diet (Anderson *et al.* 2009), type 2 diabetes (Phielix *et al.* 2008), chronic obstructive pulmonary disease (Naimi *et al.* 2011) and obesity (Anderson *et al.* 2008) may change the intrinsic properties of the mitochondria. Intrinsic changes are most often determined by normalizing the functional measure of interest to a marker of mitochondrial content. The most commonly used markers of mitochondrial content are citrate synthase (CS) activity (Mogensen *et al.* 2006a; Boushel *et al.* 2007; Fernström *et al.* 2007; Rabol *et al.* 2009a, b), mitochondrial DNA (mtDNA) (Boushel *et al.* 2007; Phielix *et al.* 2008), cardiolipin (Ritov *et al.* 2006) and activity of cytochrome *c* oxidase (COX) (Picard *et al.* 2011). The array of diverse biomarkers currently used for normalization may explain some of the controversies that exist in some of the above-mentioned areas. Hence, studies have also shown that the intrinsic mitochondrial functionality is not altered with acute

exercise (Molnar *et al.* 2006), type 2 diabetes (Boushel *et al.* 2007; Hey-Mogensen *et al.* 2010) and obesity (Ara *et al.* 2011). Thus, a determination of which biomarker of mitochondrial content is the most appropriate and valid to use is highly warranted.

The purpose of the present study was to evaluate the association and validity of CS activity, cardiolipin content, mtDNA, complex I–V protein content and complex I–IV activity as biomarkers of mitochondrial content. A secondary aim was to repeat these association analyses to investigate which of these biochemical measures could be used as a marker of muscle oxidative capacity (OXPHOS). OXPHOS was determined as the maximal coupled respiration (state 3) in permeabilized fibres.

Methods

Ethical approval

Subjects were informed orally and in writing about the purpose of the study, experimental procedures, and all its potential risks prior to providing written consent to participate. The study was approved by the local ethics committee of Frederiksberg and Copenhagen municipality (H-D-2007-0026) and was performed in accordance with the *Declaration of Helsinki*.

Subject characteristics

Sixteen subjects were recruited for this study. All subjects were healthy non-smoking males, who were not taking any medication nor had any known family history of type 2 diabetes, severe obesity, or cardiovascular diseases. In order to increase the sensitivity of the statistical analysis the recruited subjects represented a large range of physical training levels and accordingly were assumed to exhibit large variations in mitochondrial content.

Participants reported to the laboratory on two occasions. On the first occasion subjects completed an incremental cycle exercise test to determine maximal oxygen uptake ($\dot{V}_{O_{2peak}}$) (Oxycon Pro, Jaeger, Würzburg, Germany) and a dual-energy X-ray absorptiometry scan (Lunar Prodigy Advance; Lunar, Madison, WI, USA) for determination of body composition. On the second

occasion, subjects reported to the laboratory after a 12 h fast. After 30 min rest, a blood sample was taken for later analysis of plasma concentrations of glucose (Larsen *et al.* 2009), insulin and lipids (Larsen *et al.* 2011). A biopsy was taken from the vastus lateralis muscle under local anaesthesia (3–5 ml 5% lidocaine (lignocaine)) followed by 20 min rest. Thereafter subjects completed a graded cycle test to determine maximal whole body lipid oxidation (Achten *et al.* 2002).

Preparation of muscle biopsies

The muscle biopsy (approximately 200 mg) was divided into three parts. One part was prepared for respiratory measurements in saponin-permeabilized fibres, another part of the biopsy was prepared for TEM, and a third part was snap frozen in liquid nitrogen and stored at -80°C until further analysis of enzyme activities, protein content (western blotting), genomic and mitochondrial DNA content, and skeletal muscle lipids.

Mitochondrial respiratory protocols

Permeabilized fibres were prepared according to the previously described protocol (Kunz *et al.* 1993). Respiratory rates were measured using high resolution respirometers (Oxygraph-2k; Oroboros, Innsbruck, Austria) in MiR05 buffer (containing 110 mmol l^{-1} sucrose, 60 mmol l^{-1} potassium lactobionate, 0.5 mmol l^{-1} EGTA, 3 mmol l^{-1} $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 20 mmol l^{-1} taurine, 10 mmol l^{-1} KH_2PO_4 , 20 mmol l^{-1} Hepes, 1 g l^{-1} BSA, pH 7.1) at 37°C . All respiratory protocols were made in duplicates. Protocols 1–3, 4 and 5 were modified from Larsen *et al.* 2011, Larsen *et al.* 2012 and Rabol *et al.* 2010, respectively.

Protocol 1. L-Malate (2 mmol l^{-1}), ADP-Mg (5 mmol l^{-1}), glutamate (10 mmol l^{-1}) (GM_3) and cytochrome *c* ($10\text{ }\mu\text{M}$, for validation of the intactness of the outer mitochondrial membrane) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP titration in steps of 0.25 mmol l^{-1} , to induce maximal uncoupled respiration) (GM_{3u}).

Protocol 2. L-Malate (2 mmol l^{-1}), ADP-Mg (5 mmol l^{-1}), glutamate (10 mmol l^{-1}) and succinate (10 mmol l^{-1}) (GMS_3), followed by cytochrome *c* ($10\text{ }\mu\text{M}$) and finally FCCP titration (GMS_{3u}).

Protocol 3. L-Malate (2 mmol l^{-1}), ADP-Mg (5 mmol l^{-1}) and octanoyl-L-carnitine (1.5 mmol l^{-1}) (Oct_3), followed by cytochrome *c* ($10\text{ }\mu\text{M}$) and finally FCCP titration (Oct_{3u}).

Protocol 4. ADP-Mg (5 mmol l^{-1}), cytochrome *c* ($10\text{ }\mu\text{M}$), antimycin A ($2.5\text{ }\mu\text{M}$) followed by ascorbate

(2 mmol l^{-1}) and *N,N,N',N'*-tetramethyl-1,4-benzenediamine dihydrochloride (TMPD, $500\text{ }\mu\text{M}$) (TMPD + Asc).

Protocol 5. L-Malate (2 mmol l^{-1}), glutamate (10 mmol l^{-1}), pyruvate (5 mmol l^{-1}) and ADP-Mg (5 mmol l^{-1}) (PGM_3). Succinate (10 mmol l^{-1}) (PGMS_3) and octanoyl-L-carnitine (1.5 mmol l^{-1}) (PGMSOct_3). Finally, cytochrome *c* ($10\text{ }\mu\text{M}$) followed by oligomycin ($2\text{ }\mu\text{g ml}^{-1}$) to induce State 4 respiration (state 4o).

Tissue fixation and processing for TEM

Muscle biopsies were fixed as previously described (Nielsen *et al.* 2010). Briefly, biopsy specimens were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) for 24 h and subsequently rinsed four times in 0.1 M sodium cacodylate buffer. Following rinsing, muscle specimens were post-fixed with 1% osmium tetroxide and 1.5% potassium ferrocyanide ($\text{K}_4\text{Fe}(\text{CN})_6$) in 0.1 M sodium cacodylate buffer for 60 min at 4°C , and, after rinsing 3 times in H_2O , with 1% uranyl acetate in H_2O . After post-fixation, the muscle specimens were rinsed twice in H_2O at 4°C , dehydrated through a graded series of alcohol at $4\text{--}20^{\circ}\text{C}$, infiltrated with graded mixtures of propylene oxide (substituted by acetone in 2 samples) and Epon at 20°C , and embedded in 100% Epon at 30°C . In order to obtain as many fibres as possible, the ultra-thin sections were cut (using a Leica Ultracut UCT ultramicrotome; Rowako AB, Vendelsö, Sweden) in three depths separated by 150 nm. The sections were contrasted with uranyl acetate and lead citrate, and examined and photographed in a pre-calibrated Philips EM 208 electron microscope and a Megaview III FW camera (both FEI Company, Eindhoven, the Netherlands).

Stereological methods

In the sections from the three depths of each biopsy, all the longitudinally oriented fibres were included obtaining a mean of seven fibres per biopsy (range: 5–9). Intermyofibrillar (IMF) mitochondrial area and subsarcolemmal (SS) mitochondrial area per fibre surface area were estimated by point counting and direct fibre length measurement (Weibel, 1980) using 24 images obtained at $\times 20,000$ magnification in a randomized systematic order including 12 from the SS region and 6 from both the superficial and central region of the myofibrillar space. Total (IMF + SS) mitochondria was determined by recalculating SS mitochondria to a volume density by the formula: Volume beneath the surface area of a cylindrical-shaped fibre (V_b) = $R \times 0.5 \times A$, where R is fibre radius and A is the fibre surface area. Fibre radius

was assumed to be $40\ \mu\text{m}$. Two representative images of $\times 20,000$ magnification are presented in Fig. 1A and B.

IMF and SS mitochondria cristae surface areas were estimated by intersection and point counting (Weibel, 1980; Gundersen *et al.* 1988) in an average of 31 (range: 12–63) mitochondria per biopsy photographed at $\times 80,000$ magnification. One representative image of $\times 80,000$ magnification is presented in Fig. 1C. Samples

from three subjects failed quality control. Mitochondrial content and cristae surface area was therefore only determined in thirteen subjects.

CS activity

CS activity was measured as previously described (Andersen *et al.* 2003), with one minor change: a

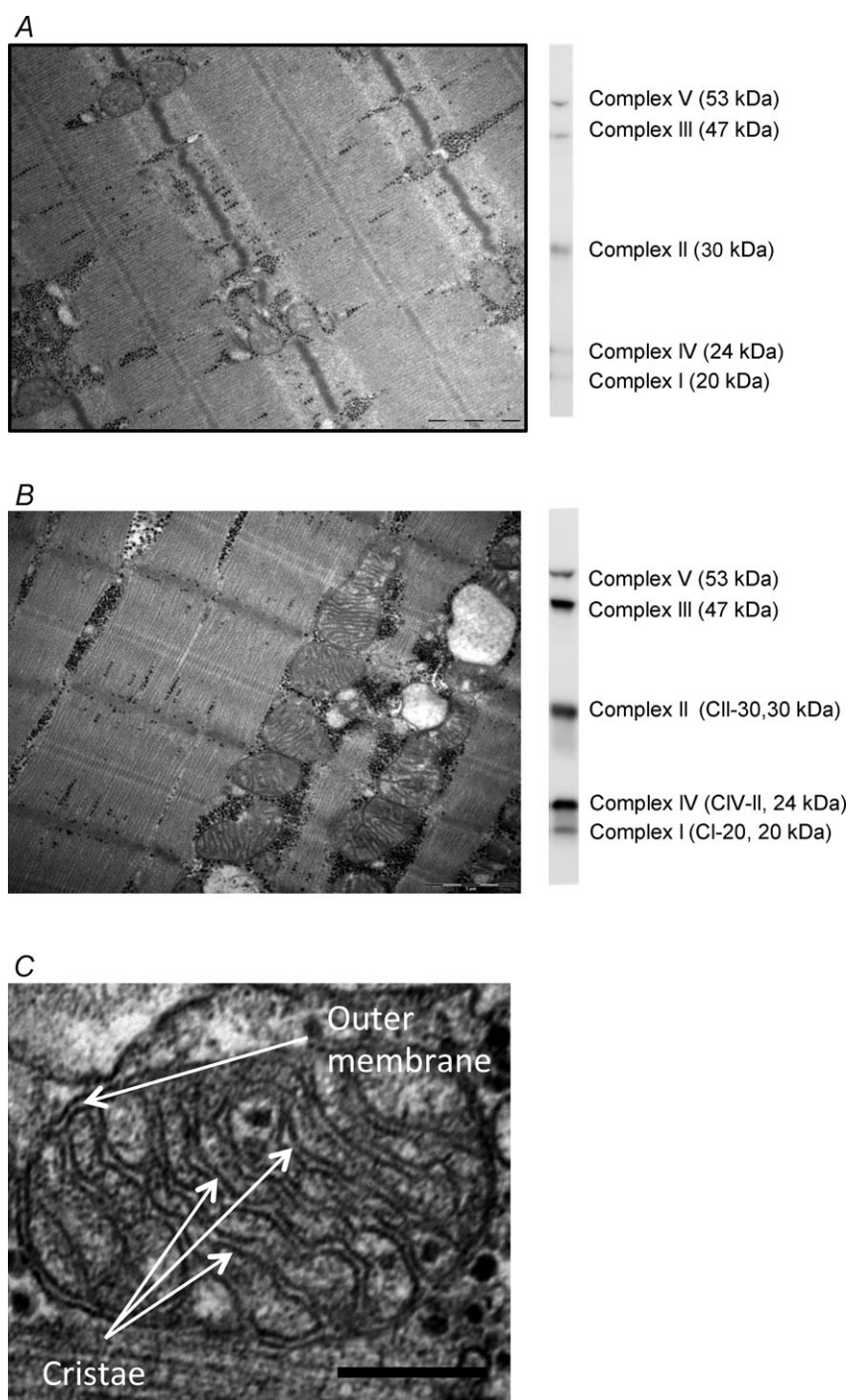


Figure 1. Representative electron microscopy images and western blots of complex I–V

Images and blots are from the two subjects with the lowest mitochondrial content (A) and second highest mitochondrial content (B). C, representative image used for measuring cristae surface area. A and B are $\times 20,000$ magnification; scale bars are $1\ \mu\text{m}$. C is $\times 80,000$ magnification and scale bar is $200\ \text{nm}$. Arrows shows examples of outer membrane and cristae.

TissueLyser II (Qiagen, Hilden, Germany) was used to make the homogenate. Enzyme activity was determined per gram wet weight (ww).

Complex I–V protein content and myosin heavy chain distribution

The homogenates for CS analysis were also used for western blotting analysis of complex I–V (Mitoprofile Human Total OXPHOS Human WB Antibody Cocktail; Mitosciences, USA) and quantification of myosin heavy chain (MHC) composition as previously described (Andersen & Aagaard, 2000; Larsen *et al.* 2011). Two representative blots of complex I–V are presented in Fig. 1A and B.

Activity of electron transport chain complex I–IV

Preparation of muscle tissue homogenates and enzyme activity measures of electron transport chain complex I–IV was carried out as previously described (Wibrand *et al.* 2010). Enzyme activity was determined at 37°C in a Varian Cary 100 Bio spectrophotometer equipped with a thermostatable multicell holder.

Quantification of genomic and mitochondrial DNA content

Total DNA was precipitated from approximately 15 mg frozen tissue and copy numbers of both genomic and mitochondrial DNA per milligram wet tissue weight were determined as previously described (Kraunsoe *et al.* 2010).

Quantification of skeletal muscle cardiolipin content

Lipids were extracted from approximately 3 mg freeze-dried muscle tissue (dw) using chloroform/methanol (Bartels *et al.* 2002). Cardiolipin was quantified with a thin-layer chromatography-based method as previously described (Ruiz & Ochoa, 1997; Bartels *et al.* 2002; Nielsen *et al.* 2002).

Statistics

Pearson correlation coefficients were calculated to investigate the correlation between the absolute values. To investigate the association in the relative variation a Lin's concordance test was performed (Lin, 1989). Lin's concordance coefficient (R_c) defines how well the relationship between two variables is represented by a line through the origin at an angle of 45 deg (slope = 1). Lin's scale is between 0 and 1 where 0.21–0.40 shows a fair concordance, 0.41–0.60 a moderate concordance,

Table 1. Subject characteristics

Subjects characteristics ($n = 16$)	mean \pm SEM	Range
Age (years)	24.3 \pm 0.9	20–32.6
BMI	23.4 \pm 0.5	19.9–27.5
Fat mass (%)	14.0 \pm 1.7	4.2–30.6
$\dot{V}_{O_2\text{peak}}$ (ml min ⁻¹ kg ⁻¹)	50.0 \pm 2.4	29.9–71.6
Maximal lipid oxidation (g min ⁻¹)	0.34 \pm 0.03	0.19–0.58
Plasma glucose (mmol l ⁻¹)	5.3 \pm 0.1	4.6–5.8
Insulin (pmol l ⁻¹)	21.5 \pm 2.3	11.0–37.7
Cholesterol (mmol l ⁻¹)	3.9 \pm 0.2	2.0–5.6
NEFA (μ mol l ⁻¹)	321 \pm 37	94–580
HDL (mmol l ⁻¹)	1.3 \pm 0.1	0.6–1.8
LDL (mmol l ⁻¹)	2.3 \pm 0.2	1.1–4.0
MHC I (%)	49.9 \pm 4.0	22.8–78.7
MHC IIa (%)	43.0 \pm 2.8	21.3–62.3
MHC IIx (%)	7.2 \pm 1.8	0–20.3

BMI: body mass index. $\dot{V}_{O_2\text{peak}}$: maximal oxygen uptake per kg body weight. NEFA: plasma non-esterified free fatty acids. HDL: plasma high density lipoproteins. LDL: plasma low density lipoproteins. MHC: myosin heavy chain isotype I, IIa and IIx.

0.61–0.80 a substantial concordance and 0.81–1.00 an almost perfect concordance (Lin, 1989). This analysis was performed using relative data (individual values were related to one particular subject values in all measures. This particular subject had the median value in the mitochondrial content measures). Only the biomarkers that were significantly correlated (Pearson correlation coefficient, $P < 0.05$) to the mitochondrial content, cristae surface area or OXPHOS were included for calculating Lin's R_c . Only the biomarkers that had R_c above 0.61 ('a substantial concordance') were considered as valid biomarkers of mitochondrial content or OXPHOS. Comparison of different respiratory states was analysed using a one-way ANOVA with repeated measures. Data are presented as mean \pm SEM. Significance was accepted at $P < 0.05$.

Results

Subject characteristics

In accordance with the predetermined recruitment criteria subjects showed large variation in $\dot{V}_{O_2\text{peak}}$ and maximal lipid oxidation, and had normal plasma glucose and insulin concentrations (Table 1). Mitochondrial fractional area ranged from 4 to 15% of the cellular volume (Table 2) and $\dot{V}_{O_2\text{peak}}$ ranged from 29.9 to 71.6 ml min⁻¹ kg⁻¹ (Table 1).

Respiration in permeabilized fibres

The different respiratory rates can be seen in Fig. 2. As expected, respiration activated with TMPD + Asc

Table 2. Correlation matrix showing the Pearson correlation coefficient (*r*) and the related *P* value

	Range	Mitochondrial content			Total cristae area			GMS ₃			PGMSOct ₃		
		<i>r</i>	<i>P</i>	<i>R_c</i>	<i>r</i>	<i>P</i>	<i>R_c</i>	<i>r</i>	<i>P</i>	<i>R_c</i>	<i>r</i>	<i>P</i>	<i>R_c</i>
Mitochondrial fractional area ($\mu\text{m}^2 \mu\text{m}^{-2}$)	0.04–0.15				0.93	<0.001	0.91	0.81	0.001	0.58	0.65	0.03	0.56
Total cristae surface area ($\text{m}^2 \text{cm}^{-2}$)	1.3–6.1							0.75	0.003	0.49	0.66	0.03	0.50
GMS ₃ ($\text{pmol s}^{-1} \text{mg}^{-1}$)	51–115										0.58	0.03	0.42
PGMSOct ₃ ($\text{pmol s}^{-1} \text{mg}^{-1}$)	46–93												
Cardiolipin ($\mu\text{g} (\text{mg dw})^{-1}$)	1.8–7.1	0.86	<0.001	0.85	0.73	0.005	0.67	0.70	0.003	0.58	0.50	0.07	
CS ($\mu\text{mol g}^{-1} \text{min}^{-1}$)	76–233	0.84	<0.001	0.80	0.72	0.005	0.64	0.66	0.006	0.58	0.52	0.055	
Complex I activity (U (g ww^{-1}))	5.6–18.2	0.78	0.002	0.77	0.76	0.003	0.71	0.52	0.048	0.41	0.34	0.25	
Complex II protein content (AU)	0.59–2.60	0.73	0.005	0.72	0.72	0.006	0.71	0.48	0.06		0.55	0.04	0.49
Complex IV activity (U (g ww^{-1}))	48–176	0.79	0.001	0.71	0.75	0.003	0.64	0.69	0.004	0.64	0.34	0.25	
Complex V protein content (AU)	0.40–2.24	0.74	0.004	0.69	0.75	0.003	0.70	0.65	0.006	0.54	0.59	0.03	0.48
Complex II activity (U (g ww^{-1}))	4.8–17.2	0.73	0.004	0.67	0.63	0.02	0.54	0.68	0.005	0.63	0.33	0.27	
Complex III activity (U (g ww^{-1}))	16–70	0.70	0.01	0.60	0.62	0.02	0.52	0.64	0.01	0.59	0.39	0.19	
SCR act. (U (g ww^{-1}))	6.8–25.9	0.69	0.01	0.60	0.59	0.03	0.56	0.55	0.03	0.43	0.30	0.31	
Complex III protein content (AU)	0.07–2.07	0.61	0.03	0.47	0.59	0.03	0.48	0.56	0.02	0.39	0.44	0.11	
Complex IV protein content (AU)	0.09–1.63	0.55	0.05	0.44	0.62	0.03	0.50	0.53	0.03	0.40	0.38	0.18	
mtDNA (copies (mg ww^{-1}))	8.32×10^7 – 34.6×10^7	0.35	0.23		0.31	0.30		0.46	0.07		0.50	0.07	
Complex I protein content (AU)	0.1–1.24	0.19	0.54		0.30	0.32		0.22	0.42		0.27	0.36	

If the association was significantly correlated ($P < 0.05$) the Lin's concordance coefficient (R_c) is also shown. Correlations are between total mitochondrial volume, total cristae area, ADP-stimulated fibre respiration using glutamate + malate + succinate as substrate (GMS₃) or ADP-stimulated fibre respiration using pyruvate + glutamate + malate + succinate + octanoyl-L-carnitine (PGMSOct₃), and the potential biomarkers of mitochondrial volume. The Lin's scale is defined as follows: 0.21–0.40 shows a fair concordance, 0.41–0.60 a moderate concordance, 0.61–0.80 a substantial concordance and 0.81–1.00 an almost perfect concordance. AU, arbitrary units; ww, wet weight; dw, dry weight; SCR act., succinate-cytochrome *c* reductase activity.

(direct electron donor to complex IV) showed the highest respiratory rate followed by uncoupled respiration using both reduced form of nicotinamide-adenine dinucleotide (NADH)- and the reduced form of flavin adenine dinucleotide (FADH₂)-generating substrates (GMS_{3u}). There was no statistical difference between GMS₃, PGMS₃ and PGMSOct₃, suggesting that GMS provides sufficient NADH and FADH₂ to reach maximal coupled respiration. The integrity of the outer mitochondrial membrane was assessed by addition of cytochrome *c* in protocols 1–3 and 5. No effect of this addition was seen (data not shown).

Correlation between the biomarkers and the mitochondrial fractional area

Several biomarkers showed a strong correlation and high concordance with mitochondrial fractional area. The correlation coefficients, *P* values and Lin concordance coefficients can be seen in Table 2 where the biomarkers are listed according to their R_c rank to the mitochondrial fractional area. Cardiolipin content

had the strongest correlation and highest R_c value of 0.85 defined as an 'almost perfect concordance' (Lin, 1989; Fig. 3A). CS activity showed the second highest correlation and concordance (Fig. 3B). CS activity, complex I, II and IV activity and complex II and V protein content all had R_c values defined as a 'substantial concordance'. Interestingly there was no correlation between mtDNA content and mitochondrial fractional area ($r = 0.35$, $P = \text{n.s.}$) (Fig. 3G). In contrast there was a very strong correlation between fibre respiration using TMPD + Asc and mitochondrial fractional area ($r = 0.94$, $P < 0.001$) (Fig. 3H). The R_c was 0.74 which is defined as a 'substantial concordance' ($R_c = 0.74$).

Correlation between the biomarkers and mitochondrial cristae surface area

There was a strong correlation between cristae surface area and mitochondrial fractional area ($r = 0.93$, $P < 0.001$). The Lin's concordance test showed an almost perfect fit

($R_c = 0.91$) suggesting that the relative variation in these two values, in this group of subjects, is closely associated.

The cristae surface area was significantly correlated with the same biomarkers as the mitochondrial content (Table 2). The biomarker that showed the strongest concordance with cristae surface area was complex I activity ($R_c = 0.71$) and complex II protein content ($R_c = 0.71$), followed by complex V protein content ($R_c = 0.70$), cardiolipin content ($R_c = 0.67$), CS activity ($R_c = 0.67$) and complex IV activity ($R_c = 0.64$). As can be seen in Table 2, the concordance was generally lower between the biomarkers and the cristae surface area when compared with mitochondrial surface area.

Correlation between the biomarkers and mitochondrial respiratory capacity

In order to investigate biomarkers of muscle OXPHOS, the highest coupled respiratory state (GMS₃) and the

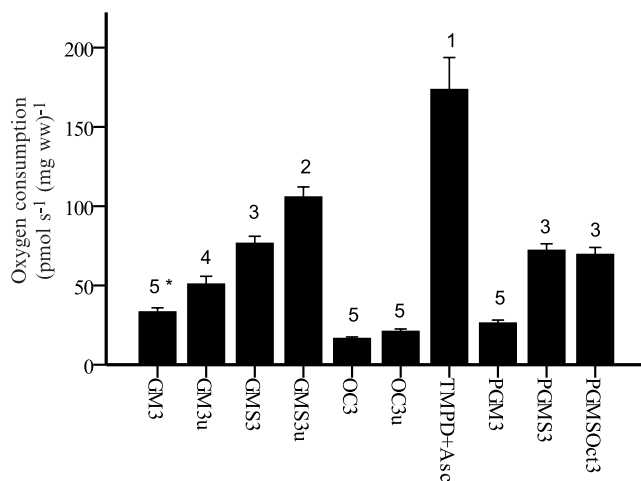


Figure 2. Fibre respiration

Oxygen consumption ($\text{pmol s}^{-1} (\text{mg ww})^{-1}$) in permeabilized fibres.

GM₃: glutamate + malate + ADP-Mg; GM_{3u}:

glutamate + malate + ADP-Mg + FCCP; GMS₃:

glutamate + malate + ADP-Mg + succinate; GMS_{3u}:

glutamate + malate + ADP-Mg + succinate + FCCP; Oct₃:

L-malate + octanoyl-L-

carnitine + ADP-Mg; Oct_{3u}:

malate + octanoyl-L-carnitine + ADP-Mg + FCCP; TMPD:

ADP-Mg + cytochrome c + anti-

mycin A + ascorbate + *N,N,N',N'*-tetramethyl-1,4-benzenediamine

dihydrochloride; PGM₃: pyruvate + malate + glutamate + ADP-Mg;

PGM_{3u}: pyruvate + glutamate + malate + ADP-Mg + succinate;

PGM_{3SOct3}:

pyruvate + glutamate + malate + ADP-Mg + succinate + octanoyl-L-

carnitine. Numbers denote the statistical rank using one-way

ANOVA with repeated measures, i.e. values denoted with the

number 3 are significantly lower than values denoted with the

number 2 and significantly higher than the values denoted with the

number 4. * $P = 0.06$ compared with GM_{3u}. Values are mean \pm SEM.

respiratory rate with the maximal substrate supply (PGMSOct₃) were chosen as measures of muscle OXPHOS (Table 2). GMS₃ had a strong correlation with mitochondrial fractional area ($r = 0.81$). The correlation between PGMSOct₃ and mitochondrial fractional area was also significant ($r = 0.65$) but not as strong as with GMS₃. GMS₃ was significantly correlated to several biomarkers showing the highest concordance to complex IV activity ($R_c = 0.64$), and complex II activity ($R_c = 0.63$) both defined as a 'substantial concordance'. Only complex II protein content ($R_c = 0.49$) and complex V protein content ($R_c = 0.48$) were significantly correlated to PGMSOct₃, both defined as having a 'moderate concordance'.

Discussion

The main finding of the present study was that several biomarkers showed a strong correlation and high concordance with the mitochondrial fractional area. The biomarker with the strongest correlation and highest relative concordance was cardiolipin content, followed by CS and complex I activities. The cristae surface area was strongly correlated and showed a strong concordance with mitochondrial fractional area, suggesting that cristae surface area per mitochondrial fractional area does not vary much in skeletal muscle between subjects. Finally, we found that complex IV activity was the biomarker that had the highest concordance with muscle OXPHOS determined as state 3 respiration using glutamate, malate and succinate as substrates, followed by complex II activity, cardiolipin content and CS activity.

Mitochondrial fractional area and cristae surface area

In the present study we determined mitochondrial fractional area and cristae surface area using two-dimensional TEM as representative measures of mitochondrial content. We reasoned that cristae surface area may be a more robust measure of mitochondrial content than mitochondrial fractional area since many of the proteins and enzymes involved in substrate oxidation, electron transport and phosphorylation are attached to or imbedded in the inner mitochondrial membrane. Furthermore, the measure of cristae area is not affected by mitochondrial shrinking or swelling. In accordance with a previous study using cat skeletal muscle (Hoppeler *et al.* 1987), we found a very strong correlation and concordance between mitochondrial fractional area and cristae surface area (Table 2). However, in contrast to our hypothesis we found that the biomarkers and respiratory measures showed a stronger correlation and higher concordances with mitochondrial fractional area than

cristae surface area (Table 2). This could be due to the fact that the estimate of mitochondrial fractional area is based on a greater diversity of mitochondrial morphologies and therefore more representative of the muscle biopsy compared to the estimate of total cristae surface area. Indeed, cristae surface area was only estimated in a subset of mitochondria (12–63 of several hundreds of mitochondrial profiles) of which the cristae could be clearly identified.

Markers of mitochondrial content

In the present study we have shown that several biochemical measures have a strong correlation and high concordance with mitochondrial fractional area (mitochondrial content). This includes cardiolipin content ($R_c = 0.85$), CS activity ($R_c = 0.80$), complex I activity ($R_c = 0.77$), complex II protein content ($R_c = 0.72$), complex IV activity ($R_c = 0.71$), complex V

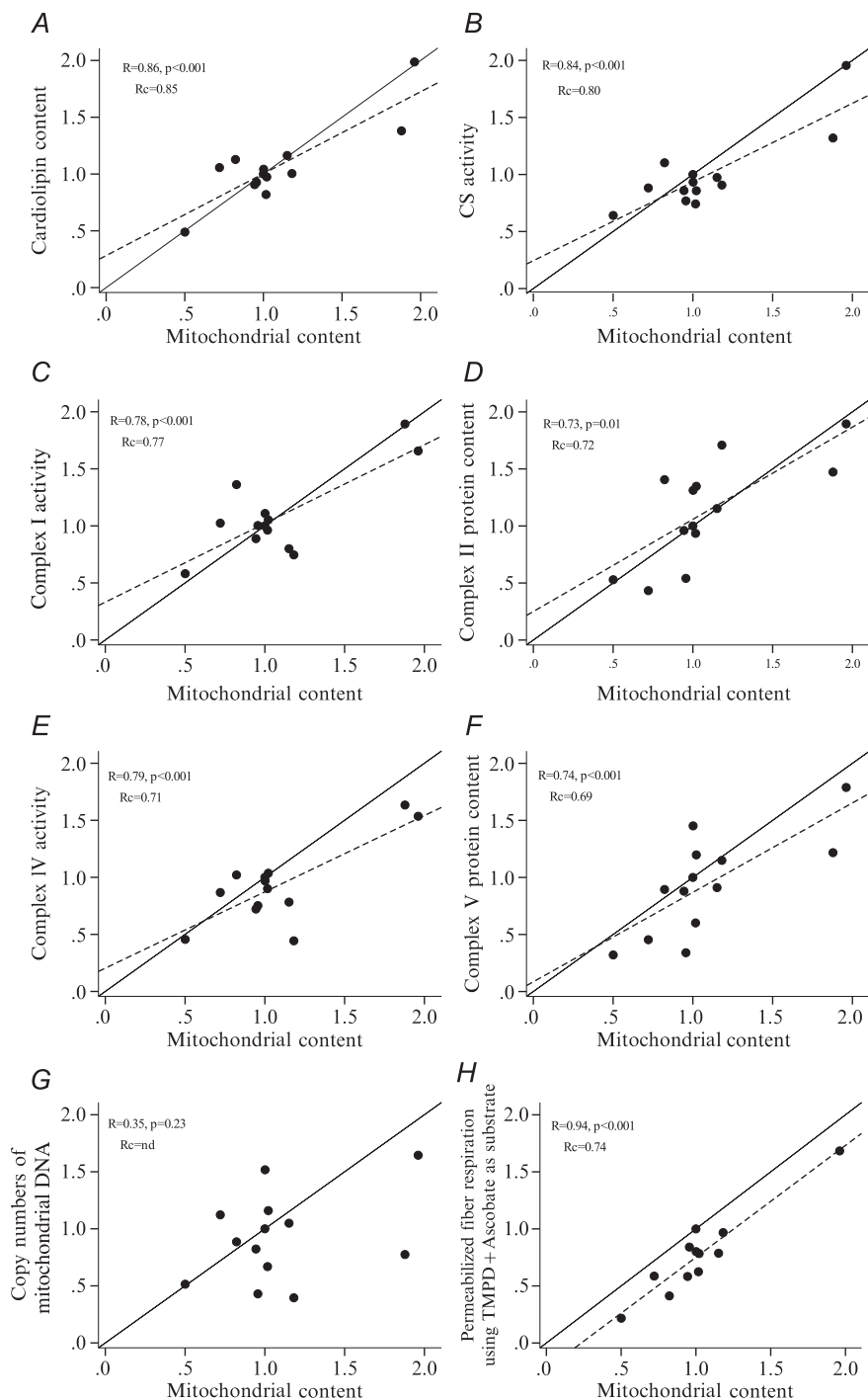


Figure 3. Correlation plots

Correlation between the relative variation of the mitochondrial content and the six biomarkers (A–F) of mitochondrial content that showed the highest Lin's concordance coefficient (R_c) and two biomarkers of certain interest (G and H).

TMPD + ascorbate are redox substrates feeding electrons directly to complex IV. Continuous lines represent the perfect linear fit (slope = 1) and dashed lines represent the actual linear fit. The linear fit was only shown when significance was present.

protein content ($R_c = 0.69$) and complex II activity ($R_c = 0.67$). These results validate the use of these biochemical measures as markers of mitochondrial content. However, depending on the experimental setup, the use of some markers may have advantages over others. If the purpose of the study is to use the biomarker as a denominator for calculating intrinsic mitochondrial functionality (i.e. respiration per mitochondrial content) it seems more appropriate to use non-enzymatic markers that are not involved in mitochondrial substrate oxidation and phosphorylation. In the present study two such measures were determined, cardiolipin and mtDNA content. Cardiolipin, which is a phospholipid located in the inner mitochondrial membrane, is not directly involved in substrate oxidation, electron transport or phosphorylation. However, it has been suggested to affect inner membrane proton leak (Hoch, 1998) and stabilizing the complexes in the electron transport chain (Pfeiffer *et al.* 2003). Previous studies have shown that cardiolipin content increases in human vastus lateralis after exercise training along with an increase in other markers of mitochondrial content (Menshikova *et al.* 2005, 2007). Thus, the present and previous studies show that cardiolipin is a very appropriate and valid biomarker of mitochondrial content.

In contrast, mtDNA content was not significantly correlated with mitochondrial content ($r = 0.35$, $P = 0.23$). This result was somewhat unexpected since mtDNA is used in various studies as a marker of mitochondrial content. Moreover, studies have shown a significant positive correlation between CS activity and mtDNA content in human vastus lateralis (Wang *et al.* 1999) and an increase in mtDNA content in response to training (Puntschart *et al.* 1995; Menshikova *et al.* 2006). However, studies have also shown that training does not increase mtDNA content in human (Menshikova *et al.* 2005; Pesta *et al.* 2011) and rodent skeletal muscle (Schultz & Wiesner, 2000) despite an increase in other markers of mitochondrial content. The inconsistency of these results is most likely due to the relatively low power used in these studies and the fact that mitochondrial DNA copy number can vary considerably (4–6 copies per cell) between individuals. Nevertheless, this further supports our finding that mtDNA content is a poor biomarker of mitochondrial content in skeletal muscle.

CS activity is a frequently used biomarker of mitochondrial content. Previous studies have validated its use as a marker of mitochondrial content showing a close association between the change in its activity and morphological change in mitochondrial content in response to electrically stimulated training in rabbit tibialis anterior muscle (Reichmann *et al.* 1985). Even though our data suggest that cardiolipin had a stronger correlation (cardiolipin content $r = 0.86$ vs. CS activity $r = 0.84$) and higher relative concordance (cardiolipin content $R_c = 0.85$

vs. CS activity $R_c = 0.80$) with mitochondrial content, taking into account the relatively low number of subjects and the variability in the measures, the small difference between these two biomarkers is not enough to claim that cardiolipin is superior to CS activity as a biomarker of mitochondrial content. However, if cardiolipin content and CS activity are to be used as a nominator for calculating intrinsic mitochondrial functionality, the use of cardiolipin content has several advantages over CS activity. In contrast to cardiolipin, CS activity can be acutely regulated by exercise (Tonkonogi *et al.* 1997). Furthermore, CS activity may contribute to the oxidation of substrates in some respiratory protocols. The advantage, however, is that CS activity is easy to measure and it can be determined in both whole tissue, in isolated mitochondrial-rich solution, and in the same solution that has been used for measuring mitochondrial respiration (Mogensen *et al.* 2006b).

An interesting finding was that there was a very strong correlation between mitochondrial content and the respiratory rate in permeabilized fibres when using TMPD + ascorbate as substrate ($r = 0.94$, $P < 0.001$, Fig. 3H). The advantage of using TMPD + ascorbate respiration in permeabilized fibres as a marker of mitochondrial content is that it can be performed on the same day as the other respiratory measures, avoiding the potential effects of long-term storage of the muscle sample (Pache & Reichmann, 1990). But most importantly, unless inhibitors of COX activity have been used, TMPD + ascorbate respiration can always be determined as the final step in a respiratory protocol. In this way, the functional measure and the measure of the mitochondrial content can be determined in the same tissue preparation. However, the disadvantage is that regardless of which protocol is being used, complex IV is always part of the mitochondrial substrate oxidation. Furthermore, when using TMPD + ascorbate as substrate the respiratory rate does not reach a steady state but rather it displays a spiked response, which can be difficult to interpret.

Biomarkers of mitochondrial oxidative capacity

A secondary purpose of the present study was to investigate which biochemical measure could be used as a biomarker of muscle OXPHOS. Muscle OXPHOS was defined as the maximal coupled respiration in permeabilized fibres. This was accomplished when using glutamate, malate and succinate as substrates. Several biochemical measures showed a strong correlation with GMS₃ but only complex IV activity ($R_c = 0.64$) and complex II activity ($R_c = 0.63$) had R_c values above 0.61 (Table 2). From our point of view complex IV activity is the most appropriate marker of muscle OXPHOS capacity, since the correlation between GMS₃ and complex II activity could be due

to the experimental conditions where succinate exerts a high control over the GM_3 respiration and is the substrate for the complex II protein. Furthermore, in contrast to complex II activity, complex IV activity was also significantly correlated with the respiratory states where succinate was not used as substrate (GM_3 $r = 0.53$, $P = 0.04$; Oct_3 $r = 0.54$, $P = 0.04$).

Conclusion

The purpose of the present study was to investigate which of the most commonly used markers of mitochondrial content had the strongest correlation with a morphological determination of the actual mitochondrial content in skeletal muscle of healthy young human subjects. We conclude the following: (1) Cardiolipin has the strongest association with the mitochondrial content and is the most valid biomarker for investigating changes in mitochondrial content and for calculating intrinsic mitochondrial functionality. (2) CS activity is also strongly associated with the mitochondrial content. The advantage of CS activity is that it is the most commonly used marker of mitochondrial content and it is easy to measure. The disadvantage is that in some functional measures the CS enzyme is involved in mitochondrial substrate oxidation. (3) mtDNA was not associated with the mitochondrial content and is therefore regarded as a poor biomarker of mitochondrial content. (4) Complex II activity and complex IV activity were strongly associated with the muscle OXPHOS determined as the maximal coupled respiratory capacity in permeabilized fibres. Complex IV activity is the most valid biomarker of muscle OXPHOS due to its consistent correlation with many of the respiratory measures in diverse protocols.

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Author contributions

The sample collection, whole body analysis, respiratory analysis in permeabilized fibres, and western blots were performed at the Center for Healthy Aging—Department of Biomedical Science, Copenhagen University, Denmark. Measures of cardiolipin content were performed in the Department of Clinical Biochemistry, Copenhagen University Hospital, Denmark. Measures of complex I–IV activity were performed at the Metabolic Laboratory, Department of Clinical Genetics, Copenhagen University Hospital, Denmark. Measures and analysis of transmission electron microscopy imaging were performed at the Department of Clinical Pathology, Odense University Hospital, Denmark and Institute of Sports Science and Clinical Biomechanics, University of Southern Denmark, Denmark. M.H.-M. contributed to the design of the experiments, the data collection, analysis and interpretation of the data, and article composition. S.L., N.S., F.D. and J.N.,

contributed to the design of the experiments, collection of data, interpretation of data and manuscript revision. R.B., J.W.H. and H.D.S. contributed to the design of the study, interpretation of data and manuscript revision. C.N.H., L.B.N. and F.W. contributed to the data collection, analysis and manuscript revision for important intellectual content. All authors approved the final version of this manuscript.

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