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Reduced mitochondrial density in the vastus lateralis muscle of patients with COPD.

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Short title: muscle mitochondrial density in COPD

ABSTRACT

Skeletal muscle dysfunction is a well-recognised hallmark of COPD, leading to exercise intolerance. The vastus lateralis of these patients is characterised by reduced mitochondrial enzyme activity, whereas this is not the case in the tibialis anterior. It is however unclear if the comprised oxidative capacity in the vastus is due to reduced mitochondrial volume density.

Therefore, muscle biopsies were obtained from the vastus lateralis of 6 COPD patients and 4 healthy age-matched controls and from the tibialis anterior of another 6 patients and 6 controls. Mitochondrial number, fractional area and morphometry, as well as Z-line width (as a surrogate marker of fibre type), were analysed using transmission electron microscopy.

Mitochondrial number (0.34 vs. 0.63 μm^{-2}) and fractional area (1.95% vs. 4.25%) were reduced in the vastus of COPD patients compared to controls, respectively. Despite a reduced mitochondrial number (0.65 vs. 0.88 μm^{-2}), mitochondrial fractional area was maintained in the tibialis.

It can be concluded that reduced mitochondrial fractional area is likely contribute to the decreased oxidative capacity in the vastus of COPD patients, whereas the maintained mitochondrial fractional area in the tibialis may explain the normal oxidative capacity.

Keywords

chronic obstructive pulmonary disease, electron microscopy, mitochondria, skeletal muscle, ultrastructure

INTRODUCTION

Peripheral muscle dysfunction is an established hallmark of chronic obstructive pulmonary disease (COPD). It severely affects patients' exercise tolerance leading to disablement and poor quality of life. Several underlying muscular metabolic derangements have been reported for the vastus lateralis muscles of patients with COPD, including a shift from oxidative type I (slow-twitch) towards glycolytic type II (fast-twitch) fibres [1, 2] and reduced activities of enzymes involved in oxidative energy metabolism [3, 4]. These enzymes, involved in the citric acid cycle and fatty acid β-oxidation, reside within the mitochondria. An increased mitochondrial fractional area (area%) has indeed been associated with an increased oxidative enzyme capacity in human skeletal muscle [5]. It can therefore be hypothesized that in COPD, the reduced oxidative enzyme capacity in the vastus lateralis is the consequence of a reduced mitochondrial area%. In COPD, the ultrastructure of muscle mitochondria has so far only been studied in the diaphragm [6]. As opposed to the vastus lateralis, the diaphragm of these patients is characterized by an increased oxidative capacity [7-9] and, strikingly, an augmented mitochondrial density was found as compared to healthy age-matched controls [6]. Likewise, because normal oxidative enzyme activities have been reported for COPD in another peripheral muscle, the tibialis anterior [10], a normal mitochondrial area% is anticipated in this muscle.

The aim of the current study was therefore to examine muscle mitochondria on the ultrastructural level in the vastus lateralis or tibialis anterior muscle of patients with COPD as compared to healthy subjects. For this, muscle biopsies were obtained and mitochondrial morphology, number and area%, along with mitochondrial morphometry were analyzed using transmission electron microscopy. Since alterations in mitochondrial area% may result in

disturbances in fuel stores a secondary aim was to examine glycogen and lipid droplets at the ultrastructural level.

METHODS

Twelve patients with mild to severe airflow obstruction and 10 healthy control subjects were studied (table 1); from 6 patients and 4 controls a vastus lateralis biopsy was obtained and a biopsy from the tibialis anterior was obtained from another 6 patients versus 6 controls. All patients had COPD according to the GOLD criteria [11]. Furthermore, patients had irreversible obstructive airway disease (less than 10% improvement of FEV_1 predicted baseline after β_2-agonist inhalation). They were in clinically stable condition and not suffering from a respiratory tract infection or an exacerbation of their disease at least 4 weeks prior to the study, they were one maintenance dose of inhaled steroids and β_2-agonist and did not receive systemic steroids. Exclusion criteria were malignancy, asthma, bronchiectasis, cardiac failure, distal arteriopathy, recent surgery, severe endocrine, hepatic or renal disorders and use of anticoagulant medication. The control subjects were healthy age-matched volunteers. Written informed consent was obtained from all subjects and the study was approved by the medical ethical committee of the University Hospital Maastricht (Maastricht, The Netherlands), consistent with the Helsinki Declaration.

Body composition

Body height and weight were assessed. Whole-body fat-free mass (FFM) was determined by bioelectrical impedance (Xitron 4000b, Xitron technologies, San Diego, California, USA) as

described previously [12]. Weight parameters were adjusted for body surface, resulting in the body mass index (BMI) and FFM index (FFMI).

Pulmonary function tests

Spirometry was used to determine, amongst others, the FEV_1, with the highest value from at least three technically acceptable assessments being used. Diffusion capacity for carbon monoxide was measured by using the single-breath method (Masterlab, Jaeger, Wurzburg, Germany). All values obtained were expressed as percentage of the predicted value [13].

Muscle biopsy and electron microscopic evaluation

Postabsorptive muscle biopsies were obtained under local anaesthesia under resting conditions. Vastus lateralis biopsies were obtained by the needle biopsy technique [14]. Tibialis anterior biopsies were obtained using a conchotome [15]. The specimens were immediately fixed in 0.1 M phosphate buffer with 2.5% glutaraldehyde at pH 7.4 and stored dark and cool in the fixation buffer. For electron microscopy, specimens were rinsed in 0.1 M phosphate buffer and post-fixed in the fixation buffer supplemented with 1% osmiumtetroxide for 1 hr, dehydrated through a graded ethanol series and embedded in epoxy resin (Glycid ether 100, Serva, Heidelberg, Germany). Appropriate locations and fibre longitudinal orientation were evaluated in toluidine blue-stained semi-thin sections from the central region of each biopsy. Ultra-thin sections from the selected areas were contrasted with uranyl acetate and lead citrate and viewed with a Philips CM 100 transmission electron microscope. Using this staining protocol mitochondria will appear as spherical-round shaped electron dense (dark grey-black objects). Mitochondrial analysis was based on the basics described previously [5, 16, 17]: Micrographs of randomly selected areas of central parts of muscle fibres were obtained at a final magnification of 1550-2650× and scanned at 2400 dpi or more.

Mitochondria were identified based on their electron dense (dark grey-black) appearance, shape and subcellular location (intermyofibrillar). A representative micrograph is shown in figure 1A. These digitized micrographs were analyzed with an interactive image analysis system (QWin v2.8, Leica Microsystems Imaging Solutions). All mitochondria entirely within the micrograph were included, 3-5 micrographs were analyzed so that at least 275 mitochondria per subject were examined. Mitochondria were outlined in an overlaying bitmap (see figure 1B for an example) which was used to compute the following parameters: least diameter, perimeter, and size (area). Subsequently, mitochondrial number was computed as the quantity of mitochondria in a given muscle fibre area. Mitochondrial area% was computed as the percentile mitochondrial area fraction of total fibre area. As a surrogate marker of muscle fibre typology [18], average z-line width was also determined for each micrograph: 10 randomly chosen straight z-lines were outlined in an overlaying bitmap and the mean width of these objects was measured. Furthermore, glycogen and lipid stores were evaluated semi-quantitatively in a blinded manner: the entire section was evaluated under the electron microscope for a general impression and then quantity scores (1-5) were assigned using the same digitized micrographs mentioned above; for each subject the scores from these micrographs were averaged.

Statistics

Data were analyzed using SPSS (Statistical Package for the Social Sciences, version 13.0 for Windows, SPSS Inc., Chicago, IL, USA). Differences between groups were analyzed with the Mann-Whitney U test and correlations with the Spearman correlation test. A two-tailed probability value of less than 0.05 was considered statistically significant. Data are presented as means ± SD.

RESULTS

Characteristics of patients and healthy controls are presented in table 1. Age and anthropometric parameters were not different between the two groups. In patients, the predicted value for FEV_1 varied between 22% and 84% and the FEV_1/FVC varied from 25% to 68%.

Electron microscopic analysis of mitochondria is presented in tables 2A (vastus lateralis biopsies) and 2B (tibialis biopsies). For both muscles, mean Z-line width, which is an indicator of fibre type, was similar between patients and controls, indicating that muscle fibre typology of the samples under examination was comparable and that the data shown are not biased by differences in muscle fibre typology. In the vastus lateralis, determinants of mitochondrial size (mitochondrial area, least diameter and perimeter) were also not different between the two groups, although there was one subject in the control group (control 1) with larger mitochondria compared to all the other subjects. Mitochondrial number as well as area% was lower in vastus lateralis muscle samples from COPD patients than from controls. Even when control 1 was omitted from the analysis, mitochondrial area% was still significantly lower in the patients ($p=0.039$). Interestingly, in tibialis samples, mitochondrial number was also lower in COPD compared to controls, whereas mitochondrial area% remained unaltered. Mitochondrial morphometry (size, diameter, and perimeter) revealed no significant differences between both groups, although mitochondrial size (area and least diameter) tended to be larger in the tibialis from COPD ($p=0.109$) as compared to controls.

In COPD, there were no significant correlations between mitochondrial area% and disease severity expressed as either the FEV_1 (figure 2) or the FEV_1/FVC (not shown). Nor where

there any significant correlations between these disease severity measures and mitochondrial number or with other mitochondrial dimensions (not shown).

Quantity scores for the presence of fuel stores are presented in table 3. The evaluation of glycogen and lipid stores in the vastus lateralis or tibialis samples revealed no significant differences between patients and healthy subjects.

DISCUSSION

The main finding of this study is the reduced mitochondrial number and mitochondrial area% in the vastus lateralis muscle of patients suffering from COPD compared to age-matched healthy controls. A reduced mitochondrial area% was, however, not observed in the tibialis anterior of these patients. This indicates that the reduced oxidative capacity in the vastus lateralis and the absence of this phenomenon in the tibialis anterior could very well be attributed to their respective mitochondrial area%.

In this study mitochondrial density was expressed either as the mitochondrial number or area%. The number is the amount of mitochondria located in a certain muscle fibre area, whereas the mitochondrial area% is the area fraction of mitochondria. In other words, a large number of small mitochondria can result in the same mitochondrial area% as a small number of large mitochondria. Moreover, changes in mitochondrial area% and not per se changes in their numbers will lead to changes in the mitochondrial capacity of the cell, reflected by altered activities of enzymes hosted by these organelles. The vastus lateralis of COPD patients is indeed characterized by reduced activities of mitochondrial enzymes involved in oxidative energy metabolism, such as citrate synthase and hydroxyacyl-CoA dehydrogenase [3, 4]. In

the current study, both the mitochondrial number and area% were reduced in the vastus lateralis of these patients and it is therefore very plausible that the observed reduced mitochondrial area% contributes to the reported reduced activities of oxidative enzymes. In contrast to the vastus lateralis, tibialis oxidative enzyme activities were found to be normal in COPD [10]. This further strengthens the concept that mitochondrial area% determines the oxidative capacity, because despite the fact that the mitochondrial number was reduced in the tibialis of COPD patients, the mitochondrial area% was found to be normal in this muscle.

The fact that tibialis mitochondrial area% in COPD patients was maintained despite reduced numbers can only be explained by the fact that the tibialis mitochondria tended to be larger in patients. If this is a compensatory mechanism and why this does not occur in the vastus lateralis remains unclear, but prone to speculations: The vastus lateralis is a muscle primarily involved in locomotion, whereas the tibialis anterior is a constantly-used posture muscle. Concomitant with their function, the tibialis anterior is composed of more fatigue-resistant type I fibres than the vastus lateralis [19]. It can be speculated that type I fibres are more capable to maintain mitochondrial area% than type II fibres. The key regulator of mitochondrial biogenesis is peroxisome proliferator-activated receptor-γ co-activator 1α (PGC1α). Its expression is highest in type I fibres [20] and its levels are therefore probably higher in the tibialis anterior than in the vastus lateralis. One of the target genes of PGC1α is mitofusin2, which, as its name indicates, plays in important role in the fusion of mitochondria and therefore plays in important role in mitochondrial maintenance [21]. Whether disturbed PGC1α and/or mitofusin expression in the vastus lateralis are indeed involved in the loss of mitochondrial area% needs further exploration.

The vastus lateralis muscles of patients with COPD are characterized by a I→IIA→IIX fibre type shift [2]. Whether fibre type distribution is also abnormal in the tibialis anterior of COPD patients is presently unknown. Nevertheless, from both muscles it is theoretically possible that for patients relatively less oxidative type I fibres were included in the analyses, resulting in the observed lower mitochondrial number. However, although it is not possible to identify fibre types from the electron microscopy images directly, based on the width of the z-lines it is possible to predict fibre types with about 83% accuracy (type I: 128 ± 10 nm; type IIA: 104 ± 8.5 nm; type IIX: 88 ± 9.1 nm) with no mismatching between types I and IIX [18]. In the current study, comparing the z-line widths of the vastus lateralis and the tibialis anterior illustrates that type I fibre proportions are indeed higher in the latter (although this was not done in the same subjects). The mean z-line width in the muscles studied was not different between patients and controls, indicating that any observed differences in mitochondrial density are, at least partly, independent of the fibre type distribution. We also previously showed that oxidative enzyme capacity within a specific fibre type is reduced in vastus lateralis biopsies of patients with COPD [2]. Even despite the fact that patients of the tibialis anterior analysis seemed more severely diseased based on the FEV_1 than those of the vastus lateralis analysis, mitochondrial area% is only reduced in the vastus lateralis. We therefore conclude that the vastus lateralis mitochondrial area% is reduced in COPD as compared to healthy subjects and that this by itself contributes to the loss of oxidative capacity in this muscle.

Reduced mitochondrial area% along with disturbed energy metabolism may lead to abnormal levels of glycogen and/or lipid stored in the muscle cell. The literature is not consistent regarding glycogen contents in peripheral muscle homogenates of COPD patients [22-24]. Recently, lipid content was examined histochemically in vastus lateralis muscle biopsies of

COPD patients and it was found to be decreased compared to healthy controls [25]. No electron microscopic data of intramuscular fuel stores has however been reported for limb muscles of COPD patients. In the current study, we found no significant differences in the quantity scores of lipid and glycogen stores between patients with COPD and healthy subjects, indicating that there was no severe depletion or accumulation of these fuels. It is however possible that the power of the current study design is not sufficient to discover more nuanced differences. Furthermore, abnormalities can be expected especially after acute exercise, when these fuels are being used, and the present biopsies were obtained in rest.

There is no golden standard for mitochondrial morphometry, but the basics are similar to our approach in that several micrographs were obtained from each sample and from these micrographs parameters such as mitochondrial count, size and area% are determined for example by point-counting using grids or by computerized morphometry as in the current report; mitochondrial volume densities and Z-lines widths reported in these studies are similar to the current findings [17, 18, 26, 27]. Two populations of mitochondria can be distinguished based on their subcellular localization; intermyofibrillar mitochondria are located between the contractile myofibrils and subsarcolemmal mitochondria are found adjacent to the sarcolemma facing the sarcoplasmic site [28]. It is currently not completely clear whether these subtypes have distinct functions within the muscle cell. It has however been suggested that subsarcolemmal mitochondria are involved in ATP production for membrane transport and cytoplasmic reactions, whereas intermyofibrillar mitochondria are more efficient at producing ATP which is used for muscle contraction [29]. Moreover, 75% of the mitochondria are located in the intermyofibrillar space [5]. For these and also some technical reasons, we focused on intermyofibrillar mitochondria in the current study.

In summary, we showed that the number of mitochondria is reduced in two limb muscles of patients with COPD. However, mitochondrial area% was reduced only in one muscle, namely the vastus lateralis. As mitochondria are the organelles in which most enzymes involved in oxidative metabolism reside, this loss of mitochondrial area% doubtlessly contributes to the impaired oxidative capacity in the vastus lateralis of patients with COPD. Therefore, to further unravel the molecular mechanism of impaired muscle oxidative capacity in COPD future studies should probably focus on key regulators of mitochondrial maintenance.

Table 1: Group characteristics

	Vastus lateralis biopsy		Tibialis anterior biopsy	
	Controls	COPD	Controls	COPD
N (male/female)	4 (3/1)	6 (4/2)	6 (6/0)	6 (6/0)
Age (yrs)	60.5 ± 3.1	60.0 ± 11.8	65.2 ± 6.6	66.5 ± 9.6
Length (m)	1.71 ± 0.05	1.69 ± 0.06	1.79 ± 0.04	1.75 ± 0.06
Weight (kg)	80.8 ± 8.3	75.8 ± 18.7	84.3 ± 5.0	66.0 ± 10.7 **
BMI (kg/m^2)	27.5 ± 1.6	26.5 ± 5.7	26.3 ± 2.0	21.5 ± 3.5*
FFM (kg)	61.0 ± 8.6	53.5 ± 6.8	81.7 ± 3.6	53.5 ± 6.0 **
FFMI (kg/m^2)	20.7 ± 2.0	18.3 ± 2.5	25.5 ± 1.3	17.6 ± 3.0 **
FEV_1 (% predicted)	99.5 ± 11.4	53.7 ± 22.9 *	111.7 ± 10.6	35.4 ± 11.6 **
FVC (% predicted)	109.3 ± 13.3	88.7 ± 21.3	117.3 ± 2.0	84.6 ± 19.5 **
FEV_1/FVC (%)	73.3 ± 0.9	47.7 ± 13.8 *	73.0 ± 8.9	32.8 ± 8.7 **
DL_{CO} (% predicted)	114.0 ± 11.7	86.1 ± 19.3 *	123.4 ± 20.8	65.8 ± 39.6 *
Smokers N (y/n/ex-)	0/0/4	0/0/6	1/2/3	1/1/4

Values are mean ± SD. BMI, body mass index; FFM, fat-free mass; FFMI, fat-free mass index; FEV_1, forced expiratory volume in one second; FVC, forced vital capacity; DL_{CO}, diffusion capacity for carbon monoxide. Significantly different compared to controls: * $p<0.05$; ** $p\leq0.01$.

Table 2A. Mitochondrial parameters in vastus lateralis biopsies

	total n of mitochondria counted	mean mitochondrial number (n/µm^2)	mean mitochondrial area%	mean mitochondrial size (µm^2)	mean mitochondrial least Ø (µm)	mean mitochondrial perimeter (µm)	mean z-line width (nm)
Control 1	393	0.45	6.82	0.154	0.34	1.53	99
Control 2	516	0.56	2.69	0.048	0.17	0.86	84
Control 3	531	0.95	3.63	0.038	0.16	0.74	74
Control 4	318	0.57	3.84	0.068	0.24	1.02	96
Mean ± SD	440 ± 102	0.63 ± 0.22	4.25 ± 1.79	0.077 ± 0.053	0.23 ± 0.08	1.04 ± 0.35	88 ± 11
Patient 1	310	0.21	1.62	0.077	0.23	1.08	84
Patient 2	363	0.41	1.98	0.048	0.19	0.87	104
Patient 3	275	0.31	1.07	0.034	0.16	0.72	96
Patient 4	309	0.36	1.54	0.043	0.18	0.82	86
Patient 5	279	0.41	2.81	0.072	0.19	1.11	108
Patient 6	299	0.32	2.67	0.083	0.24	1.15	91
Mean ± SD	306 ± 32 *	0.34 ± 0.07 *	1.95 ± 0.68 *	0.060 ± 0.020	0.20 ± 0.03	0.96 ± 0.18	95 ± 10

Table 2B. Mitochondrial parameters in tibialis anterior biopsies

	total n of mitochondria counted	mean mitochondrial number (n/µm^2)	mean mitochondrial area%	mean mitochondrial size (µm^2)	mean mitochondrial least Ø (µm)	mean mitochondrial perimeter (µm)	mean z-line width (nm)
Control 5	320	0.73	3.68	0.054	0.20	0.91	116
Control 6	323	0.83	4.78	0.057	0.20	0.91	92
Control 7	302	0.78	4.23	0.054	0.20	0.88	123
Control 8	318	1.08	8.58	0.079	0.24	1.10	105
Control 9	296	0.92	3.65	0.040	0.18	0.78	102
Control 10	270	0.95	4.73	0.050	0.18	0.88	93
Mean ± SD	305 ± 20	0.88 ± 0.13	4.94 ± 1.85	0.056 ± 0.013	0.20 ± 0.02	0.91 ± 0.1	105 ± 13
Patient 7	448	0.65	3.93	0.058	0.20	0.94	110
Patient 8	288	0.59	3.85	0.065	0.22	1.01	120
Patient 9	525	0.62	4.93	0.080	0.24	1.08	109
Patient 10	270	0.70	4.81	0.068	0.23	0.99	97
Patient 11	278	0.71	6.25	0.088	0.25	1.14	105
Patient 12	294	0.63	2.64	0.042	0.18	0.76	89
Mean ± SD	351 ± 108	0.65 ± 0.05 **	4.4 ± 1.22	0.067 ± 0.016	0.22 ± 0.02	0.99 ± 0.13	105 ± 11

Values are mean ± SD; area%: fractional area; Ø: diameter; Significance of difference compared to controls: * $p<0.05$; ** $p<0.01$.

Table 3A. Fuel stores in vastus lateralis biopsies

	glycogen particle levels (1-5)	lipid droplet levels (1-5)
Control 1	2.5	3.4
Control 2	4.3	1.7
Control 3	3.6	1.3
Control 4	3.7	2.6
Mean ± SD	3.5 ± 0.8	2.3 ± 0.9
Patient 1	4.7	1.2
Patient 2	2.9	2.1
Patient 3	3.6	1.3
Patient 4	3.6	3.9
Patient 5	3.8	1.7
Patient 6	3.8	1.4
Mean ± SD	3.7 ± 0.6	1.9 ± 1

Table 3B. Fuel stores in tibialis anterior biopsies

	glycogen particle levels (1-5)	lipid droplet levels (1-5)
Control 5	1.9	2.4
Control 6	2.9	1.4
Control 7	3.5	1.5
Control 8	3.2	2.0
Control 9	2.9	1.8
Control 10	0.7	0.5
Mean ± SD	2.5 ± 1	1.6 ± 0.6
Patient 7	2.0	1.2
Patient 8	3.0	1.9
Patient 9	1.5	1.9
Patient 10	3.1	1.6
Patient 11	3.8	1.7
Patient 12	3.8	1.4
Mean ± SD	2.9 ± 1	1.6 ± 0.3

LEGENDS

Figure 1. A) Representative micrograph of a central region in a fibre of the human vastus lateralis (Control 3). Z = z-line; L = lipid droplet; G = glycogen stores; M = mitochondria. B) The corresponding overlaying bitmap with the outlined mitochondria. Only mitochondria entirely in the field were included. Likewise, a bitmap was made with outlined z-lines (not shown). Bitmaps were used to automatically compute the parameters presented in table 2.

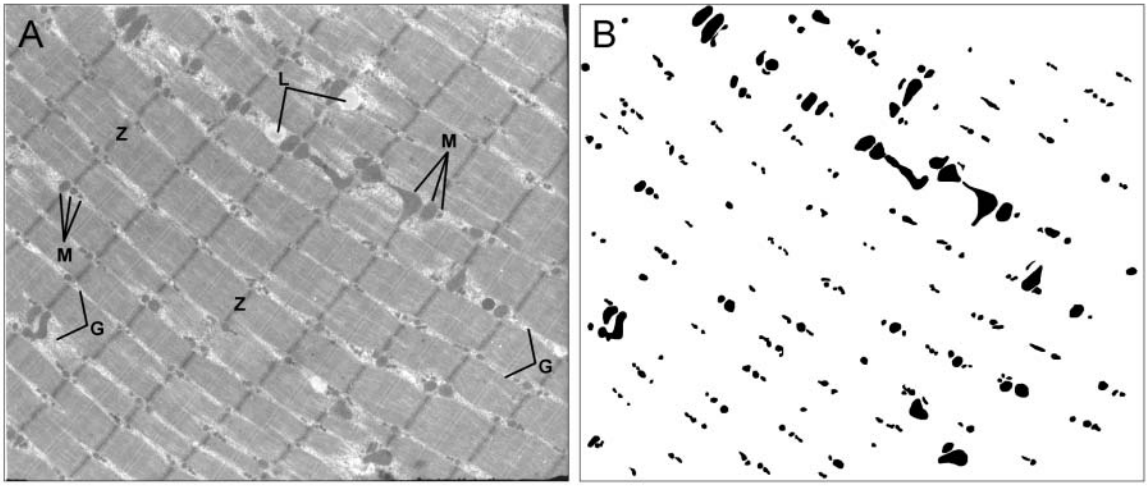
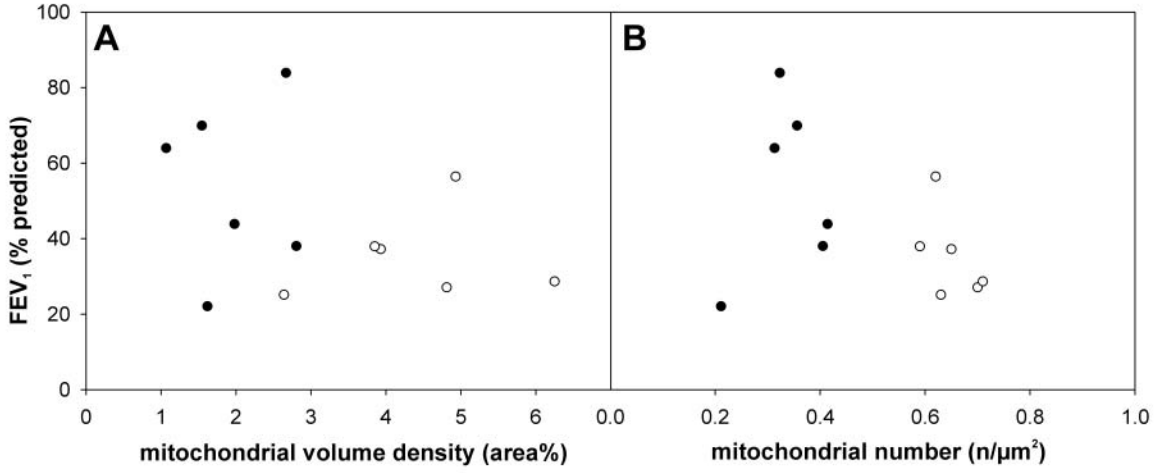


Figure 2. Relations between disease severity and mitochondrial volume density (A) and number (B) in vastus lateralis (●) biopsies and tibialis anterior (○) biopsies from patients with COPD. There were no significant correlations. FEV_1, forced expiratory volume in one second.


A
B
100
80
60
40
20
0
FEV1 (% predicted)
0
1
2
3
4
5
6
mitochondrial volume density (area%)
0.0
0.2
0.4
0.6
0.8
1.0
mitochondrial number (n/μm2)

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