

Mitochondrial DNA disturbances and deregulated expression of oxidative phosphorylation and mitochondrial fusion proteins in sporadic inclusion body myositis

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Abstract

Sporadic inclusion body myositis (sIBM) is one of the most common myopathies in elderly people. Mitochondrial abnormalities at the histological level are present in these patients. We hypothesize that mitochondrial dysfunction may play a role in disease aetiology. We took the following measurements of muscle and peripheral blood mononuclear cells (PBMCs) from 30 sIBM patients and 38 age- and gender-paired controls: mitochondrial DNA (mtDNA) deletions, amount of mtDNA and mtRNA, mitochondrial protein synthesis, mitochondrial respiratory chain (MRC) complex I and IV enzymatic activity, mitochondrial mass, oxidative stress and mitochondrial dynamics (mitofusin 2 and optic atrophy 1 levels). Depletion of mtDNA was present in muscle from sIBM patients and PBMCs showed deregulated expression of mitochondrial proteins in oxidative phosphorylation. MRC complex IV/citrate synthase activity was significantly decreased in both tissues and mitochondrial dynamics were affected in muscle. Depletion of mtDNA was significantly more severe in patients with mtDNA deletions, which also presented deregulation of mitochondrial fusion proteins. Imbalance in mitochondrial dynamics in muscle was associated with increased mitochondrial genetic disturbances (both depletion and deletions), demonstrating that proper mitochondrial turnover is essential for mitochondrial homeostasis and muscle function in these patients.

Key words: deletions, depletion, mitochondria, mitofusin-2, OPA1, sporadic inclusion body myositis.

INTRODUCTION

Sporadic inclusion body myositis (sIBM) is the most common inflammatory myopathy in individuals aged >50 years [1] and one of the most important myopathies associated with ageing [2]. With a male:female ratio of 3:1, it is a rare disease (ORPHA611) and its prevalence varies from 4.7 per million in the Netherlands to 14.9 per million in western Australia [3]. These rates continue

to increase, probably due to improved diagnostic protocols and increased ageing of the population. This myopathy is a devastating condition, causing slow, progressive muscle weakness and wasting, with quadriceps and finger flexors being the muscles most typically affected. Nevertheless, the clinical presentation varies considerably. This muscle weakness usually leads to falls and difficulty in standing. Around 60% of the patients also present with dysphagia. With the wide spectrum of clinical

Abbreviations: CK, creatine kinase; COX, cytochrome c oxidase; CS, citrate synthase; H&E, haematoxylin and eosin; HAE, hydroxyalkenal; IBMFRS, Inclusion Body Myositis Functional Rating Scale; MDA, malondialdehyde; MFN2, mitofusin 2; MRC, mitochondrial respiratory chain; NSE, non-specific esterase; OPA1, optic atrophy 1; ORO, Oil Red O; PAS-D, periodic acid–Schiff diastase stain; PBMC, peripheral blood mononuclear cell; RT-PCR, reverse transcriptase PCR; ROS, reactive oxygen species; SDH, succinate dehydrogenase; sIBM, sporadic inclusion body myositis; TRC, tyrosine-rich crystalloids; VDACL1, voltage-dependent anion channel 1.

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manifestations in sIBM, muscle biopsy continues to be the gold standard for diagnosis [4].

The histological features observed in muscle biopsies of sIBM patients include: (i) inflammatory changes with predominant CD8+ T-cell infiltrates and the expression of MHC-I antigens by non-necrotic muscle fibres, (ii) different degrees of degenerative changes in muscle fibres and the presence of rimmed vacuoles composed mainly of β -amyloid, phosphorylated tau and caveolin proteins, among others, and (iii) mitochondrial abnormalities characterized by the presence of ragged-red fibres, cytochrome *c* oxidase (COX)-negative and succinate dehydrogenase (SDH)-positive muscle cells [5,6], all of which are widely associated with mitochondrial dysfunction and ageing [1,2,7]. However, the distribution of these histological features is not homogeneous, and they may not be simultaneously present, particularly in the early stages of the disease. For all these reasons, and due to its slow progression, diagnosis may be delayed for 5–10 years.

Mitochondria are the cell's powerhouse. They are responsible for most of the energy supply, metabolic reactions, calcium homeostasis and cell respiration. However, under pathological conditions, mitochondria are the main centres for reactive oxygen species (ROS) production and for triggering apoptosis. Mitochondria are essential for cell bioenergetics, especially in highly energetic tissues such as muscle, in which mitochondrial dysfunction may become evident much earlier. Molecular mitochondrial alterations have recently been found in sIBM by down-regulated expression of mitochondrial respiratory chain (MRC) complex I subunits and mitochondrial DNA (mtDNA) variations [7]. In addition, mtDNA deletions have also been reported in sIBM patients [6,8], but not correlated with abnormal mitochondrial function or dynamics. Despite mitochondrial abnormalities having classically been described at a histological level, mitochondrial dysfunction in sIBM patients has scarcely been assessed at a molecular level.

Mitochondrial dynamics is a recently discovered mechanism responsible for mitochondrial turnover and renewal [9]. Mitochondria constitute a complex network that is constantly undergoing fusion and fission processes to exchange genetic and structural components, which include mtDNA and MRC machinery. Deregulation of mitochondrial dynamics has been associated with disease, but has not previously been described in sIBM patients [10–16].

Although the pathological features of sIBM have been widely described, its aetiology remains unknown. It has been proposed that its development could be due to a complex interaction of environmental agents, accelerated ageing and genetic susceptibility [2,17]. As muscle tissue has a high dependence on ATP to exert its function, mitochondrial alterations in sIBM could be one of the factors involved in triggering muscle weakness and degeneration, because these alterations have been related to muscle disease by many authors [18–20]. For an in-depth evaluation of the pathological characteristics of the disease, we describe the mitochondrial phenotype at a genetic, molecular and functional level in sIBM patients.

To determine potential disease biomarkers we assessed abnormal mitochondrial fingerprints, in the target tissue of the disease (muscle) and with less invasive approaches using peri-

pheral blood mononuclear cells (PBMCs). This PBMC model is widely feasible for the evaluation of mitochondrial function [21–23]. Thus, we designed the present study to evaluate mitochondrial dysfunction in both muscle and PBMCs of sIBM patients to correlate dysfunction severity with genetic and molecular mitochondrial alterations, and determine their potential association with the deregulation of mitochondrial dynamics.

MATERIALS AND METHODS

Study design

We performed a single-site, cross-sectional, case–control, observational study.

Study population, diagnosis, clinical data and sample collection

A total of 30 patients with sIBM who had attended the Internal Medicine Department of the Hospital Clinic of Barcelona (Spain) over the last 20 years were prospectively and consecutively included. These patients were age and gender paired with 38 controls. All muscle biopsies were performed for diagnostic purposes indicated for muscle weakness or raised creatine kinase (CK) levels. Surgical muscle biopsies were obtained by trained physicians, and the samples were processed routinely in the laboratory as described elsewhere [24]. For histological studies fresh muscle samples were frozen in cooled isopentane, sectioned by cryotome at -30°C and stained with different reagents for diagnostic purposes: haematoxylin and eosin (H&E), tyrosine-rich crystalloids (TRCs), non-specific esterase (NSE), periodic acid–Schiff diastase stain (PAS-D), Oil Red O (ORO), acid and alkaline phosphatase, NADH, COX, SDH and ATPase at pH 4.3, 4.6 and 9.4, as reported elsewhere [24]. In addition some immunohistochemistry reactions such as class I antigens from the MHC as well as p62 were performed. The same expert pathologist (J.M.G.) read all the samples. Leftover biopsy material from both sIBM patients and individuals with no histological myopathy was included as patient and control samples, respectively. Histological data were collected to study inflammation and the presence of ragged-red fibres, COX-negative and SDH-positive fibres, as well as the number of vacuolated muscle cells. Diagnosis of sIBM was considered as definite or probable according to the criteria of the European Neuromuscular Centre [1,25]. Samples from 23 muscle biopsies from sIBM patients and 18 from controls were included following these criteria. In parallel, blood samples were collected from 14 sIBM patients and 20 control individuals free of muscle disease. Exclusion criteria were: age <40 years, muscle disease in the case of controls, mitochondrial disease, and family history of hereditary mitochondrial pathology, HIV infection or drug abuse. All individuals were informed, and signed written consent was obtained for inclusion in this study, which was approved by the Ethical Committee of our hospital, following the Declaration of Helsinki. All sIBM patients answered the Inclusion Body Myositis Functional Rating Scale (IBMFERS) test, which is a validated, disease-specific test to assess disease severity.

A database was created to collect epidemiological, clinical and histological data, which were further complemented with experimental results.

Leftover samples from mandatory muscle biopsy diagnosis were included in optimal cutting temperature compound (OCT) and immediately frozen and stored at -80°C until homogenization (5% w/v) with mannitol to perform experimental studies.

Around 20 ml of peripheral blood was also obtained by antecubital vein puncture and collected in EDTA tubes. PBMCs were isolated using Ficoll density gradient centrifugation, divided into aliquots and stored at -80°C until analysis.

Mitochondrial genetic studies

Total DNA from muscle biopsies and PBMCs was obtained by the standard phenol–chloroform extraction procedure. The assessment of mtDNA deletions was performed in muscle samples by long-range PCR using Phusion High-Fidelity PCR Master Mix with GC Buffer (F-532L, ThermoFisher Scientific) and the following primers: forward 5'-TTAGCAAGGGAAGTACTCCCA-3' and reverse 5'-CGGATACAGTTCACCTTAGCTACCCCAAGTG-3'. The methodology of this procedure was performed as previously described [26–28]. The PCR products were electrophoresed in a 0.8% agarose gel with ethidium bromide to detect different sizes in the mitochondrial genome (see Supplementary Figure S1).

To evaluate mtDNA content, fragments of the mitochondrially encoded 12S rRNA gene and the nuclear-encoded RNase-P gene were amplified separately in triplicate by quantitative reverse transcriptase PCR (RT-PCR) using Applied Biosystems technology [29]. The relative mtDNA content was expressed as the ratio mtDNA 12S rRNA:nDNA RNase-P.

Mitochondrial transcript quantification

To evaluate mtRNA content in both muscle and PBMCs, total RNA was first extracted using the TriPure procedure and immediately retro-transcribed into cDNA using random hexamer primers, following previously described methodology [30]. The mtRNA:nRNA content was then measured by RT-PCR as previously described for mtDNA and expressed in the same units (mtRNA 12S rRNA:nRNA RNase-P).

Mitochondrial protein quantification

Mitochondrial protein quantification was assessed in muscle and PBMCs by the quantification of two mitochondrial complex IV protein subunits: COX-II (encoded by the mitochondrial genome) and COX-IV (encoded by the nuclear genome). These proteins were assessed through immunoblotting using SDS/7/13% PAGE and immunodetection using previously described reagents and antibodies [22]. Mitochondrial values normalized to nuclear-encoded COX proteins were expressed as the COX-II:COX-IV ratio, and absolute values of COX-II and COX-IV were normalized with a loading control (α -tubulin for muscle samples and β -actin for PBMC samples) and expressed as the COX-II: α -tubulin and COX-IV: α -tubulin ratios in muscle or the COX-II: β -actin and COX-IV: β -actin ratios in PBMCs. COX-II and COX-IV values were also normalized by mitochondrial mass [through constitutive voltage-dependent anion channel

1 (VDAC1) expression] and expressed as COX-II:VDAC1 and COX-IV:VDAC1 ratios in both tissues (see Supplementary Figure S2) [31].

MRC complex I and complex IV enzyme activity

MRC complex I (EC 1.6.5.3) and IV (COX – EC 1.9.3.1) enzyme activities were measured spectrophotometrically according to the methodologies of Spinazzi et al. [32] in both muscle homogenates and PBMCs. Specific enzymatic activities were expressed in absolute values as nanomoles of substrate consumed or product generated per minute per milligram of cell protein (nmol/min per mg protein) or in relative values with respect to mitochondrial content normalized by citrate synthase (CS) activity (COX:CS).

MRC complex assembly

Total oxidative phosphorylation rodent WB Antibody Cocktail (MS604, Abcam) was used to detect one subunit from each MRC complex (I–V): NDFUFB8 from complex I, SDHB from complex II, UQCRC2 from complex III, MTCO1 from complex IV and ATP5A from complex V. These subunits are labile when the respiratory complex to which they belong is not assembled and a decrease in this subunit would manifest this phenomenon. To normalize the levels of expression of these five proteins from the MRC we analysed using the SYPRO technique for the total protein content and the VDAC1 levels for the mitochondrial mass in the same membrane.

Mitochondrial mass analysis

Mitochondrial mass was analysed in both muscle and PBMCs by spectrophotometric measurement of CS (EC 4.1.3.7) enzyme activity and, in parallel, by the measurement of VDAC1 protein levels by Western blotting. CS is a mitochondrial enzyme of the citrate cycle widely considered to be a reliable marker of mitochondrial content, and VDAC1 [22] is an outer membrane anion channel also considered to be a mitochondrial mass marker [22]. CS activity was expressed as nmol/min per mg of protein and VDAC1 expression was normalized by α -tubulin in muscle and by β -actin in PBMCs, both of which are validated proteins for assessing total cell loading mass and expressed as the VDAC1: α -tubulin ratio in muscle or the VDAC-1: β -actin ratio in PBMCs.

Oxidative stress assay

Lipid peroxidation (an indicator of oxidative damage produced by ROS in cellular lipid compounds) was quantified in muscle and PBMCs using the Oxys Research kit (Deltaclon) through the spectrophotometric measurement of malondialdehyde (MDA) and 4-hydroxyalkenal (HAE), both products of fatty acid peroxide decomposition and normalized by protein content (μM MDA + HAE per mg of protein) [33].

Expression of mitochondrial fusion proteins

Mitochondrial dynamics were assessed in muscle tissue by the evaluation of both optic atrophy 1 (OPA1) and mitofusin 2 (MFN2) expression at two different levels: transcript and protein quantification. For the assessment of transcript levels of OPA1 and MFN2, mRNA, total RNA extracted by TriPure was reverse

transcribed with the SuperScript III reverse transcriptase kit (Invitrogen). Quantitative PCRs were performed with ABI Prism 7900HT real-time PCR equipment (Applied Biosystems) using materials and reagents previously described [34,35]. All measurements were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA content. Protein levels of OPA1 and MFN2 were assessed in parallel using Western blotting on SDS/7/8% PAGE and posterior immunodetection using a BD antibody (612606) and an Abcam antibody (ab56889), respectively. In the case of OPA1, results were expressed as the ratio between the long and the short forms of the protein (OPA1 long:short), because this ratio decreases in abnormal mitochondrial dynamic states. In the case of MFN2, absolute values were normalized by total cell loading mass and expressed as the ratio MFN2: α -tubulin.

Statistical analysis

Statistical analysis was performed using the SPSS, version 20.00 to search for differences depending on group assignment (patients vs controls or presence vs absence of mtDNA deletions) using the non-parametric Mann–Whitney U-test for independent samples. Results were expressed as means \pm S.E.M.S or as a percentage of increase/decrease with respect to controls that were arbitrarily assigned as 0%. In all cases, $P < 0.05$ was considered statistically significant.

RESULTS

Clinical and histological data

According to the design of the study, no statistical differences were observed with regard to age and gender between sIBM patients and controls. Clinical, epidemiological, histological and mtDNA deletion data of both patients and controls are summarized in Table 1 and histological data examples are compiled in Figure 1. At the time of study inclusion the sIBM patient cohort scored 26.61 ± 1.19 out of 40 with the IBMFRS test, indicating moderate-to-advanced sIBM clinical severity.

MtDNA deletions

When assessing for the presence of mtDNA deletions in muscle from sIBM patients, 57% of the sIBM cohort presented multiple mtDNA deletions, whereas the rest showed no alterations in this parameter (see Supplementary Figure S1).

MtDNA content

MtDNA content was significantly decreased by 36% in muscle from sIBM patients compared with controls (641.6 ± 141.2 vs 979.7 ± 111.3 , respectively, $P < 0.05$) (Figure 2). Similarly, mtDNA was decreased, albeit not significantly, by 14.5% in PBMCs (Figure 2).

Mitochondrial transcript quantification

No variation was found in the levels of mtRNA in either muscle or PBMCs (Figure 2) of sIBM patients compared with controls.

Table 1 Clinical, epidemiological and histological data of patients with sIBM and controls included in the present study

Muscle biopsies	sIBM (n=23)	Controls (n=18)
Clinical and epidemiological data		
Age (years) ^a	68.64 ± 2.87	63.22 ± 2.55
Male:female ratio	0.64	0.38
IBMFRS score ^a	$26.61 \pm 1.19/40$	–
mtDNA deletions (%) ^b	57	–
Histological data (%)		
Rimmed vacuoles ^b	61.20	–
Ragged-red fibres ^b	60	–
COX-negative fibres ^b	36.36	–
SDH-positive fibres ^b	44	–
MHC-I expression ^b	57.14	–
Inflammation ^b	67.74	–
PBMC samples		
Clinical and epidemiological data		
Age (years) ^a	68.43 ± 3.28	68.55 ± 1.41
Male:female ratio	1	1.85
IBMFRS score ^a	$25.85 \pm 1.31/40$	–

^aData presented as means \pm S.E.M.s.

^bData presented as percentage.

Mitochondrial protein quantification

As shown in Figure 2, muscle from sIBM patients presented an increase of 25% in the COX-II:COX-IV ratio with respect to controls, suggesting increased expression of mitochondrial versus nuclear COX subunits (see Supplementary Figure S2). It is interesting that in PBMCs this ratio showed a statistically significant decrease of 45.46% (0.42 ± 0.05 vs 0.77 ± 0.09 , $P < 0.05$; Figure 2). In addition, in PBMCs COX-IV protein levels normalized by total cell mass showed a strong trend to be increased by 67.85% in sIBM patients compared with controls (0.47 ± 0.08 vs 0.28 ± 0.03 , $P = 0.064$; Figure 2), suggesting increased nuclear versus mitochondrial COX protein expression. This abnormality became statistically significant when COX-IV values were normalized by mitochondrial mass (COX-IV:VDAC-1), which showed a 172.72% increase in sIBM patients compared with controls (1.80 ± 0.35 vs 0.66 ± 0.01 , $P < 0.05$; Figure 2).

Mitochondrial complex I and IV activity

Measurements of respiratory chain activities showed no differences in complex I activity in muscle of patients and controls, in either absolute or relative units (data not shown). However, complex IV activity was abnormally decreased in both muscle and PBMCs of sIBM patients with respect to controls (Figure 2). Thus, taking into account absolute values, a decrease of 13.55% was found in muscle (Figure 2). These differences became statistically significant in muscle when complex IV activity was normalized to mitochondrial mass (CS), by showing a 30.31% decrease in sIBM patients compared with controls (0.26 ± 0.02 vs 0.33 ± 0.03 , $P < 0.05$; Figure 2). On analysing this parameter in PBMCs, complex IV activity was also significantly decreased by 31.4% in absolute values in patients compared with controls (24.32 ± 3.1 vs 35.45 ± 3.4 , $P < 0.05$; Figure 2) and by 28.21%

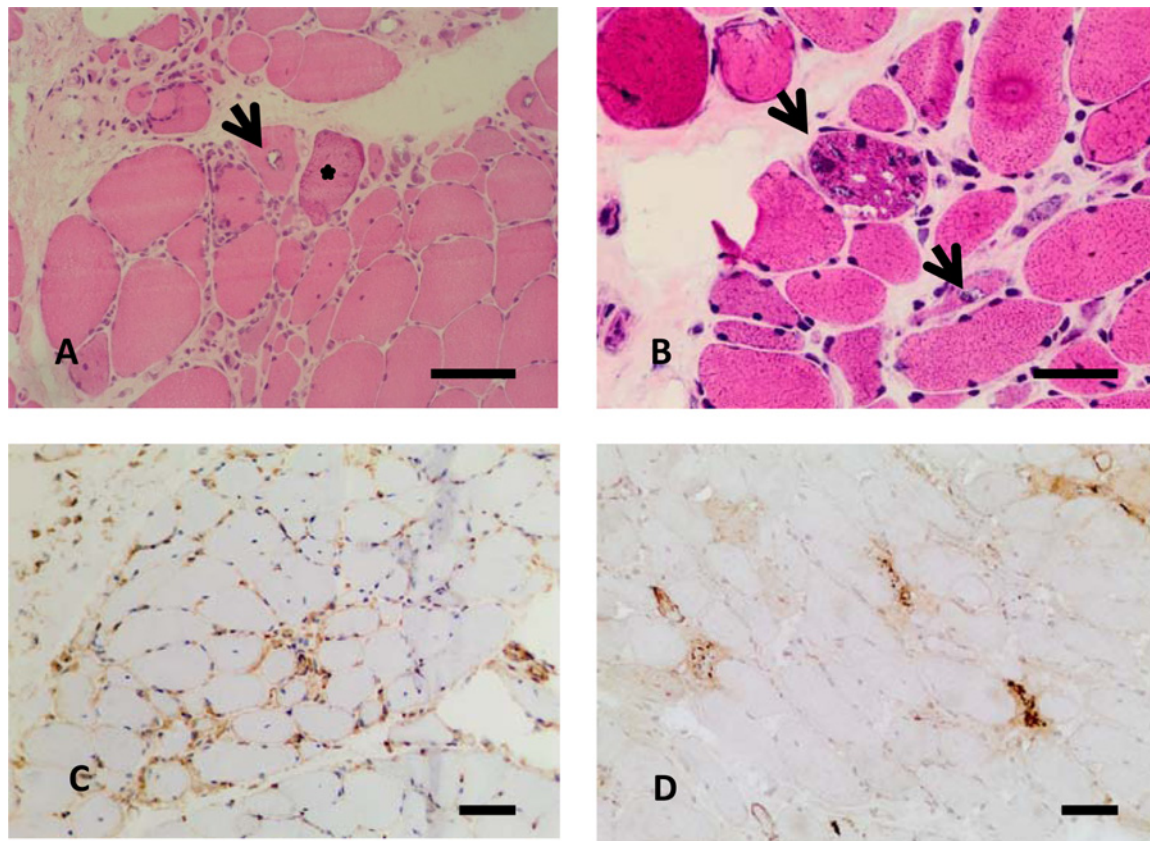


Figure 1 Images from the histological staining of sIBM muscle tissue

(A, B) H&E in frozen muscle tissue: marked variability in fibre size. Some inflammatory cells are observed in the endomysium, together with rimmed vacuoles (arrows) and a typical ragged-red fibre (star). (C) Almost universal sarcolemmal positivity of class I antigen from the MHC, a typical feature in sIBM. (D) Positivity of p62 in rimmed vacuoles, suggestive of muscle degeneration. Scale bar=50 μ m.

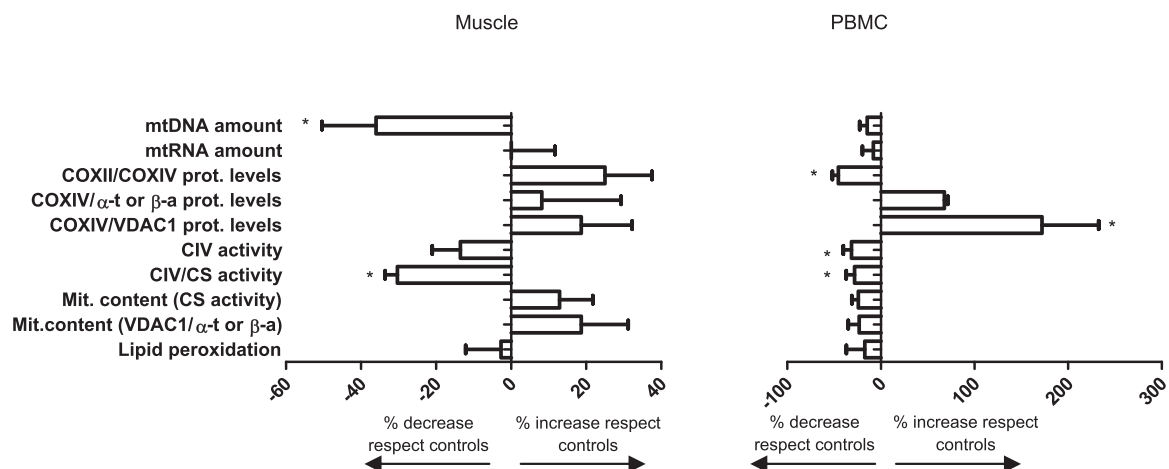


Figure 2 Mitochondrial parameters in muscle tissue and PBMCs from sIBM patients compared with controls

Mitochondrial parameters in muscle tissue and PBMCs from patients with sIBM expressed as percentage of increase/decrease compared with controls. A significant decrease in mtDNA amount and complex IV activity normalized to mitochondrial content can be observed in muscle, and also a significant decrease in COX-II:COX-IV ratio, complex IV absolute activity and complex IV activity normalized to mitochondrial content can be observed in PBMCs. In this muscle tissue, a significant increase in COX-IV subunit normalized to mitochondrial content can be observed as well as other non-significantly altered parameters. α -t, α -tubulin; β -a, β -actin; C-IV, MRC complex IV. * : $p < 0.05$.

when this parameter was normalized to the mitochondrial mass (0.28 ± 0.1 vs 0.39 ± 0.1 , $P < 0.05$; Figure 2).

MRC complexes assembly

The analysis of the subunit levels for each complex of the MRC (I–V) that correlates with the proper assembly of the respiratory chain complexes did not show differences between sIBM patients and controls, normalized to either total protein or VDAC1 content. It is interesting that the complex V subunit presented a trend towards a decrease in sIBM patients, but this difference did not reach statistical significance (Figure 3).

Mitochondrial mass content

Mitochondrial mass, assessed by both the quantification of CS enzyme activity and VDAC1 protein content, was increased in muscle of sIBM patients compared with controls (12.9% in CS activity and 18.7% in VDAC1: α -tubulin protein levels; see Figure 2) and decreased in PBMCs of sIBM patients compared with controls (by 24.18% in CS activity and 23.26% in VDAC1: β -actin protein content; see Figure 2). However, none of these results was statistically significant.

Oxidative stress levels

No differences were found in lipid peroxidation, an indicator of oxidative damage produced by ROS in lipid compounds, between sIBM patients and controls in muscle or PBMCs (see Figure 2).

Quantification of mitochondrial dynamics

Transcript levels of OPA1 showed a significant 37% reduction in muscle of sIBM patients compared with controls (0.59 ± 0.11 vs 0.94 ± 0.19 , $P < 0.05$; Figure 4). OPA1 long:short protein isoform ratios, although not significant, were decreased by 45% in muscle of sIBM patients compared with controls (0.55 ± 0.17 vs 1 ± 0.16 , $P < 0.083$; Figure 4). In the case of MFN2 transcripts, muscle of sIBM patients showed a 31% reduction. With regard to MFN2 protein expression, a strong trend towards a decrease was observed in sIBM patients, with 54% compared with controls (1.93 ± 0.3 vs 4.17 ± 0.1 , $P = 0.096$; Figure 4). The differences in MFN2 levels in either transcript or protein content were not statistically significant.

Correlation of mitochondrial parameters with regard to the presence/absence of mtDNA deletions

Last, the sIBM cohort was divided into two groups on the basis of the presence or absence of mtDNA deletions for further exploration of mitochondrial homeostasis, depending on this condition with respect to controls. The mtDNA content was slightly decreased in sIBM patients with mtDNA deletions compared with those without (Figure 5). These differences became statistically significant in sIBM patients with deletions with respect to the control group (498.50 ± 178.70 vs 979.70 ± 111.38 , $P < 0.05$). MFN2 levels showed a similar pattern to those of mtDNA content, showing the lowest content in sIBM patients with mtDNA deletions and the highest in controls (1.16 ± 0.24 vs 4.17 ± 0.96 , $P < 0.05$), with sIBM patients without mtDNA deletions presenting an intermediate value of expression (Figure 5).

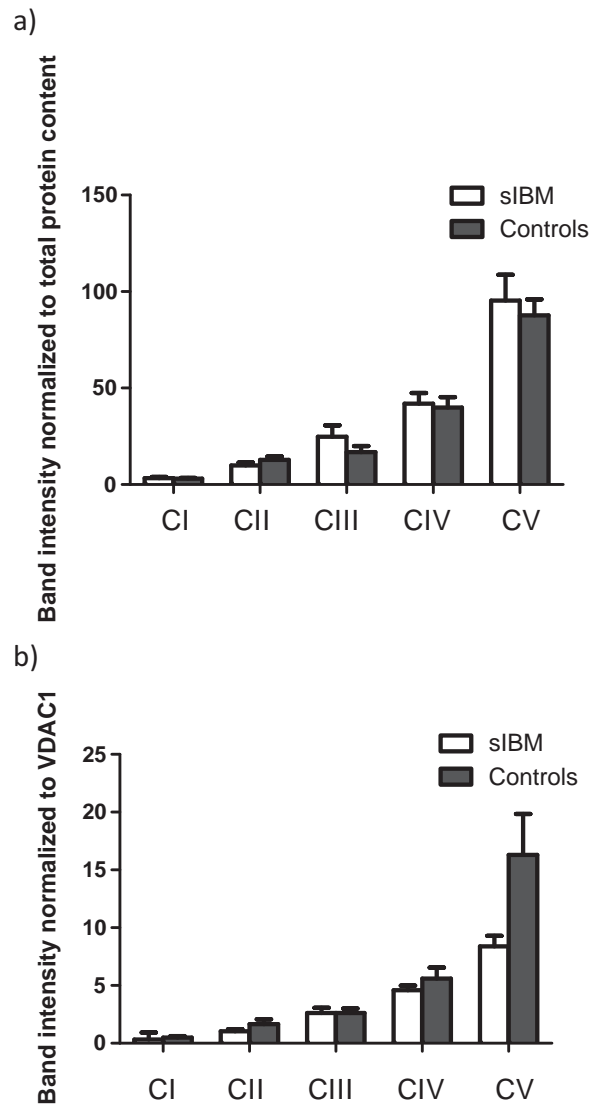


Figure 3 MRC complex assembly through the measurement of protein expression level of labile subunits of each complex in muscle tissue from sIBM patients compared with controls

No significant differences with regard to levels of specific subunits for each MRC complex were found between muscle from sIBM patients and muscle from controls, normalized to (a) total protein content and (b) the nuclear-encoded mitochondrial protein VDAC1. A trend towards increase is observed in the complex V subunit when normalized to VDAC1 which, although not significant, may give us an idea of complex V disassembly. This multiplex Western blotting measures the protein expression level of a subunit of each MRC complex (I–V), labile when the complex is not properly assembled; the following were measured: NDFUF8 from complex I (CI; 20 kDa), SDHB from complex II (CII; 30 kDa), UQCRC2 from complex III (CIII; 48 kDa), MTCO1 from complex IV (CIV; 40 kDa) and ATP5A from complex V (CV; 55 kDa).

DISCUSSION

Mitochondrial abnormalities in muscle from sIBM patients are accepted in the scientific community as a result of histological evidence [7,36]. However, little research has been performed to characterize potential functional or molecular mitochondrial

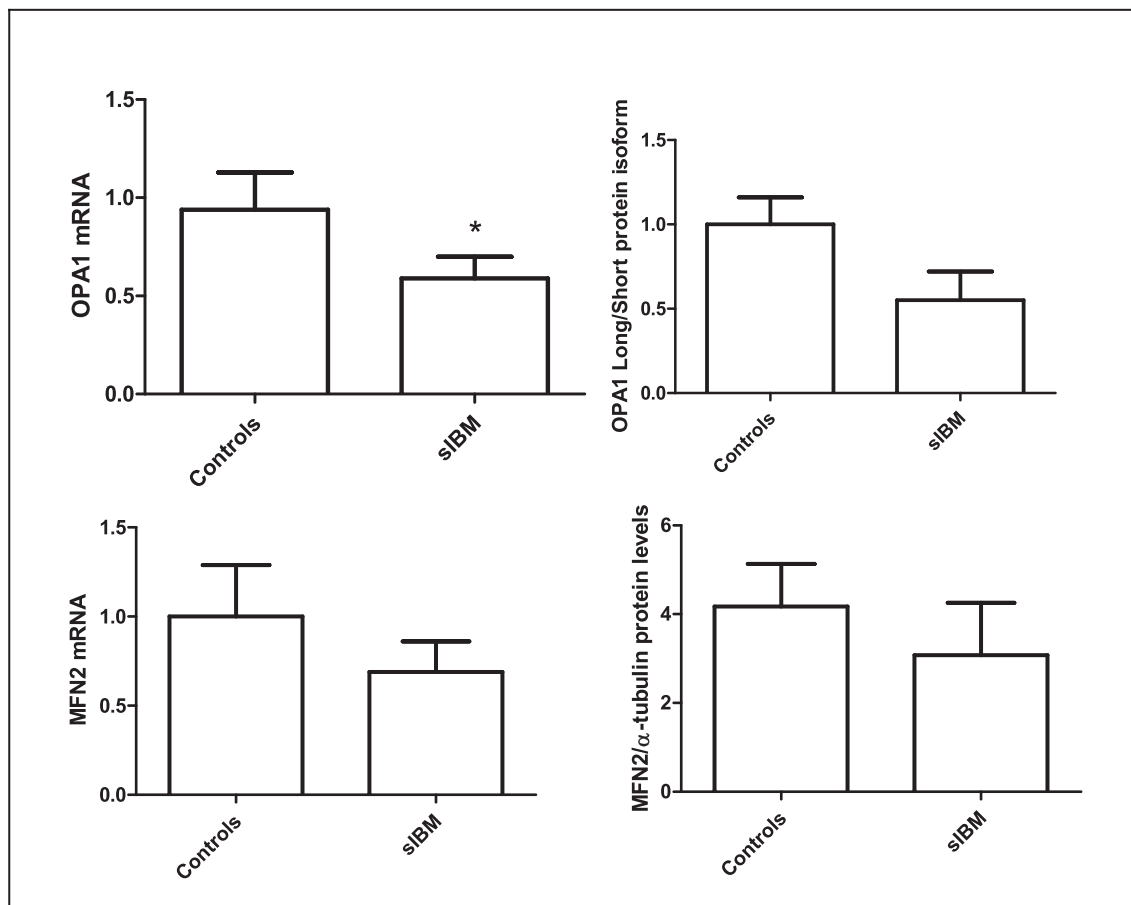


Figure 4 Mitochondrial dynamics in muscle from sIBM patients

MFN2 and OPA1 were assessed in muscle from sIBM patients at the transcript and protein levels. Significant differences were found when OPA1 mRNA levels were compared between the two groups. The other parameters showed a non-significant trend to be decreased in the sIBM respective controls. * : $p < 0.05$.

disrupted pathways [6–8]. In the present study, we evaluated mitochondrial dysfunction at a genetic and molecular level, and studied its potential association with mitochondrial dynamic deregulation. We compared mitochondrial status in muscle and PBMCs from sIBM patients with that of controls, first to evaluate its involvement in disease aetiology and, second, to determine whether mitochondrial alterations are confined to muscle tissue (the target tissue of the disease) or whether these alterations extend to other body tissues, which may allow less invasive approaches and potential follow-up as a putative biomarker.

With regard to experimental data, sIBM patients showed a decrease in mtDNA content. This mtDNA depletion was statistically significant in muscle from sIBM patients, because it is post-mitotic tissue that is prone to store mitochondrial deficiencies. On the other hand, mtDNA depletion was only mildly manifested in PBMCs, probably due to their higher renewal capacity and shorter mean lifespan. Although mtDNA depletion was found in both tissues, no significant changes were observed in mtRNA levels of either muscle or PBMCs [37].

On analysing MRC function, no differences were found in complex I activity in muscle from sIBM patients compared with

controls. Otherwise, MRC complex IV deficiency was present in both tissues (muscle and PBMCs) of sIBM patients. Absolute values of complex IV activity in muscle showed a trend towards a decrease, but, on normalizing these absolute values by mitochondrial mass, complex IV deficiency became significant. These phenomena may be due to the increase in mitochondrial biogenesis detected in muscle of sIBM patients as compensating for mitochondrial dysfunction. This increase in muscular mitochondrial content, characteristic of primary mitochondrial diseases [38], is observed in parallel in sIBM patients as an increase in CS activity and VDAC1:β-actin protein levels, as well as by the presence of ragged-red fibres. Similarly, mitochondrial protein synthesis tended to increase, albeit not significantly, in muscle from sIBM patients (COX-II:COX-IV), probably as a consequence of the trend of this homeostatic muscle to increase mitochondrial biogenesis. As mentioned previously, PBMCs present a shorter mean lifespan, thereby hindering the development of homeostatic mechanisms such as increasing mitochondrial content to deal with mitochondrial dysfunction. Thus, in PBMCs, complex IV activity was found to be significantly decreased in both absolute and relative values to mitochondrial

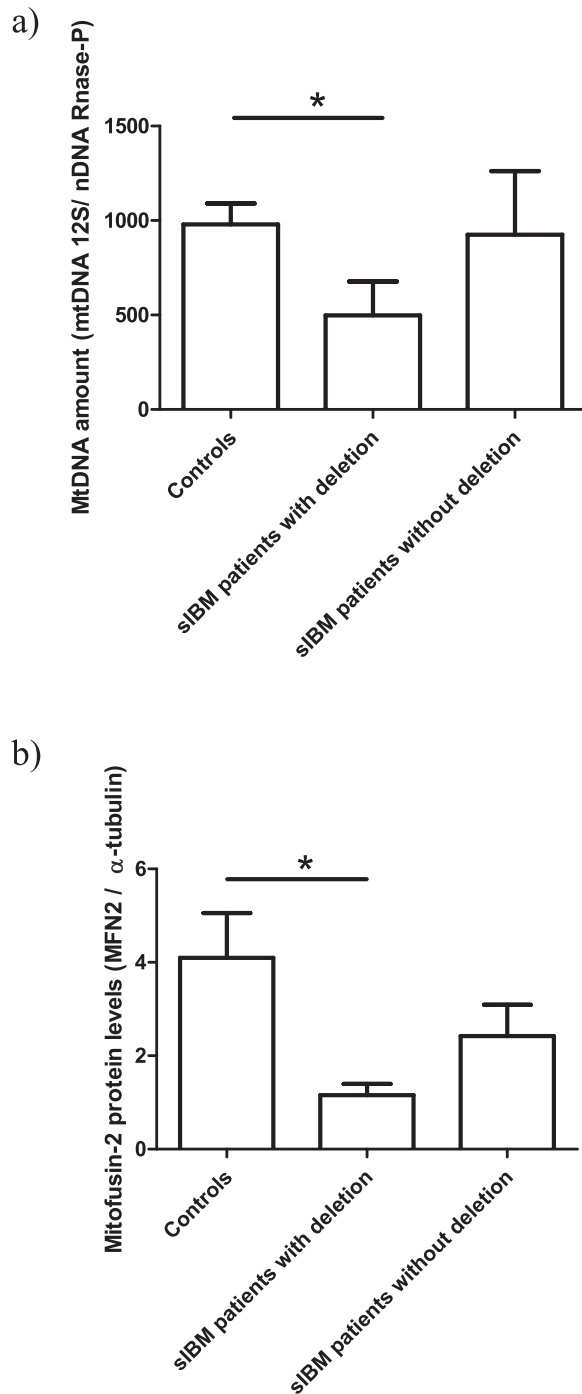


Figure 5 MtDNA amount and MFN2 protein levels in muscle of sIBM patients with and without mtDNA deletions and controls
 Comparison of (a) mtDNA amount and (b) MFN2 levels among patients with sIBM with and without mtDNA deletions and controls. The patients with sIBM and mtDNA deletions showed significantly reduced muscle levels of mtDNA and MFN2 protein levels with respect to the controls. The patients with sIBM and no mtDNA deletions presented an intermediate value, suggesting the implication of mtDNA deletions in other mitochondrial alterations or the involvement of mitochondrial dynamics in mtDNA homeostasis. * : $p < 0.05$.

mass, which additionally showed trends towards a decrease, according to poor homeostatic compensation. Unlike in muscle,

in PBMC nuclear-encoded protein subunits of COX (COX-IV) were slightly increased to compensate for COX dysfunction. The trends towards up-regulation of mitochondrially encoded proteins in muscle or nuclear-encoded proteins in PBMCs, to compensate for MRC dysfunction, may depend on the levels of endoplasmic reticular stress of both cell types, which has been reported to be altered in muscle [39,40].

Mitochondrial dysfunction in sIBM patients is not caused by abnormal assembly of MRC complexes, at least as assessed through the measurement of protein levels of specific subunits that are labile in cases of complex instability. However, there was a strong trend of the complex V subunit, when normalized to mitochondrial mass, to be decreased. This decrease disappeared when the complex V subunit content was normalized to total cell protein. This could be explained by the slight increase in mitochondrial mass that underlies sIBM. Further analysis of complex V activity could be useful to elucidate whether this complex is altered in this disease.

Oxidative stress does not seem to play a role in sIBM pathogenesis, because lipid peroxidation levels are not increased in muscle or PBMCs from sIBM patients, at least in the present study cohort.

Overall, these data describe an altered mitochondrial phenotype in muscle and PBMCs of sIBM patients, with both tissues presenting a different lesion profile, probably due to different compensatory mechanisms related to the disease. It is interesting, regardless of the homeostatic attempt to preserve mitochondrial function, that COX enzymatic deficiency is present in both tissues as a common pathogenic parallelism. Thus, it seems that mitochondrial dysfunction in sIBM is not confined only to the target tissue of the disease, muscle, but may also extend to other body tissues. Whether COX/CS activity could be used as a prognostic or severity biomarker of the disease should be assessed in further studies. Alternatively, PBMC mitochondrial alterations may be interpreted as a deregulation of the immune system, non-specific for sIBM, as stated before for other diseases with inflammatory components [41–45]. Regardless of the interpretation, mitochondrial impairment is present in both PBMCs and muscle tissue of sIBM patients.

Mitochondrial dynamics are the mechanism for this organelle recycling and turnover. Imbalance of proper ratios of fusion and fission events has recently been associated with disease [13,46,47]. Mitochondrial dynamics were severely altered in muscle of sIBM patients as demonstrated by the decreased expression of OPA1 and MFN2 transcript and protein levels. Deregulation of the mitochondrial fusion:fission ratio leads to abnormal mitochondrial turnover, which is essential for the recycling of damaged and dysfunctional mitochondria [48], such as those characteristic of sIBM patients.

It is interesting that reduced expression of MFN2 in sIBM patients is associated with abnormal mitochondrial genetics (both deletions and depletion). Previous studies have reported the strong dependence of mtDNA maintenance on proper mitochondrial dynamics in physiological conditions [10]. This is the first study to report this association in the context of sIBM disease.

Whether mitochondrial genetic and molecular alterations lead to abnormal mitochondrial dynamics or, on the contrary, to an

imbalance in mitochondrial dynamics underlying the mitochondrial genetic and molecular disturbances is still a matter of debate. In any case, both adverse conditions seem to go hand in hand with the development of sIBM disease.

In summary, in the present study we demonstrate that mitochondria are impaired in sIBM, not only at histological but also at genetic, molecular and functional levels, confirming the involvement of mitochondria in the aetiology of this disease. The lesion is characterized by different patterns in both muscle and PBMCs of sIBM patients, demonstrating that mitochondrial injury is not exclusive to the target tissue of the disease but is also present in peripheral tissues. It is interesting that mitochondrial COX activity normalized by mitochondrial mass is altered in both tissues, highlighting the relevance of this parameter, and the need for further studies on this as a putative candidate diagnostic tool or therapeutic target. In addition, deregulation in mitochondrial dynamics seems to be crucial for mtDNA stability, not only in healthy conditions [10] but also in sIBM disease. Deregulation of mitochondrial dynamics in muscle leads to increased genetic alterations such as mtDNA depletion and multiple deletions, demonstrating that mitochondrial turnover is essential for proper mitochondrial health and, furthermore, for muscle function. Although further investigation is needed to demonstrate whether mitochondrial alterations are primary or secondary in sIBM, the present study provides more in-depth understanding of the role of mitochondria in this disease. It also provides knowledge related to the mechanisms implicated in order to help develop new diagnostic tools or therapeutic targets for this disease, in the absence of non-invasive diagnostic tools or effective therapy.

CLINICAL PERSPECTIVES

- The better understanding of how mitochondria is in sIBM would be useful at clinical level at different levels: to confirm that mitochondrial changes seen in the muscle biopsy of sIBM patients is a solid marker to help diagnose this disease.
- This information could be useful to develop new therapeutical strategies to treat muscle dysfunction in sIBM patients.
- The knowledge about the pathogenic mechanisms of the disease allow us to elaborate a more accurate description of the disease for the patient, as they could be able to know about the disease they're suffering.

AUTHOR CONTRIBUTION

J.M. Grau, along with F. Cardellach and G. Garrabou, conceived the study and supervised the data collection and analysis. J.M. Grau was responsible for the diagnosis and inclusion of all patients, performed the IBMFRS test and collected all the clinical data with the help of C. Morén. M. Catalán-García was responsible for the experimental analysis of the amounts of mtDNA and mtRNA (with the help of M. Bañó and J. Enrich-Bengoá), for the analysis of the MRC complex IV activity and CS activity (with the help of A. Hernando), for the analysis of the oxidative stress and mitochondrial protein synthesis (with the help of M. Guitart-Mampel and I. González-Casacuberta), and for the analysis of mitochondrial mass

by Western blotting (with the help of D.-L. Juárez). MtDNA deletions were assessed by our collaborators S. Emperador and J. Montoya, and mitochondrial dynamics-related proteins were analysed through collaboration with A. Díaz-Ramos and A. Zorzano. E. Tobías provided technical assistance for reactive preparation. M. Catalán-García created a database to collect all clinical and experimental parameters and performed the statistical analysis of the data, under the supervision of G. Garrabou and C. Morén. All authors, especially G. Garrabou, J.M. Grau, J. Montoya and A. Zorzano, participated in the revision of the manuscript, adding new concepts of high relevance.

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