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Impaired Mitochondrial Function and Dynamics in the Pathogenesis of FXTAS

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Abstract Mitochondrial involvement plays an important role in neurodegenerative diseases. At least one-third of adult carriers of a *FMR1* premutation (55-200 CGG repeats) are at risk of presenting an adult-onset neurodegenerative disorder known as fragile X-associated tremor/ataxia syndrome (FXTAS). In an attempt to provide new insights into the mechanisms involved in the pathogenesis of FXTAS, we characterized mitochondrial function and dynamics by the assessment of oxidative respiratory chain function, mitochondrial content, oxidative stress levels, and mitochondrial network complexity. Regarding mitochondrial function, we found that mitochondrial respiratory capacity is compromised in skin fibroblasts whereas in blood, no differences were observed between the FXTAS and control groups. Furthermore, fibroblasts from FXTAS patients presented altered mitochondrial architecture, with more circular and less interconnected mitochondria being observed. Mitochondrial function and dynamics deregulation and characteristic of neurological disorders are present in FXTAS patients. These features might be limiting temporal and spatial bioenergetics cells supply and thus contributing to disease pathogenesis.

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Introduction

Fragile X-associated tremor/ataxia syndrome (FXTAS, OMIM #300623) is a late-onset neurodegenerative disorder that appears in at least one third of adult carriers of an *FMR1* premutation (55-200 CGG repeats) [1]. Clinical symptoms appear in patients over 50 years of age, and usually begin with an action tremor. Afterwards, different findings including ataxia, loss of sensation in the distal lower extremities, and autonomic dysfunction may occur and gradually progress [2, 3]. The neuropathological hallmark in FXTAS is the presence of intranuclear inclusions in neurons and astrocytes throughout the central nervous system, which are synuclein negative but do contain the expanded *FMR1* messenger RNA (mRNA) as well as several proteins that are expected to exacerbate cellular dysregulation [4]. These inclusions resemble the aggregates seen observed in other protein-mediated disorders such as Huntington disease (HD), Parkinson disease (PD), or Alzheimer disease (AD), in which similarly, neural cells from these patients have characteristic neurotoxicity effects [5].

Neurodegenerative diseases are a heterogeneous group of disorders characterized by gradually progressive, selective loss of anatomically or physiologically related neuronal systems, in which mitochondrial dysfunction and oxidative stress occur early in the majority of them [6]. Mitochondria are key regulators of cellular energy production and also play critical roles in cell cycle progression, differentiation, immune responses, lipid and calcium homeostasis, and apoptotic cell death [7, 8]. This range of mitochondrial functions is intimately linked to the mitochondrial architecture. Mitochondria are dynamic organelles that are constantly undergoing fusion and

fission events to adapt to cellular energetic demands and maintain the integrity of the mitochondrial network. Thus, it is not surprising that both mitochondrial dysfunction and aberrant mitochondrial network complexity have been associated with an increasing number of diseases such as PD, AD, and HD [9, 10].

Regarding FXTAS, mitochondrial dysfunction has been described in cultured fibroblasts and in postmortem brain tissue from FXTAS patients [11–15]. Although several studies support a role for mitochondrial dysfunction in FXTAS pathogenesis, mitochondrial dynamics has been poorly studied.

In an attempt to provide new insights into the mechanisms involved in the pathogenesis of FXTAS, in this study, we report the characterization of mitochondrial function and dynamics in patients with FXTAS with respect to control individuals.

Material and Methods

Subjects

Patients with FXTAS and control subjects with normal CGG repeat size (≤40 CGGs) were recruited for this study. All patients with FXTAS belong to fragile X syndrome families and were molecularly diagnosed at the Biochemical and Molecular Genetics Department of the Hospital Clinic of Barcelona, Spain, and clinically evaluated at the Neurology Service of the Hospital Santa Pau and Santa Creu of Barcelona. FXTAS group encompasses patients who met criteria in any of the three categories of involvement: definite, probable, and possible [3]. From the six FXTAS patients recruited (three males and three females), two out of three females were subjected to hysterectomy and one of them also presented hypothyroidism and fibromyalgia. No other comorbidities were reported. Control cohort consisted of healthy partners or family members of the FXTAS patients.

All participants provided written informed consent for testing and for the use of their phenotypic/clinical and genetic data. The study was approved by the Ethics Committee of the Hospital Clinic of Barcelona. The study was carried out in accordance with the Declaration of Helsinki (2013).

Blood Cells Isolation

Peripheral blood mononuclear cells (PBMC) were obtained from six FXTAS patients and six controls by a Ficoll density gradient centrifugation procedure as described elsewhere [16]. PBMCs were resuspended in phosphate saline buffer (PBS) and further divided and stored into aliquots at −80 °C until analysis.

Fibroblasts Culture

Skin biopsies were obtained using a 3-mm punch from three patients with FXTAS and three control individuals recruited from the Dermatology Department of the Hospital Clínic of Barcelona. The biopsy was diced under sterile conditions and then plated at 37 °C with a 5 % CO_2 atmosphere in T25 flasks in minimum essential media (MEM) 13 % containing 500-ml MEM (Gibco®) and 75-ml newborn calf serum (Gibco®) supplement with 0.30 ml penicillin (Gibco®) and 0.30 streptomycin (Gibco®). Trypan blue exclusion test was used to quantify the number cell viability and cell counts using a hemocytometer.

Mitochondrial Respiratory Function in PMBCs

Global and specific mitochondrial respiratory chain (MRC) complex I-stimulated oxygen consumption was measured by polarography at 37 °C in fresh PMBCs using a two-chamber Clark oxygen electrode (Hansatech Instruments Limited) as previously described [17]. Data recording was performed using the O_2 view software version 1.0.0.1. (Hansatech Instruments Limited). The oxygen uptake rates of mitochondria were evaluated in PBMCs as described previously [18]. Results were expressed as nanomoles of oxygen per minute and milligram of protein (nmol of O_2/min mg protein).

Mitochondrial Respiratory Function in Fibroblasts

To determine oxygen consumption of skin fibroblasts from FXTAS and control individuals, a million of living fibroblast cells were obtained and resuspended in cold mitochondrial respiration medium, MiR05 (Oroboros Instruments). High-resolution respirometry was performed at 37 °C by polarographic oxygen sensors in a two-chamber Oxygraph-2 k system according to manufacturer's instructions (Oroboros Instruments). The oxygen uptake rates of mitochondria were evaluated in fibroblasts as described previously [19]. Data recording was performed using the DatLab software v5.1.1.9 (Oroboros Instruments). Following manufacturer's recommendations, specific oxygen uptakes rates were obtaining by subtracting the rates from antimicine as is considered unspecific oxygen consumption. Oxygen consumption were normalized for the number of cells, thus, results are expressed as picomoles of oxygen per milliliter (pmol O_2/mL).

Mitochondrial Mass in PBMCs and Fibroblasts

To assess mitochondrial number, the spectrophotometric measurement of citrate synthase (CS) activity (EC 4.1.3.7) was performed in the presence of acetyl-CoA and oxaloacetate by monitoring the reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) at 412 nm. The absorbance changes were

followed in a HITACHI U2900 spectrophotometer using the UV-Solution software version 2.2. Data points were measured every 15 s during 4 min. CS activity was expressed as nanomoles of product per minute and milligram of protein (nmol/min mg protein).

Measurement of Reactive Oxygen Species Production

Lipid peroxidation was measured as an indicator of oxidative damage of reactive oxygen species (ROS) into cellular lipid compounds in PMBCs and skin fibroblasts using the BIOXYTECH® LPO-586™ by the spectrophotometric measurement of malondialdehyde (MDA) and 4-hydroxyalkenal (HAE) levels, according to the manufacturer's instruction (Oxis International Inc.). Fatty acid peroxide decomposition products were considered as indicators of ROS production. Results were normalized by protein content and expressed as micromolar of MDA and HAE per milligram of protein (μM MDA + HAE/mg protein).

Mitochondrial Network Complexity Analysis in Cultured Skin Fibroblasts

Cultured skin fibroblasts were washed with PBS before fixation with 4 % paraformaldehyde for 15 min. Fixed cells were washed, permeabilized with 0.1 % Triton X-100 incubated in blocking solution (1 % bovine serum albumin). Immunochemistry for the mitochondrial network complexity was performed using three different labeling: Tom20 antibody (Santa Cruz Biotechnology), Wheat Germ Aglutinin Alexa Fluor 594 conjugate probe (Life Technologies), and TOPRO3 IODIDE probe (Life Technologies) as previously described [20]. Samples were analyzed by confocal microscopy using a Leica TCS SL laser scanning confocal spectral microscope (Leica Microsystems Heidelberg GmbH.). A minimum of 10 fibroblasts from each individual were visualized and analyzed using the ImageJ software (www.fiji.sc) and a macro of instructions previously described [20]. Mitochondria

network of each cell was then subjected to particle analysis and aspect ratio (AR = major axis/minor axis) and circularity (4π·area/perimeter 2) parameters were measured. Form factor (FF) was calculated as the inverse of circularity. AR and FF values are considered parameters of mitochondria health. An AR value of 1 indicates a perfect circle, and as mitochondria elongates and become more elliptical AR increases. An FF value of 1 corresponds to a circular unbranched mitochondrion, while higher FF values indicate a longer more branched mitochondrion.

Statistical Analysis

Results were expressed as mean ± standard error means (SEM). Statistical analysis was performed using the nonparametric Mann–Whitney U test using commercially available software (SPSS-PC, version 18.0; SPSS Inc., Chicago, IL, USA). Significance was accepted for asymptotic bilateral P values below 0.05.

Results

All subjects included in this study were unrelated *FMR1* premutation carriers ascertained through fragile X syndrome families that presented with FXTAS symptoms, including tremor and/or ataxia and controls in a similar age group range. Table 1 summarizes clinical and molecular data of individuals recruited in this study.

In order to assess mitochondrial function, mitochondria respiration was determined in PBMCs and skin fibroblasts from controls and patients with FXTAS. While results obtained from PBMCs did not show statistically significant differences between groups, data from fibroblasts revealed a significant decline in the oxidative phosphorylation (OXPHOS) capacity in patients with FXTAS (Table 2). In particular, the basal oxygen consumption in intact cells (routine) were statistically significantly reduced in patients

Table 1 Characteristics of the individuals recruited in this study

		Controls	FXTAS
Peripheral blood mononuclear cells	Age (years)[a]	61.17 ± 1.96	67.83 ± 5.62
	Sample size (n)	6	6
	Females (n)	3	3
	Males (n)	3	3
	(CGG) n[a]	≤35	109.5 ± 12.96
Fibroblasts	Age (years)	75 ± 9.07	60.67 ± 5.84
	Sample size (n)	3	3
	Females (n)	1	2
	Males (n)	2	1
	(CGG) n[a]	≤30	103 ± 16.64

[a] Results are expressed as mean values ± standard error mean

Table 2 Rates of oxygen uptake in skin fibroblasts. Results are expressed as mean values ± standard error mean

	Oxygen consumption (pmol O_2/s ml)				
	Routine respiration	Stimulated by complex I	Stimulated by complex I + II	Inhibiting complex I	Inhibiting complex I + II
Control	43.01 ± 5	22.45 ± 6.2	34.42 ± 9.2	25.39 ± 4.6	3.48 ± 1.7
FXTAS	13.98 ± 6	2.81 ± 1.0	3.43 ± 3.3	5.39 ± 5.1	2.80 ± 1.4
p value*	$p = 0.05$	$p = 0.05$	$p = 0.05$	$p = 0.05$	–

*Significance was accepted for asymptotic bilateral p values ≤0.05

with FXTAS relative to controls when using endogenous substrates ($P = 0.05$). Furthermore, the oxidative capacities under stimulation of mitochondrial complex I with NAD-linked substrates (glutame/malalte/piruvate oxidation (GMPox)) as well as under the combined stimulation of mitochondrial complexes I and II by the addition of the FAD-linked substrate succinate (glutame/malalte/piruvate/succinate oxidation (GMPSox,)) were also statistically significantly reduced in patients with FXTAS compared with controls ($P = 0.05$). Finally, the inhibition of the complex I with rotenone, indicative of complex II-stimulated oxygen consumption, showed a significant reduction in the oxygen uptake in patients with FXTAS compared to controls ($P = 0.05$). The residual oxygen consumption determined by the inhibition of both complexes I and II, considered unspecific oxygen consumption and thus used as an internal methodological control, showed similar rates in both groups (Table 2).

The activity of CS enzyme was determined in PMBCs and in cultured skin fibroblasts. Results showed that patients with FXTAS have similar mitochondrial content relative to controls in both tissues (Supplementary Table 1); thus, the reduction in the OXPHOS capacity detected in skin fibroblasts could not be explained by lower mitochondria content.

Oxidative stress measured by the rate of lipid peroxidation did not show statistically significant differences between patients and controls. However, ROS production was 20 % enhanced in FXTAS PMBCs and 30 % in cultured skin fibroblasts from patients with FXTAS with respect to the control group (Fig. 1).

Finally, mitochondrial network complexity assessed in cultured skin fibroblasts revealed a more disorganized mitochondrial network in patients with FXTAS (Fig. 2a, b). The average circularity was significantly higher in patients with FXTAS ($p = 0.05$) (Fig. 2c), indicating that mitochondria in these individuals are less elongated than those of controls. Furthermore, AR and FF parameters associated with mitochondria length and branching, respectively, were decreased in fibroblasts from patients with FXTAS when compared to controls (Fig. 2d, e). Additionally, the positive significant correlation between mitochondria AR and FF detected in control fibroblasts, indicative of mitochondria health status, was lost in FXTAS patients (Fig. 2f).

Discussion

In this study, we assessed mitochondrial function, biogenesis, and dynamics in FXTAS patients and controls. Our results showed that mitochondrial respiratory capacity and dynamics are compromised in skin fibroblasts whereas in blood, no differences were observed between groups. Since blood is not a highly energy-dependent tissue, it may be argued that this cell type is not suitable for assessing metabolic mitochondrial function, at least in FXTAS patients.

In cultured skin fibroblasts from patients with FXTAS, characterization of mitochondrial function showed a statistically significant decrease in basal mitochondrial respiration and a reduction of oxidative activities related to the stimulation of mitochondrial complexes I and II (Table 2). These changes were not attributable to a lower density of mitochondria per cell as confirmed by the analysis of CS activity. Our results are in agreement with previous reports in which a lower OXPHOS capacity was described in cultured skin fibroblasts from FXTAS patients [11, 12]. Moreover, we demonstrated a reduced capacity of basal mitochondrial respiration and no significant alterations in residual oxygen consumption (Table 2). Based on these results, we can conclude that the changes observed in mitochondrial oxygen measures in cultured skin fibroblasts from FXTAS patients are specifically mitochondrial and not due to alteration of other cellular
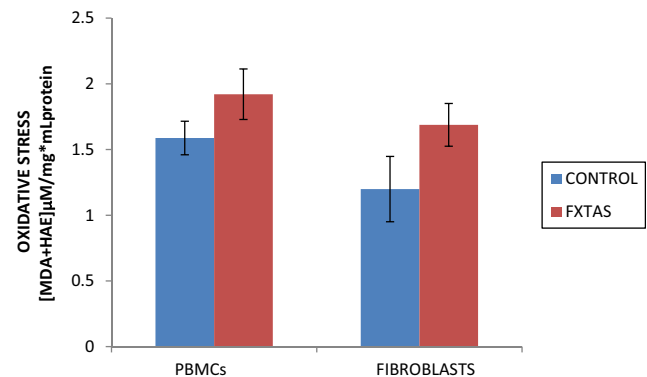

Fig. 1 Quantification of ROS production in patients with FXTAS and control individuals from PBMCs and skin fibroblasts. Results are expressed as micromolar means ± SEM of MDA and HAE per milligram of protein (µM MDA + HAE/mg protein)

Fig. 2 Morphological analysis and mitochondrial architecture in fibroblasts from patients with FXTAS and control individuals. **a**, **b** Images of mitochondria stained with Tom20 antibody (*green*). A more disorganized mitochondrial network with increased circular mitochondria is shown in FXTAS cells. **c**–**f** Mitochondrial network complexity in skin fibroblasts from patients with FXTAS and control individuals. Circularity, aspect ratio, and form factor, parameters associated with mitochondria length and branching, are represented as mean ± SEM. Statistically significant difference was observed in circularity parameter in fibroblasts from FXTAS (*n* = 3) relative to control (*n* = 3). The positive correlation between mitochondria aspect ratio and form factor in skin fibroblasts from controls was lost in patients with FXTAS
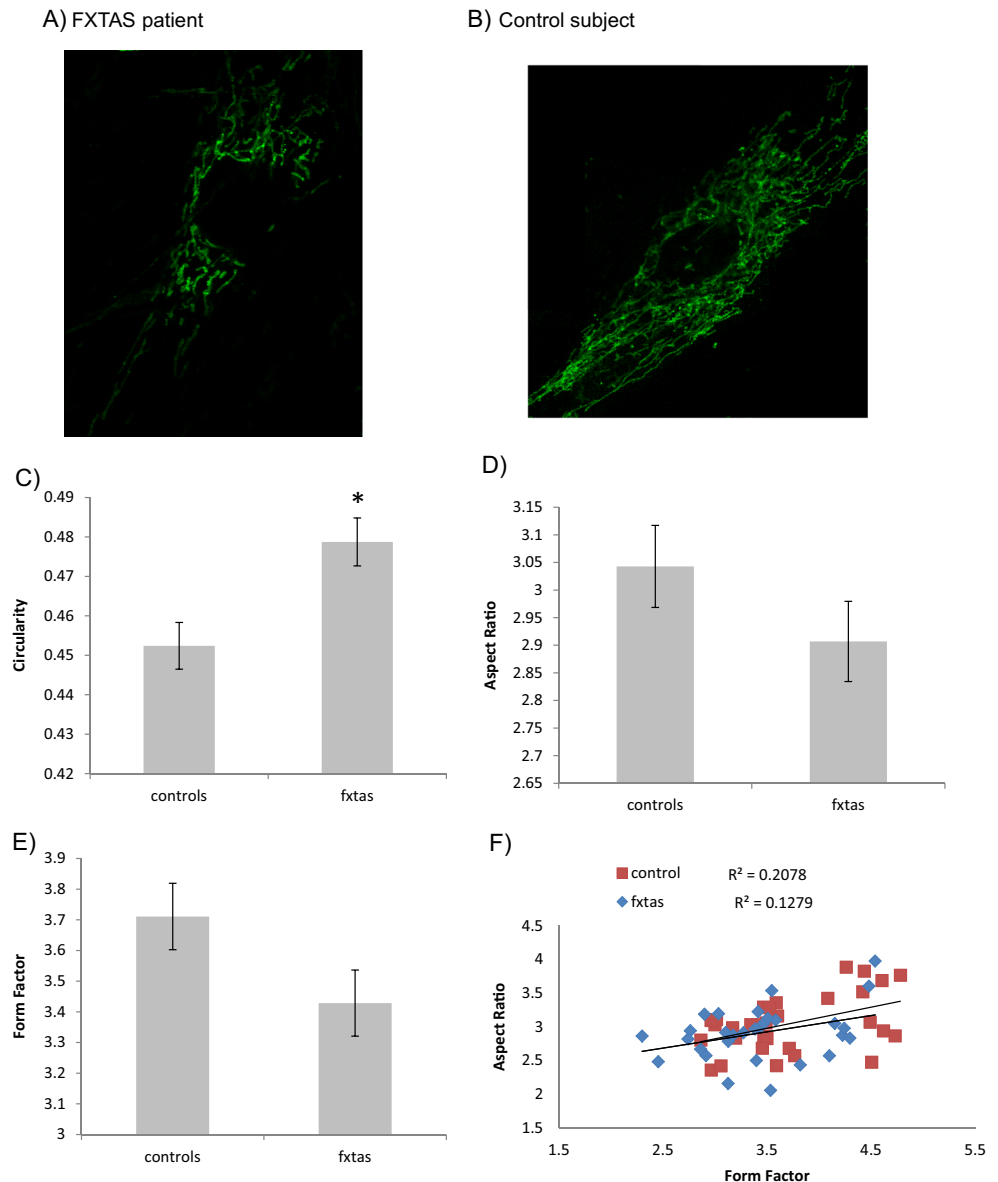
oxygen-consuming processes such as catalase or autophagy activities. Collectively, our data point to a compromised bioenergetic capacity in skin fibroblasts of FXTAS patients. This, in turn, might lead to a lack of energy in times of higher demand such as, for example, under stress conditions; especially in tissues highly dependent on oxidative metabolism as neurons [21].

Inefficiencies of OXPHOS capacity enhance ROS generation, which are a collection of highly unstable molecules that attack cellular structures and cause oxidative stress damage, triggering apoptotic mechanisms [9]. The measurement of ROS production by rates of lipid peroxidation in PBMCs and skin fibroblasts showed oxidative stress in patients with FXTAS, albeit without statistical significance in any of the tissues assessed (Fig. 1). These results do not seem to correlate with OXPHOS capacity, where only impairment in

fibroblasts has been identified. Differential OXPHOS/ROS rates in both cell types might be a result of the distinct cell life span and turn-over, oxidative energy-dependence, and embryologic origin of these tissues which may make fibroblasts more prone to OXPHOS/ROS deregulation. Likewise, previous studies have described enhanced ROS production in skin fibroblasts from patients with FXTAS by measuring other oxidative stress markers [11, 12]. An imbalance of net ROS production can cause oxidative damage which is known to have a major role in cognitive decline related to normal aging as well as in other neurodegenerative diseases such as AD or PD [22]. However, the exact mechanisms of excessive accumulation of ROS in these patients affected with neurodegenerative diseases still remain elusive. The free radical theory of aging states that a vicious cycle of free radical damage harming mtDNA and OXPHOS machinery

would lead to the feedforward production of more ROS [23]. Nevertheless, the assessment of oxidative stress in the in vivo human brain is still a challenge.

Mitochondria form interconnected networks, the biogenesis and structure of which are highly influenced by the needs of the cell [8]. The shape and cellular distribution of the mitochondrial network is maintained largely by the conserved activities of mitochondrial fission, fusion, motility, and tethering [8]. Through these mechanisms, mitochondria continuously merge and divide to share solutes, metabolites, genomes, and proteins to promote mitochondrial removal and turn-over. Understanding how mitochondrial dynamics is linked to cellular pathophysiology is currently the subject of intense study in a wide range of disorders [7]. Impairments in the regulation and function of mitochondria network connections have been associated with aging and disease, including metabolic disorders, cancer, and neurodegeneration. Mitochondrial dynamics in FXTAS has been poorly investigated. To our knowledge, only Kaplan and collaborators [13] have described a deficit in the dynamics of mitochondrial movement in hippocampal neurons in a mouse model of FXTAS. These deficits were found in early development, suggesting that they may be a primary etiologic process in disease development and that they might contribute as a risk factor for developing FXTAS. In agreement, Napoli and collaborators have recently reported mitochondrial network disruption in young asymptomatic FMR1 premutation carriers, presenting more fragmented and disorganized mitochondrial network [24]. Our study showed that skin fibroblasts from patients with FXTAS also present an alteration of the mitochondrial network (Fig. 2a, b). More circular and thus less elongated mitochondria were found, suggesting reduced fusion processes and defective mitochondrial intercommunication. Moreover, the loss of a positive correlation between mitochondria elongation and branching in FXTAS showed less interconnected mitochondria. While Napoli and co-workers reported a strong direct correlation between CGG repeat number and degree of the mitochondrial network fragmentation [24], the parameters of mitochondrial health measured in our study failed to evidence this correlation in patients with FXTAS. Nevertheless and due to the limited number of samples studied, a size effect can not be completely discarded.

It is known that the maintenance and operation of the mitochondrial network is crucial in mitochondrial bioenergetics. In fact, alteration of mitochondrial dynamics can cause OXPHOS defects due to the inability of the mitochondrial network to respond to physiological requirements [25]. These observations together with our results suggest that the aberrant mitochondrial architecture observed in skin fibroblasts from FXTAS patients triggers mitochondrial dysfunction.

Overall, mounting evidence indicates a role of mitochondria in the pathological process of neurodegenerative diseases, albeit, it is unclear whether mitochondrial impairment is actually involved in its onset and progression or is a consequence of neurodegeneration. In FMR1 premutation carriers, altered bioenergetics and mitochondrial network has been evidenced in fibroblasts from young individuals (29 years) [24] and in hippocampal neurons from P1 postnatal mice [13]. Furthermore, fibroblasts from older (>60 years) permutation carriers without FXTAS also exhibit mitochondrial bioenergetics dysfunction, albeit is more severe in those with FXTAS [11]. Interestingly, mitochondrial impairment increases longitudinally in FMR1 premutation carriers not only according to disease onset, but also according to age. Thus, the potential effect of mitochondrial aging in FMR1 premutation carriers may also be playing a role in observed features. However, in all the reported studies, age and gender-matched controls always showed healthier mitochondrial status than patients, confirming mitochondrial involvement in the disease. Altogether, mitochondrial dysfunction might appear as an early event in FMR1 premutation carriers and it turns more enhanced in FXTAS patients, suggesting that mitochondrial impairment is an incipient pathological process of FMR1 premutation and is directly involved in the pathophysiology of FXTAS.

This study has two main limitations. First, the small sample size might explain the lack of significance in some of the analyses. Second, skin fibroblasts might not constitute as an appropriate model due to clonal selection and drift in culture, and thus, it is likely that cells with lower viability were less represented. On the other hand, skin fibroblasts form dynamic cell-cell contacts similar to neurons and also reflect age-dependent cumulative cell damage. Regarding FXTAS, it has been demonstrated that fibroblast exhibit overexpression of FMR1 transcript mimicking the levels observed in neurons [12].

Mitochondrial function and dynamics must be tightly regulated in order to temporally and spatially satisfy the bioenergetic needs of any cell type, especially neurons, which almost entirely depend on mitochondrial function to satisfy energetic demands. In the present study, we found that mitochondrial respiration as well as architecture are altered in skin fibroblasts from FXTAS patients, indicating that both are implicated in the pathophysiology and/or etiology of neurological symptoms.

Compliance with Ethical Standards

Conflict of Interest Statement All authors have read the journal's policy on conflicts of interest. The authors declare no conflict of interest.

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