Mitochondrial structural alterations in fibromyalgia: a pilot electron microscopy study

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Abstract

Objective

The pathogenesis of fibromyalgia (FM), characterised by chronic widespread pain and fatigue, remains notoriously elusive, hampering attempts to develop disease modifying treatments. Mitochondria are the headquarters of cellular energy metabolism, and their malfunction has been proposed to contribute to both FM and chronic fatigue. Thus, the aim of the current pilot study, was to detect structural changes in mitochondria of peripheral blood mononuclear cells (PBMCs) of FM patients, using transmission electron microscopy (TEM).

Methods

To detect structural mitochondrial alterations in FM, we analysed PBMCs from seven patients and seven healthy controls, using TEM. Patients were recruited from a specialised Fibromyalgia Clinic at a tertiary medical centre. After providing informed consent, participants completed questionnaires including the widespread pain index (WPI), symptoms severity score (SSS), fibromyalgia impact questionnaire (FIQ), beck depression inventory (BDI), and visual analogue scale (VAS), to verify a diagnosis of FM according to ACR criteria. Subsequently, blood samples were drawn and PBMCs were collected for EM analysis.

Results

TEM analysis of PBMCs showed several distinct mitochondrial cristae patterns, including total loss of cristae in FM patients. The number of mitochondria with intact cristae morphology was reduced in FM patients and the percentage of mitochondria that completely lacked cristae was increased. These results correlated with the WPI severity. Moreover, in the FM patient samples we observed a high percentage of cells containing electron dense aggregates, which are possibly ribosome aggregates. Cristae loss and possible ribosome aggregation were intercorrelated, and thus may represent reactions to a shared cellular stress condition. The changes in mitochondrial morphology suggest that mitochondrial dysfunction, resulting in inefficient oxidative phosphorylation and ATP production, metabolic and redox disorders, and increased reactive oxygen species (ROS) levels, may play a pathogenetic role in FM.

Conclusion

We describe novel morphological changes in mitochondria of FM patients, including loss of mitochondrial cristae. While these observations cannot determine whether the changes are pathogenetic or represent an epiphenomenon, they highlight the possibility that mitochondrial malfunction may play a causative role in the cascade of events leading to chronic pain and fatigue in FM. Moreover, the results offer the possibility of utilising changes in mitochondrial morphology as an objective biomarker in FM. Further understanding the connection between FM and dysfunction of mitochondria physiology, may assist in developing both novel diagnostic tools as well as specific treatments for FM, such as approaches to improve/strengthen mitochondria function.

Key words

fibromyalgia, mitochondria, transmission electron microscopy, morphology, cristae, ribosome aggregation

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Introduction

Fibromyalgia (FM) is a debilitating condition characterised by chronic widespread pain and a broad spectrum of other somatic as well as psychological manifestations, including fatigue, muscle pain, irritable bowel syndrome, connective tissue and joint pain, sleep disturbances and many more.

The pathogenic mechanisms of FM remain elusive. Environmental stress factors such as infection and trauma (*e.g.* motor vehicle accidents) may trigger the onset of FM, but the precise pathogenetic pathways are not clear (1-4). Mitochondria are essential for energy/ATP production through oxidative phosphorylation (OXPHOS), and the generation/breakdown of many metabolites. The critical role of mitochondria in cellular energy production and metabolism implies the possibility of a pathogenetic role for mitochondrial malfunctions in FM (5-7).

Oxidative stress, mitochondrial dysfunction, and inflammation have been implicated as possible contributing factors in the pathogenesis of FM (8). Elevated oxidative stress, arising from damaged mitochondria, may impact on widespread pain and sensitisation (9). Recent studies have demonstrated decreased levels of ATP and coenzyme Q10, alongside increased ROS production in peripheral blood mononuclear cells (PBMCs) and in skin cells taken from FM patients, indicating heightened cellular oxidative stress. Utilising EM, it was possible to document the presence of autophagosomes containing mitochondria in PBMCs (8, 10). Findings included reduced mitochondrial mass, downregulated mitochondrial biogenesis genes (PGC-1α, TFAM, NRF1), reduced mitochondrial DNA content, and reduced respiratory complex activity (11). Muscle tissue analysis in FM patients has also shown changes in mitochondria morphology and numbers (12, 13).

FM-associated symptoms such as fatigue, exercise intolerance, and myalgia are also common in primary mitochondrial diseases, typically stemming from mitochondrial dysfunction due to nuclear or mitochondrial DNA mutations (14, 15). The current pilot study aimed at investigating mitochondrial structural changes in PBMCs of FM patients *versus* healthy controls by employing transmission electron microscopy (TEM).

Patients and methods

Ethical statements

The study protocol was approved by the Weizmann Institutional Review Board (IRB) according to the principles of the Declaration of Helsinki (0625-19-TLV). All patients signed an informed written consent.

Patients and controls

Patient enrolment and blood collection

We studied 7 FM patients, all of whom fulfilled ACR Fibromyalgia diagnostic criteria, and 7 healthy controls. Inclusion criteria were females diagnosed with primary FM according to the American College of Rheumatology (ACR) 2016 diagnostic criteria (16), Age ≥18 years old, BMI <30. Exclusion criteria included male gender, known diagnosis of inflammatory joint disorder (*e.g.* rheumatoid arthritis, spondyloarthropathy, systemic lupus erythematosus, etc.), BMI ≥30, diabetes mellitus, active malignancy, active and/or chronic infection, current psychotic disorders, pregnancy, and patients unable to provide informed consent.

All patients and control subjects had taken no Cannabis products during at least 4 years before blood collection. All samples were from women who had not reported having contracted COVID-19 and had not received COVID-19 vaccine. Participants were asked to fill out questionnaires according to the study protocol, which included demographic data: age, sex, occupation, employment and disability status, weight, height, smoking status, use of medications and supplements (including coenzyme Q-10), co-morbidities, previous medical history, widespread pain index (WPI) documenting the extent of widespread pain, symptoms severity score (SSS) documenting the severity of associated symptoms, validated Hebrew version of the fibromyalgia impact questionnaire (FIQ) (17, 18), visual analogues scale (VAS) and a validated Hebrew version of the fibro-

myalgia fatigue scale (19, 20). Healthy control subjects had no signs/symptoms of FM and were free of any medication (except of one participant). The clinical characteristics of each group are shown in Table I.

Sample collection and PBMC preparation

Patients and controls were recruited from social media and from the Rheumatology Clinic at the Tel Aviv Sourasky Medical Center. The study protocol involved a single visit on behalf of each participant. After signing informed consent, coagulated blood of patients and healthy control subjects were obtained. Three tubes (of 9 ml) with EDTA and one tube for Erythrocyte Sedimentation Rate (ESR), total volume of approximately 30 CC of blood was collected. ESR test was conducted at the collection site (Rheumatology Department), according to standard clinical procedure. PBMCs were isolated from blood samples through a Ficoll-Paque Plus (catalog no. GE17-1440-03; Sigma) gradient centrifugation on the day of sample collection. Isolated cells were fixed with 4% paraformaldehyde, 2% glutaraldehyde in 0.1 M cacodylate buffer containing 5 mM $CaCl_2$ (pH 7.4) for 2 hours at room temperature and then transferred to 4°C for 24 hours. Cells were washed 3 times with 0.1 M cacodylate buffer. Cells were transferred from the hospital to the Weizmann Institute of science in a cooler with ice for processing and analysis.

Transmission electron microscopy (TEM)

PBMCs were postfixed with 1% osmium tetroxide (EMS, Hatfield, PA) supplemented with 0.5% potassium hexacyanoferrate trihydrate and potassium dichromate in 0.1 M cacodylate (1 hr), stained with 2% uranyl acetate (EMS) in water (1 hr), dehydrated in graded ethanol solutions and embedded in Agar 100 epoxy resin (Agar scientific Ltd., Stansted, UK). Ultrathin sections (70-90 nm) were obtained with a Leica EMUC7 ultramicrotome and transferred to 200 mesh copper transmission electron microscopy grids (SPI, West Chester, PA). Grids were stained with lead citrate and examined with a Tecnai SPIRIT transmission electron microscope (Thermo Fisher Scientific). Digital electron micrographs were acquired with a bottom-mounted Gatan OneView camera.

High resolution TEM (HR TEM) and High-angle-annular dark-field STEM Energy Dispersive X-Ray Spectroscopy (HAADF STEM EDS) analysis was done on FEI Tecnai G2 F20 S/TEM microscope equipped with Energy dispersive spectrometer (EDAX) with retractable, liquid N2 cooled, Si(Li) detector.

Morphometric analysis

Morphometric analyses of TEM images were performed with the Fiji Software on a sample of 30 systematically, uniformly, and randomly selected images. The total area of the cell was calculated, as well as the nucleus area. After that, all the mitochondria in the cell were measured for certain properties, and then each one was typed according to its cristae structure. The total area of the cytosol was calculated as the cell area minus the nucleus area. All samples were normalised to the cytosol area. The mitochondria were classified to types, as shown in Figure 2A. Approximately 5000 mitochondria were analysed from all cells.

Statistical analysis

All results are expressed as mean ± SD unless stated otherwise.

We used a linear mixed model in all the statistical analyses using R software. Significance codes: $p<0.001$ (***), $p<0.01$ (**), and $p<0.05$ (*) were considered statistically significant. Analysis of electron dense aggregates was done by Welch two sample t-test.

Table I. Demographic and clinical parameters of patients and healthy controls.

Variables	Controls (n=7)	FM patients (n=7)
Age (years)	35.4 ± 11.1	41.8 ± 12
Duration of disease from the moment of diagnosis (years)	- 6 ±3	.8
BMI (kg/m^2)	21.6 ± 3.2	25.4 ± 2.8
FIQ total score (0-100)	-	77.2 ± 18.5
VAS pain (0-100)	-	76.4 ± 21.6
Fatigue scale (0-72)	-	31 ± 9.7
WPI (0-19)	-	12.4 ± 3.9
WPI regions (0-5)	-	4.7 ± 0.7
SSS (0-12)	-	9.5 ± 1.9
BDI (0-105)	24 ± 3.2	37.2 ± 10.4*
ESR (mm/hr) *n=6 for control group (0-30 normal range)	13.1 ± 8.2	13 ± 8.6

*All values are shown as means ±. *p value is significant when <0.05.
FM: fibromyalgia; BMI: body mass index; FIQ: Fibromyalgia Impact Questionnaire; VAS: Visual Analogue Scale; WPI: widespread pain index; SSS: symptoms severity score; BDI: Beck depression inventory; ESR: erythrocyte sedimentation rate.

Results

General characteristics of population

Demographic and clinical characteristics of FM patients and controls are shown in Table I. Age, BMI, and ESR were statistically similar in groups ($p>0.05$). The mean duration of disease in the FM group was 6±3.8 years. BDI score was significantly higher in patients with FM than controls ($p<0.05$).

FM patient PBMCs possess mitochondria with partial or no cristae

TEM analysis of PBMCs taken from FM patients or healthy controls show different cristae patterns (Fig. 1). Healthy control PBMCs show mitochondria with typical visible cristae (top images), whereas FM patient PBMCs show mitochondria with partial or no cristae (bottom images).

FM patient samples possess less mitochondria as compared to samples from healthy controls

TEM analysis of PBMCs showed different cristae patterns, which were classified into three types, as previously

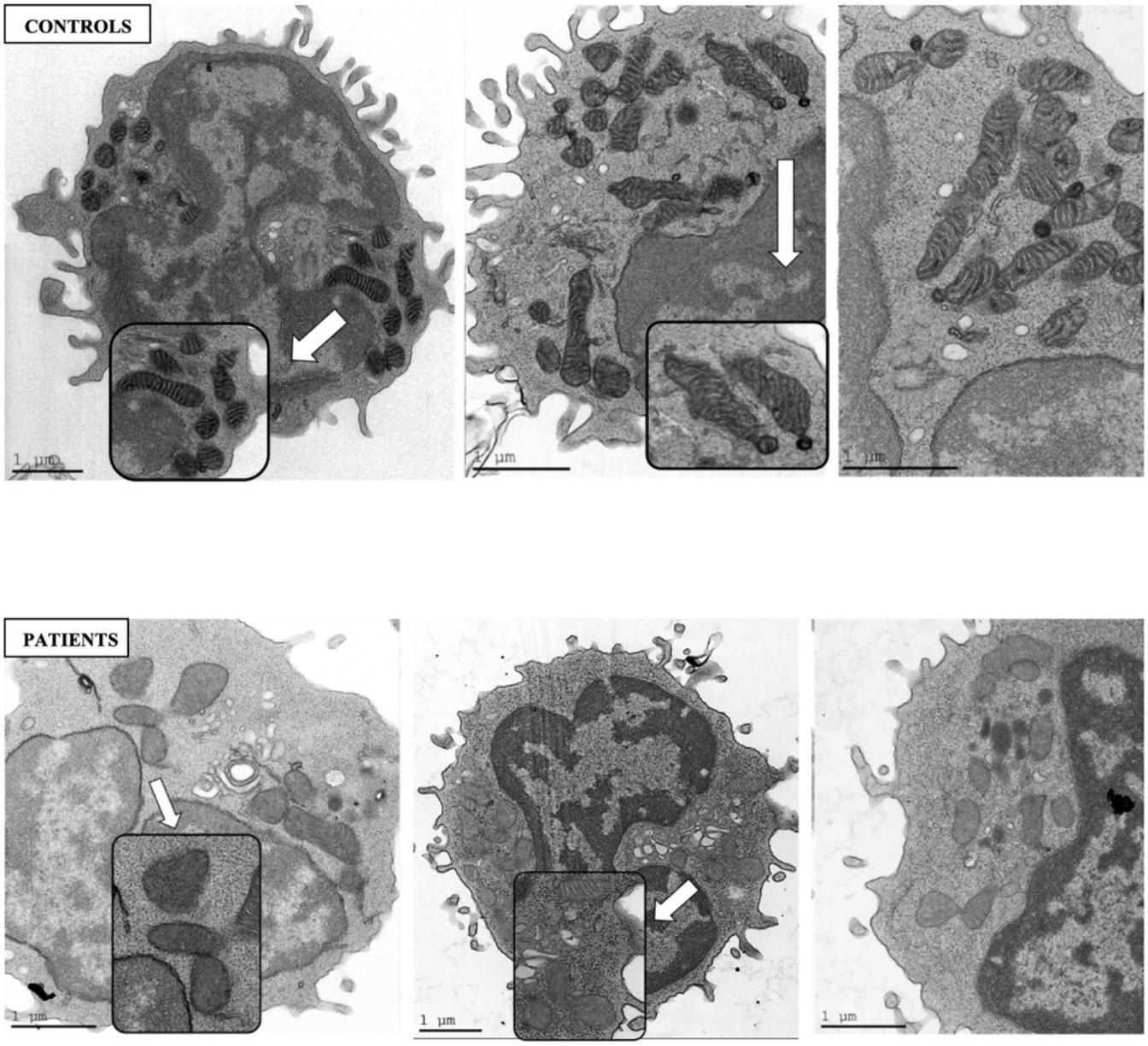

Fig. 1. Changes in cristae structure of mitochondria of FM patients in PBMCs. TEM images showing the structure of PBMCs from healthy controls and FM patients (each image represents a different participant).
Top: Controls PBMCs show mitochondria with typical visible cristae (a high magnification image appears on the right).
Bottom: FM patients PBMCs show mitochondria with partial or no cristae (a high magnification image appears on the right). Bar = 1 µm.

shown (20) (Fig. 2A). To determine the mitochondria content in these cells, we summed the area of all mitochondria of each cell and then normalised it to the cytosol area (as explained in the *Materials and methods* section). To distinguish between mitochondria with any visible crista appearance (which may represent the more functional ones) to the type 3 mitochondria, we analysed the area of mitochondria types 1+2. We observed a decrease in total mitochondria content of type 1 and 2 in FM patients as compared with healthy controls ($p<0.05$) (Fig. 2E).

FM patient samples possess more mitochondria that lack cristae

The mean area of type 1 mitochondria from the total mitochondria area was reduced in patient samples as compared to healthy samples by 14.3 % (~0.246 median value compared to 0.39; $p=0.0202$ (Fig. 2C)). This reduction was accompanied by a trend-increase in mitochondria with partial cristae (type 2) and of mitochondria without cristae (type 3) in FM cells, as compared to cells from healthy controls ($p<0.1$; Fig. 2B).
Interestingly, we found a ~8.3% increase in type 3 mitochondria in FM patient cells, which was "complementary" to the reduction in type 1 mitochondria (0.26 median in FM patient cells as compared to 0.17 in healthy cells).
Since there were many cells in the FM group without any mitochondria of type 1, we performed an analysis to quantify these cells. The number of patient cells with no mitochondria of type 1 was 16.7% higher than in healthy controls (Fig. 2D; $p=0.00013$). The median percentage of cells in the healthy group was 6.7% compared to 23.3% in patients.

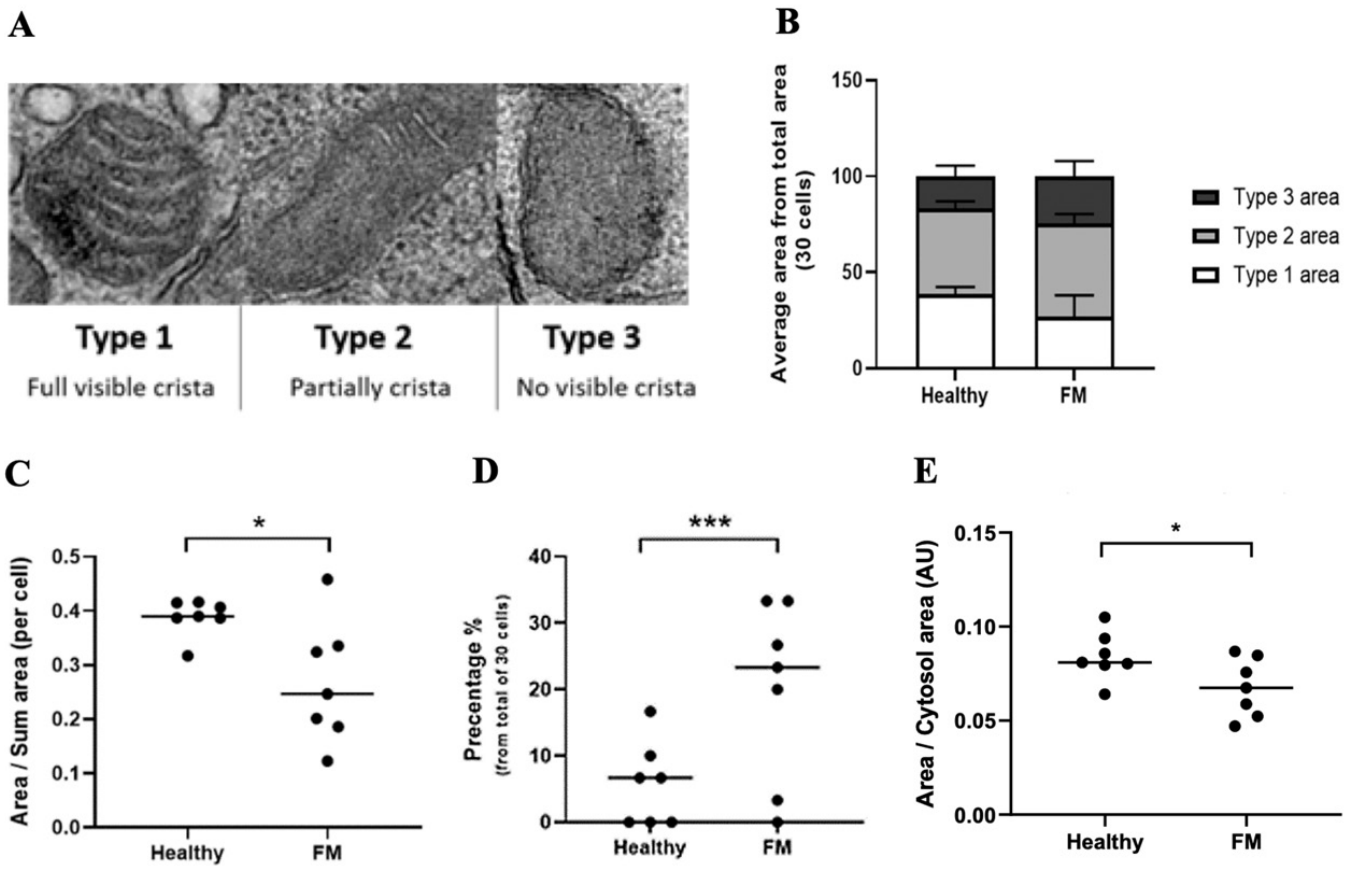

Fig. 2. Mitochondria content in PBMCs from FM patients (n=7) compared to healthy controls (n=7).
A: Classification of mitochondria to types according to crista appearance by TEM. **B**: Average mitochondria area according to types from the total mitochondria area (of all 3 types); error bars represent SD. The value is the mean areas/total area of 30 cells. **C**: Sum area of mitochondria type 1; mitochondria average area according to type classification measured by total area of mitochondria specific type from the total area content (of all 3 types). **D**: Percentage of cells without any mitochondria of type 1, measured from a total of 30 cells. **E**: Mitochondria content: sum area of mitochondria types 1 and 2 (normalised to the cytosol); the value is the mean areas/cytosol of 30 cells. Black lines represent the median. Each dot represents a different patient.
Significance codes: $p<0.05$ (*), $p<0.001$ (***) was considered statistically significant.

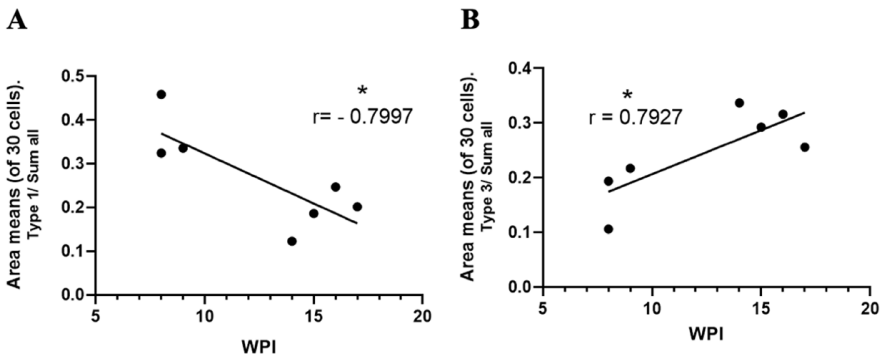

Fig. 3. Correlations of mitochondria analysis with patient's clinical data.
A: Ratio of type 1 mitochondria area (from the total area of all types) *versus* widespread pain index (WPI). **B**: Ratio of type 3 mitochondria area (from the total area of all types) versus WPI. Each dot represents a patient. The correlations were established by calculating r Pearson's correlation coefficient (set as meaningful if r>0.7 or r<-0.7). Lines represent regression lines. $p<0.05$ (*).

The increase in cristae-less mitochondria is in correlation with the clinical widespread pain index (WPI) of FM patients

Widespread pain index (WPI) was correlated with both type 1 and type 3 mitochondria ratio areas. We found a negative correlation with type 1 ratio area and a positive correlation with type 3 ratio area (Fig. 3A and B, respectively). We did not find correlations with other parameters (like VAS score, FIQ, BDI and other parameters as shown in Table I).

FM patient cells possess increased levels of electron dense aggregates

In some FM samples, we observed a high percentage of cells with increased levels of electron dense aggregates (Fig. 4A). In an attempt to determine the identity of these aggregates, high resolution TEM imaging with HAADF STEM Energy Dispersive X-Ray Spectroscopy (EDS) were done. EDS measurements showed that those agglomerates made mostly from C, Os and Pb, where Os and Pb are coming from the TEM sample prep (Fig 4B). HR TEM revealed changes in the dispersion of these particles (Fig. 4C). Based on

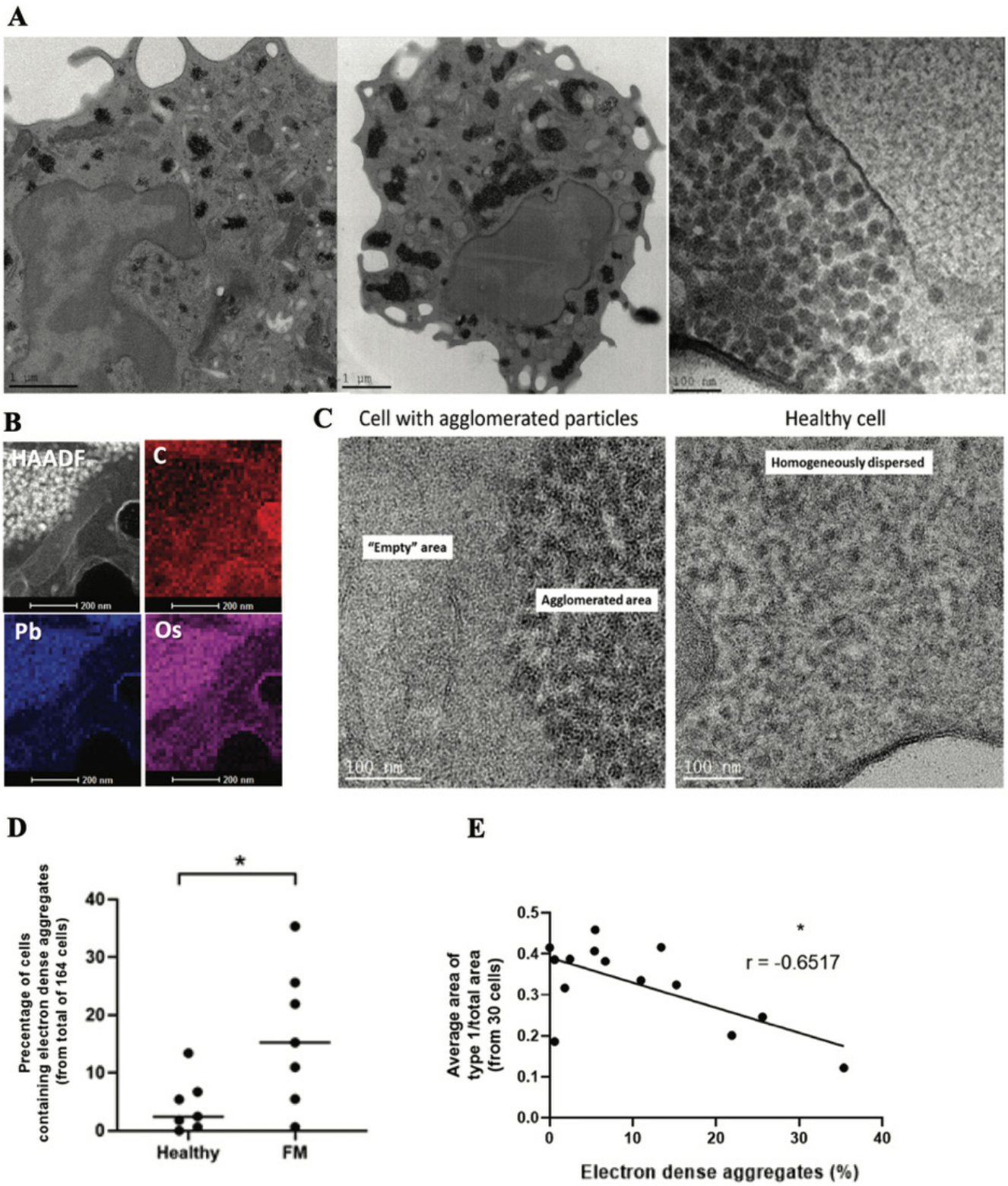

Fig. 4. FM patient cells possess increased levels of electron dense aggregates.
A: Pictures taken by transmission electron microscopy (TEM) from FM patient PBMCs, showing accumulation of electron dense aggregates. Right: high magnification of the particles. Visible mitochondria observed only in the initial stage (left picture). **B**: HAADF STEM EDS elemental maps; **C**: TEM images show a comparison between two cells at the same magnification: Right image, healthy cell with the particles distributed homogeneously. Left image, patient cell with agglomerated particles. Size of particles was similar for both types of cells. **D**: Percentage of PBMCs containing electron dense aggregates in FM patients (n=7) *vs*. healthy controls (n=7). Calculation was made from a total of 164 cells. Each dot represents a participant. Black lines represent the median analysed by Welch Two Sample t-test. $p<0.05$ (*). **E**: Mean type 1 area/total area *vs*. percentage of cells with electron dense aggregates (from a total of 164 cells). Each dot represents a patient. The correlations were established by calculating r Pearson's correlation coefficient (set as meaningful if r>0.7 or r<-0.7). Lines represent regression lines. $p<0.05$ (*).

these findings and on their size (~22 nm) and shape, we speculate that these are ribosome aggregates.

This phenomenon was also observed in healthy samples, but with a low percentage of appearance, with a mean of 4.7% of cells compared to a mean of 36.5%, 25.6%, and 21.9% in FM patients. Percentages were calculated according to the presence or lack of the particles from a total of 164 cells, as shown in Fig. 4D (changes were observed with a median of 2.4 in healthy controls *vs*. 15.2 in patients). Interestingly, no clearly visible mitochondria were found in cells showing these particles, and there was a negative correlation between type 1 mitochondria ratio area and the amount of the aggregates (Fig. 4E).

Discussion

In the current pilot study, we describe novel morphological changes in mitochondria of FM patients, which primarily include loss of mitochondrial cristae. These findings highlight the possibility that mitochondrial alteration/malfunction may play a causative role in the cascade of events leading to chronic pain and fatigue in FM. Further understanding the connection between FM and mitochondrial morphology, function, and turnover (biogenesis/mitophagy), may assist in developing both novel diagnostic tools as well as specific treatments for FM.

Total mitochondria content with full/partial cristae (types 1 and 2) was reduced in cells from FM patients. Mitochondria of type 2 have some cristae, and thus may retain functionality, compared to type 3 mitochondria which demonstrate total loss of cristae. Reduced mitochondrial functionality may imply a reduced ability to handle stressful conditions and balance cellular energy and metabolic demands.

Intriguingly, despite the small sample size, we were able to demonstrate significant correlations between mitochondrial cristae appearance and the cardinal clinical measure of the widespread pain index (WPI), representing the key element of the FM syndrome – widespread pain. While these correlations cannot be interpreted as proving a pathogenetic role of mitochondrial morphological aberrations in FM, they do imply that the findings represent a factor which is associated with the clinical severity of FM and hence not just an epiphenomenon.

In the context of mitochondria types, we saw a decrease in type 1 mitochondria (full crista) in the FM group accompanied by an increase of the other types (2+3). The ways in which mitochondrial dynamics and cristae shape affect oxidative metabolism, respiratory efficiency, electron transport chain (ETC) activity and redox state have been previously described (21, 22).The cristae are the principal site of oxidative phosphorylation (OXPHOS) and comprise a regulated submitochondrial compartment specialised for ATP production (23). Thus, partial or full loss of cristae integrity, as seen in many of the FM patient cells, may potentially lead to destabilisation of super complexes and subsequent impairment in mitochondrial respiration, as seen in aging. In addition, can affect other important functions of the mitochondria.

Recent studies have shown that dynamic changes in mitochondrial architecture have a major impact on cell's ability to modify energy production and to survive stress (24-28). Mitochondrial cristae undergo remodeling (structural modifications) upon different physiological or pathological stresses, as changes in energy substrate availability, bioenergetic states, and in response to multiple metabolic stresses, including hypoxia, glucose depletion, increased ROS, or other toxicity, that require mitochondrial adaptation and reprograming (29, 30). Under normal conditions, tubular or lamellar mitochondrial cristae have an orderly arrangement. However, under different physiological and pathological conditions, the number, size, shape, and distribution of cristae are affected to varying extents (31-33).

Mitochondrial dysfunction can lead to an increase in ROS generation, triggering inflammatory responses through NLRP3 inflammasome activation and releasing cytotoxic mediators like cytochrome c and mtDAMPs-mtDNA. Mitochondrial dysfunction was also previously linked to neuronal activity and chronic pain (5, 34, 35). These processes may exacerbate the condition by further damaging mitochondria. The observed cristae appearance in mitochondria may be indicative of a "hibernation-like state." A previous study proposed that the energy metabolism system in FM could be in a state akin to hibernation, characterised by reduced energy metabolism (36). It is hypothesised that mitochondrial down-regulation and hypometabolism are adaptive responses, allowing the organ to survive stressful events, similar to the hibernation observed in certain animals (37).

Mitochondrial dysfunction, coupled with decreased ATP production, may compel cells to adapt and enter a "metabolic dormancy". This state of hypometabolism is marked by a down-regulation of translation processes and ribosomal aggregation. The loss of cristae might not only result from mitochondrial dysfunction but could also be a part of the "hibernation" process, similar to ribosome aggregation. This could potentially include an arrest in the synthesis of mitochondrial proteins related to cristae formation. Notably, mitochondrial dysfunction may be pathogenetically linked to a broad array of FM symptoms in various organs and systems, including muscles, nerves as well as the gastrointestinal tract.

Our study suggests that the observed electron dense aggregates in FM patients are likely ribosomes, based on their location, structural similarities, and measured dimensions. These aggregates could potentially serve as a novel biomarker for FM or a specific subgroup of patients. Future research should investigate the functional parameters of mitochondria in FM patients, including changes in protein expressions related to cristae formation.

Further investigation is needed to clarify the specificity of our findings to FM. For example, comparing FM patients to those with other chronic pain conditions like rheumatoid arthritis (RA). This comparison may reveal unique FM characteristics and aid in diagnostic development. Furthermore, it is imperative to conduct additional inves-

tigations with a larger participant cohort. Nonetheless, we believe that our results are significant as they represent an objective pathology found in the mitochondria of FM patients, which is a rare and noteworthy finding. This objective evidence of mitochondrial structural alterations in FM patients, such as the loss of mitochondrial cristae align with previous reports linking mitochondrial alterations/dysfunction with FM.

In conclusion, the findings of the current pilot study indicate the possibility of structural mitochondrial pathology in fibromyalgia patients. While the functional implication of this finding remains to be determined, it carries potential significance both as a biomarker, and in contributing to a better understanding of the underlying pathogenesis of fibromyalgia.

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