

Mitochondrial dysfunction in fibroblasts of Multiple System Atrophy

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ABSTRACT

Multiple System Atrophy is a severe neurodegenerative disorder which is characterized by a variable clinical presentation and a broad neuropathological spectrum. The pathogenic mechanisms are almost completely unknown. In the present study, we established a cellular model of MSA by using fibroblasts' primary cultures and performed several experiments to investigate the causative mechanisms of the disease, with a particular focus on mitochondrial functioning.

Fibroblasts' analyses (7 MSA-P, 7 MSA-C and 6 healthy controls) displayed several anomalies in patients: an impairment of respiratory chain activity, in particular for succinate Coenzyme Q reductase ($p < 0.05$), and a reduction of complex II steady-state level ($p < 0.01$); a reduction of Coenzyme Q10 level ($p < 0.001$) and an up-regulation of some CoQ10 biosynthesis enzymes, namely COQ5 and COQ7; an impairment of mitophagy, demonstrated by a decreased reduction of mitochondrial markers after mitochondrial inner membrane depolarization ($p < 0.05$); a reduced basal autophagic activity, shown by a decreased level of LC3 II ($p < 0.05$); an increased mitochondrial mass in MSA-C, demonstrated by higher TOMM20 levels ($p < 0.05$) and suggested by a wide analysis of mitochondrial DNA content in blood of large cohorts of patients.

The present study contributes to understand the causative mechanisms of Multiple System Atrophy. In particular, the observed impairment of respiratory chain activity, mitophagy and Coenzyme Q10 biosynthesis suggests that mitochondrial dysfunction plays a crucial role in the pathogenesis of the disease. Furthermore, these findings will hopefully contribute to identify novel therapeutic targets for this still incurable disorder.

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1. Introduction

Multiple System Atrophy (MSA) is a severe neurodegenerative disorder which affects several areas of the Nervous System. MSA is clinically characterized by variable degrees of parkinsonism, cerebellar ataxia, dysautonomia and pyramidal features. A parkinsonian (MSA-P) and a cerebellar (MSA-C) subtype can be distinguished according to the predominant symptomatology [1,2].

No effective therapies are available, although many pharmacological strategies are under investigation [3].

The heterogeneity of MSA clinical presentation correlates with the numerous regions of the nervous system which are affected, including striato-nigral pathway, cerebellum and ponto-cerebellar fibers, brainstem and autonomous nervous system. Neuropathologically, the disease is mainly characterized by the finding of protein aggregates (glial cytoplasmic inclusions, GCIs) in oligodendrocytes. As alpha-synuclein is the main component of these inclusions, MSA is classified, together with Parkinson's disease and Dementia with Lewy Bodies, as an alpha-synucleinopathy [4,5].

The pathogenic mechanisms of the disease are almost completely unclear, although several hypotheses have been proposed. In particular, most of the studies have focused on alpha-synuclein over-expression or cell-to-cell transfer, inflammation and mitochondrial functioning [5].

Mitochondrial dysfunction has been demonstrated to be implicated in synucleinopathies. Although most of the studies have focused on Parkinson's disease [6], Multiple System Atrophy has been investigated, too. Previous studies assessed the activity level of respiratory chain in MSA patients and healthy controls, detecting a reduced complex I activity in patients' skeletal muscle [7] but not in platelets or substantia nigra [8]. No significant differences were observed in the amount of mtDNA deletions or rearrangements in substantia nigra of MSA patients compared to controls [9]. More interest was raised by a study which suggested a causative role of mutations in *COQ2*, encoding the second enzyme involved in Coenzyme Q10 (CoQ10) biosynthesis [10]. However, this result was not confirmed by other investigators [11–13] and the issue remains controversial. Of note, recessive *COQ2* mutations cause primary CoQ10 deficiency leading to an infantile encephalomyopathy/nephropathy with cerebellar atrophy and other primary CoQ10 deficiency syndromes are characterized by cerebellar ataxia as main clinical feature [14,15]. Finally, a reduction of CoQ10 amount selectively in cerebellum of MSA patients, but not in other brain areas, was reported in two independent studies [16,17] and a reduced CoQ10 level was described in patients' plasma, too [18–20].

The purpose of the present work is to investigate the pathogenic mechanisms of MSA by using fibroblasts derived from patients and controls, with a particular focus on mitochondrial functioning. In particular, we focused our attention on respiratory chain function and oxygen consumption, mitochondrial mass (in both fibroblasts and peripheral blood cells), mitophagy, autophagy, CoQ10 amount and biosynthesis.

2. Materials and methods

2.1. Ethical issues

All the experiments were performed in compliance with the Helsinki declaration and informed consent was obtained from all the subjects involved in the study.

2.2. Patients selection and skin biopsies withdrawal

Skin biopsies were obtained from 7 MSA-P patients, 7 MSA-C patients and 6 age-matched healthy controls. Seventeen samples were obtained from the Italian movement disorders outpatient service of Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico (Milan), where biopsies were withdrawn from the ventral surface of the right

arm through a 5 mm punch. Three fibroblast cell lines were obtained from the Italian Telethon Network of Genetic Biobanks [21]. Details about age and sex of each subject are reported in Table S1.

Diagnosis of probable MSA was performed in compliance with widely accepted clinical criteria (Gilman et al., 2008) [22].

2.3. PBCs samples

DNA extracted from peripheral blood cells of patients affected with MSA was obtained from three Italian movement disorders outpatient services. Details about the composition of each group are reported in Table S2.

2.4. Fibroblasts culture

Fibroblasts were isolated from skin biopsies and expanded in Dulbecco's modified eagle medium supplemented with fetal bovine serum (15%), penicillin/streptomycin (1%) and amphotericin B (1%).

2.5. Spectrophotometric analyses

Protein extraction was performed by sonication (50 W for 10 s, 3 times) after resuspending cell pellets in proper buffer (pH 7.2). Lysates were centrifuged at 750g for 10 min and supernatant was recovered. Lowry method was used for protein quantification. A Lambda 2 Parkin Elmer spectrophotometer was used to assess enzymatic activities. Analyses were performed at specific wave lengths for each enzymatic activity after preparing proper solutions as previously described [23] with mild changes. NADH dehydrogenase (340 nm): H₂O (455 µl), K-phosphate pH 7.5 0.1 M (340 µl), K₃[Fe(CN)₆] 17 mM (100 µl), NADH 2 mM (100 µl), homogenate (5 µl). NADH ubiquinone 1 reductase (340 nm): H₂O (610 µl), K-phosphate pH 7.5 0.1 M (200 µl), albumin 1% (100 µl), NADH 2 mM (70 µl), homogenate (10 µl), sodium azide 100 mM (10 µl), CoQ1 6 mM (5 µl); activity measured after rotenone administration (1 mM, 5 µl) was subtracted. Succinate dehydrogenase (SDH) (600 nm): K-phosphate pH 7 0.1 M (500 µl), 2,6 dichloroindophenol 0.5 mM (200 µl), H₂O (130 µl), succinic acid 200 mM (100 µl), KCN 30 mM (50 µl), homogenate (20 µl). Succinate CoQ Reductase (600 nm): K-phosphate pH 7 0.1 M (500 µl), 2,6 dichloroindophenol 0.5 mM (200 µl), H₂O (127 µl), succinic acid 200 mM (100 µl), KCN 30 mM (50 µl), homogenate (20 µl), CoQ1 15 mM (3 µl). NADH cytochrome C reductase (550 nm): H₂O (490 µl), K-phosphate pH 7.5 0.1 M (250 µl), NADH 2 mM (120 µl), cytochrome C 1 mM (100 µl), KCN 30 mM (20 µl), homogenate (20 µl). Succinate cytochrome C reductase (550 nm): K-phosphate pH 7.5 0.1 M (500 µl), H₂O (300 µl), cytochrome C 1 mM (100 µl), succinate 400 mM (50 µl), homogenate (30 µl), KCN 30 mM (20 µl). Cytochrome oxidase (550 nm): H₂O (680 µl), K-phosphate pH 7 0.1 M (200 µl), reduced cytochrome C 1% (100 µl), homogenate (20 µl). Citrate synthase (412 nm): H₂O (800 µl), DTNB 1 mM (100 µl), oxaloacetic acid 10 mM (50 µl), AcetylCoA 10 mM (30 µl), homogenate (20 µl). Experiments were performed at 30 °C. Analyses were performed through a Parkin Elmer software. Measurements were normalized over the activity level of citrate synthase, a stable matrix mitochondrial enzyme; this latter step was performed in order to normalize respiratory chain activity over mitochondrial mass.

2.6. High resolution respirometry

A high-resolution respirometry Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria) was used to assess oxygen consumption in fibroblasts [24]. For each analysis, 800,000 cells were resuspended in proper medium (MiRO5) and injected into the instrument's chambers. Oxygen flux was measured at basal level and after the progressive administration of several compounds: digitonin (4.1 µM), malate (2 mM) and glutamate (10 mM), ADP (1–5 mM),

succinate (10 mM), FCCP (stepwise injections up to plateaux), rotenone (0.5 μ M) and antimycin A (2.5 μ M). The value obtained after Antimycin A administration was subtracted to all other values. Values are expressed as pmol/(s $\times$ mL).

2.7. Assessment of extracellular lactate levels

Extracellular lactate level was assessed through the commercially available kit Lactate-Glo™ Assay (Promega). Fibroblasts were plated at a confluency of 25,000 cells/cm². After 24 h, medium was changed with DMEM high glucose (not supplemented with FBS). After 2 h, medium was removed and used for analyses. The experiment was performed according to the manufacturer's instructions and a GloMax Discover instrument (Promega) was used to assess luminescence.

2.8. Assessment of mitophagy

Mitophagy evaluation was performed as described by Hsieh et al. [25].

Fibroblasts were collected at basal condition and after being incubated with the mitochondrial membrane depolarizer carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP) (40 μ M) for 6 h and 14 h. After protein extraction a Western Blot was performed (see Paragraph 2.9 for details) to assess the amount of three mitochondrial structural proteins (SDHA, TOMM20 and COX IV) at different conditions (basal, 6 h and 14 h). As described [25], the reduction of mitochondrial markers at 14 h, but not at 6 h from treatment, reflects mitochondrial degradation and the efficiency of the mitophagic machinery.

2.9. Western blot

Proteins were extracted by resuspending cell pellets in H₂O and protease inhibitors, sonicating 2 times for 15 s at 50 W and centrifuging for 10 min at 750g. Lowry method was used for protein quantification. Samples were prepared in Novex Bolt LDS sample buffer and Novex Bolt Reducing Agent and were boiled for 3 min. Proteins (10–30 μ g) were loaded in Precast Bolt® Bis-Tris Plus Gels 4–12% and run for 30 min at 200 V in Novex Bolt 1X MES/SDS Running buffer. Transfer to nitrocellulose membrane was performed by a Trans-Blot® Turbo™ system (BioRad). Membranes were blocked in Odyssey® Blocking Buffer (LI-COR) and incubated with secondary fluorescent antibodies (IRDye®). Protein bands were visualized by an Odyssey Fc device, model 2800 (LI-COR Biosciences). Bands intensity was quantified by an Image Studio Lite Ver 5.2 software.

For CoQ10 biosynthesis enzymes, protein extraction was performed by sonication after resuspending cell pellets in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA) with protease inhibitors. Bradford system was used for protein quantification. 10 μ g of proteins were loaded for each sample and a Novex 10–20% Glycine Gel was used for electrophoresis. Proteins were then transferred to a PVC membrane. 5% milk was used for membranes blocking. Protein bands were visualized through chemiluminescence, by using ECL reagents. Bands intensity was quantified by ImageJ.

Antibodies details are reported in Table S3.

2.10. Assessment of mtDNA content by RT-PCR

mtDNA content was assessed through RT-PCR (on genomic DNA) by the simultaneous detection of a mitochondrial gene and a nuclear reference gene as previously described [26]. Data were analyzed by the $\Delta\Delta$ Ct method. The mitochondrial/nuclear probes combinations MT-CYTB/APP and MT-ND4/RNaseP were used. RNaseP assay was purchased by ThermoFisher Scientific (catalog number: 4403328). MT-CYTB, APP and MT-ND4 primers and probes were custom designed (details about primers and probes sequences are reported in Table S4).

2.11. CoQ10 dosage

Extraction of Coenzyme Q from cultured cells was performed as described by Takada et al. [27].

Quantification of CoQ10 was performed by HPLC analysis using a two pump Agilent 1100 series system. Absorbance (275 nm) was monitored with a photodiode array detector (Agilent 1100 series system). 20 μ l of ethanolic extract was chromatographed on a C18 column (Kinetex, Phenomenex, 2.6 μ m, 100 $\times$ 4.6 mm), using a mobile phase consisting of ethanol:water (97:3, v/v) at a flow rate of 0.4 ml/min. The concentrations of CoQ10 were obtained by comparison of the peak areas with those of standard solutions.

2.12. Statistical analyses

Experiments involving two biological groups were analyzed by two-tailed unpaired Student's *t*-test. Experiments involving more than two groups were analyzed by ANOVA with Tukey post-hoc test. The number of biological replicates for each experiment are reported in figure legends. Significance levels: * = *p* < 0.05; ** = *p* < 0.01; *** = *p* < 0.001.

3. Results

3.1. Complex II activity is reduced in MSA fibroblasts

Spectrophotometric analysis of all respiratory enzymes provided evidence for a reduced activity of succinate CoQ reductase (complex II) in patients compared to controls (*p* < 0.05). Furthermore, the activity level of succinate dehydrogenase (complex II initial step) and succinate cytochrome *c* reductase (complexes II + III) showed a trend of reduction, although not reaching statistical significance (Fig. 1A, Fig. S1A).

In order to evaluate whether the impaired respiratory chain activity was also accompanied by decreased oxygen consumption in MSA cells, we performed high resolution respirometry on all cell lines at basal level and after the serial administration of specific compounds. No significant results were detected (mostly because of a remarkable variability), but some hints support the findings observed at spectrophotometry. At basal level, a trend of increased O₂ consumption was observed in patients. After injecting malate, glutamate and ADP (in order to stress complex I activity) no major differences were observed between groups. Differently, after the administration of succinate (to stress also complex II activity), FCCP (to assess maximal respiration) and complex I inhibitor rotenone (to assess complex I-independent maximal respiration), oxygen consumption rate displayed a trend of reduction in patients (Fig. 1B, Fig. S1B).

The assessment of extracellular lactate levels in culture media did not show major differences between patients and controls (Fig. 1C, Fig. S1C).

3.2. Complex II amount is reduced in MSA fibroblasts

In order to evaluate whether the observed enzymatic impairment was due to a reduced protein amount, we assessed the quantity of respiratory chain complexes I, II (both SDHA and SDHB), III, IV and V through Western Blot. Data (normalized for Actin and for the mitochondrial structural protein TOMM20) demonstrated a generalized reduction of complexes II, III and IV. However, statistical significance was detected only for SDHA (Fig. 2A–C, Fig. S2A–B).

3.3. Mitochondrial mass in MSA patients

In order to assess mitochondrial mass three independent indicators were analyzed: the amount of the mitochondrial structural protein TOMM20 (Western Blot), the activity level of the mitochondrial matrix enzyme citrate synthase (spectrophotometry) and mtDNA content (RT-

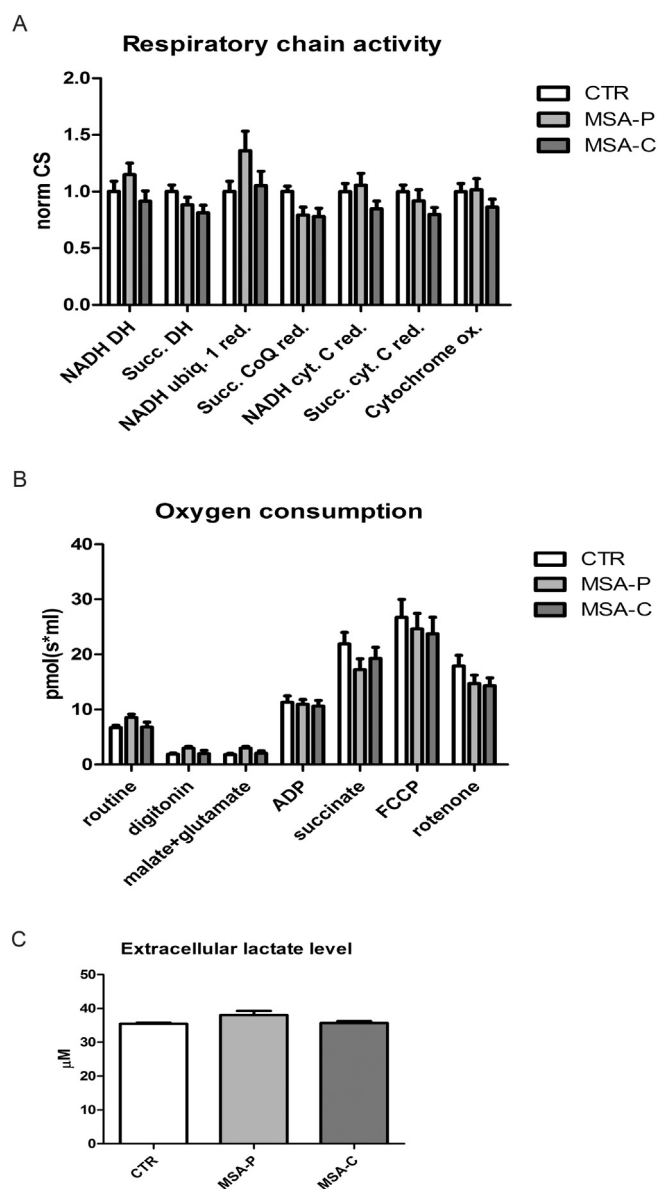


Fig. 1. Mitochondrial activity in fibroblasts. A) Graph showing respiratory chain enzymatic activities in control, MSA-P and MSA-C fibroblasts, assessed through spectrophotometric analysis (experiments in duplicate). B) Graph showing oxygen consumption in control, MSA-P and MSA-C fibroblasts, assessed through high resolution respirometry (Oxygraph) (experiments in triplicate). C) Graph showing extracellular lactate levels (experiments in duplicate). Values are expressed as mean $\pm$ SEM.

PCR using two independent mitochondrial/nuclear probes' combinations: MT-CYTB vs APP and MT-ND4 vs RNaseP). TOMM20 amount was significantly up-regulated in MSA-C patients compared to MSA-P ($p < 0.05$), while no significant differences were detected between patients and controls (Fig. 2D–F, Fig. S2C–E). A similar trend was detectable for citrate synthase activity, but not for mtDNA content, which displayed high variability among samples. This prompted us to assess mtDNA content in blood of a large number of MSA patients.

DNA samples were obtained from three independent Italian movement disorders outpatient services. In order to avoid the variability due to different DNA extraction and storage methods, each cohort was analyzed separately. Interestingly, groups 1 and 2 showed a trend of increased mtDNA content in MSA-C compared to MSA-P, which is consistent with the observed increased TOMM20 level in MSA-C fibroblasts. The same trend was not observed in group 3 (Fig. 3).

3.4. Mitophagy in MSA fibroblasts

To investigate whether impaired mitophagy could contribute to the mitochondrial functional defect, we explored the mitophagic pathway by assessing the amount of the mitochondrial proteins SDHA, COXIV and TOMM20 at basal level and after 6 and 14 h from the administration of the mitochondrial membrane uncoupler CCCP. CCCP is a compound that triggers mitophagy, thus inducing a reduction of mitochondrial structural proteins. In line with previous reports [25], we observed a reduction of all mitochondrial markers at 14 h in control cell lines. Differently, the trend of reduction at 14 h was not observed in patients, supporting the hypothesis of a reduced mitophagic flux (significant results for COXIV) (Fig. 4, Fig. S3).

To investigate whether the observed mitophagic impairment was due to a more generalized autophagic defect, we assessed the amount of the autophagy-related proteins LAMP1, p62 and LC3 II at basal level, after CCCP treatment (6 and 14 h) and after the administration of bafilomycin (an inhibitor of the fusion between the autophagosome and the lysosome). A reduced LC3 II basal level was detected in patients (basal LC3 II level, without bafilomycin), while no major differences were observed when assessing bafilomycin or CCCP related autophagic flux (bafilomycin treated/untreated ratio and CCCP treated/untreated ratio respectively) (Fig. 5, Fig. S4).

3.5. Coenzyme Q10 amount and biosynthesis are dysregulated in MSA fibroblasts

As Coenzyme Q10 deficiency has been suggested to be implicated in MSA pathogenesis, we assessed the amount of this molecule in all cell lines through HPLC. Interestingly, we observed a significant reduction of CoQ10 ($p < 0.001$) in patients (both in MSA-P and MSA-C) (Fig. 6A, Fig. S5A).

To further investigate this reduction, we assessed the amount of CoQ10 biosynthesis enzymes PDSS1, PDSS2, COQ2, COQ4, COQ5, COQ6, COQ7, ADCK3/COQ8A and COQ9 through Western Blot in fibroblasts and detected a significant up-regulation of two of these enzymes, suggesting a compensatory up-regulation: COQ5 in both MSA-P and MSA-C and COQ7 in MSA-C (Fig. 6B, Fig. S5B, Fig. S6).

4. Discussion

The present study mainly aimed at investigating mitochondrial functioning in fibroblast primary cultures of MSA patients and healthy controls and showed a dysregulation of various mitochondrial pathways.

Spectrophotometric analyses pointed out an impairment of some enzymatic activities, mainly those related to respiratory chain complex II (trend for succinate dehydrogenase and for succinate cytochrome C reductase and significant results for succinate CoQ reductase). Respirometry analyses supported this finding because, although not reaching statistical significance, a trend of reduced O_2 consumption was detected in those measurements that involve complex II (i.e. complexes I + II, maximal respiration rate and complex I-independent maximal respiration rate). Interestingly, animals treated with 3-nitropropionic (3-NP) acid [28–30], which affects mitochondrial activity by inhibiting SDH [31], have been used to model MSA (although some key features of the disease are missing). 3-NP acid, also used to model Huntington's disease, is characterized by the property to induce damage to striatum [32], a brain region severely affected in MSA, too. In this view, it can be hypothesized that striatal degeneration in MSA may be somehow interconnected with SDH functional defect. The lack of respiratory chain activity defects in autaptic samples from MSA patients [8,17] requires further investigation.

The reduction of CoQ10 in fibroblasts of both MSA-P and MSA-C patients supports the hypothesis of a possible role of this molecule in the pathogenesis of MSA, suggested by previous genetic and

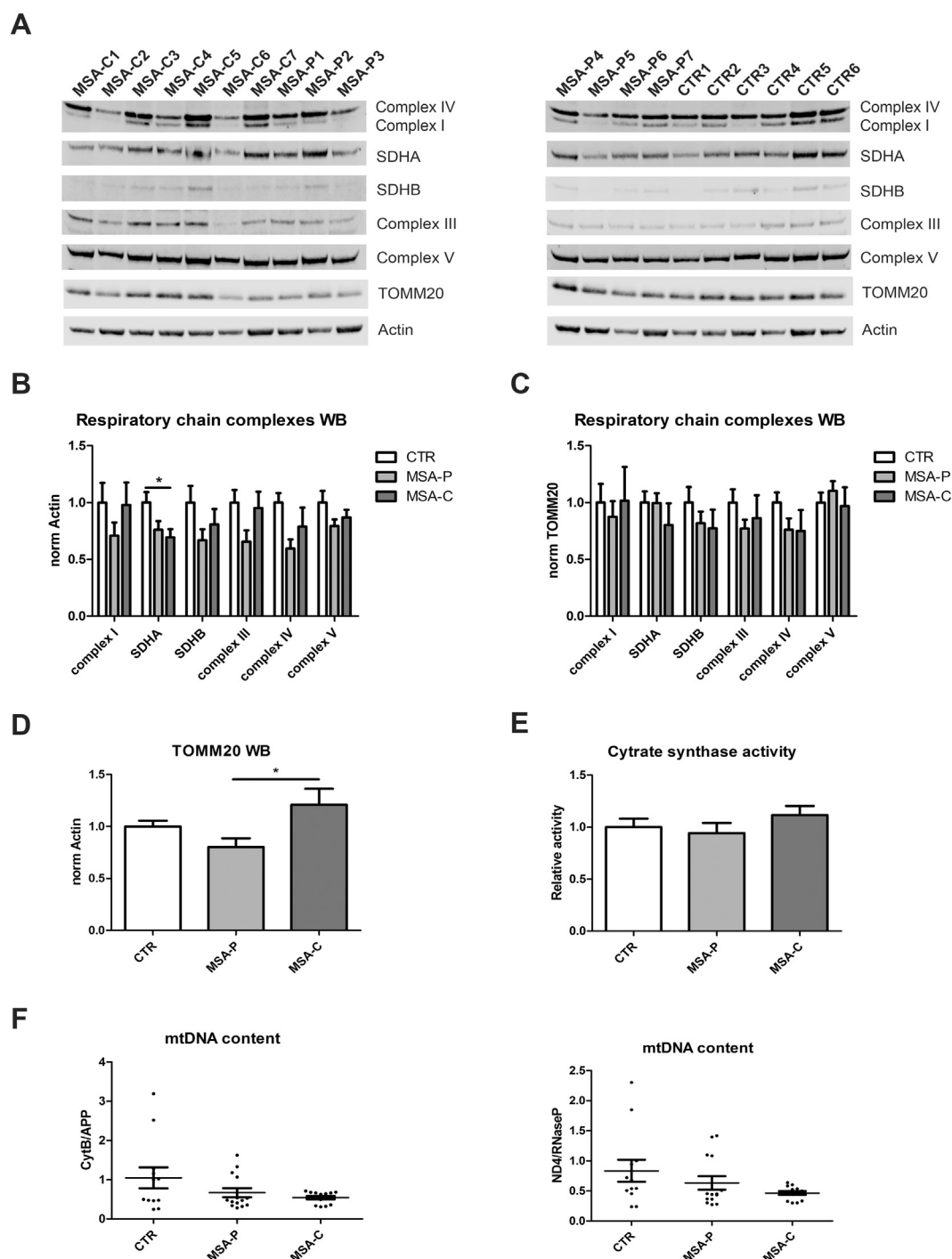


Fig. 2. Mitochondrial markers in fibroblasts. A) WB assessing the amount of respiratory chain complexes I, II (SDHA and SDHB), III, IV and V, TOMM20 and Actin in control, MSA-P and MSA-C fibroblasts. B) Graph showing quantification (normalized for Actin) of WB assessing respiratory chain complexes I, II (SDHA and SDHB), III, IV and V in control, MSA-P and MSA-C fibroblasts (triplicate). C) Graph showing quantification (normalized for TOMM20) of WB assessing respiratory chain complexes I, II (SDHA and SDHB), III, IV and V in control, MSA-P and MSA-C fibroblasts (triplicate). D) Graph showing quantification (normalized for Actin) of WB assessing TOMM20 amount in control, MSA-P and MSA-C fibroblasts (triplicate). E) Graph showing the activity level of the mitochondrial matrix enzyme citrate synthase in control, MSA-P and MSA-C fibroblasts (duplicate). F) Graph showing mitochondrial DNA content, assessed by RT-PCR, in control, MSA-P and MSA-C fibroblasts (duplicate). Values are expressed as mean $\pm$ SEM. * = $p < 0.05$.

neuropathological studies [10,16,17]. However, the cause of decreased CoQ10 amount is still unclear. The detailed analysis of CoQ10 biosynthetic pathway showed an up-regulation of two enzymes, which may reflect a compensatory response mediated by a feedback mechanism in fibroblasts.

In the perspective of identifying a common mechanism underlying these different observations, it is interesting to point out that an

association between CoQ10 deficiency and reduced complex II amount and activity has already been reported in other disorders, namely in ataxia with oculomotor apraxia type 1 [33]. This association is intriguing but the cause is still unknown. A possible explanation, already suggested by previous studies [33], is that SDHA and specific CoQ10 biosynthesis enzymes are co-regulated by the same molecular mechanism, namely the NRF1/NRF2 pathway; another possibility is that

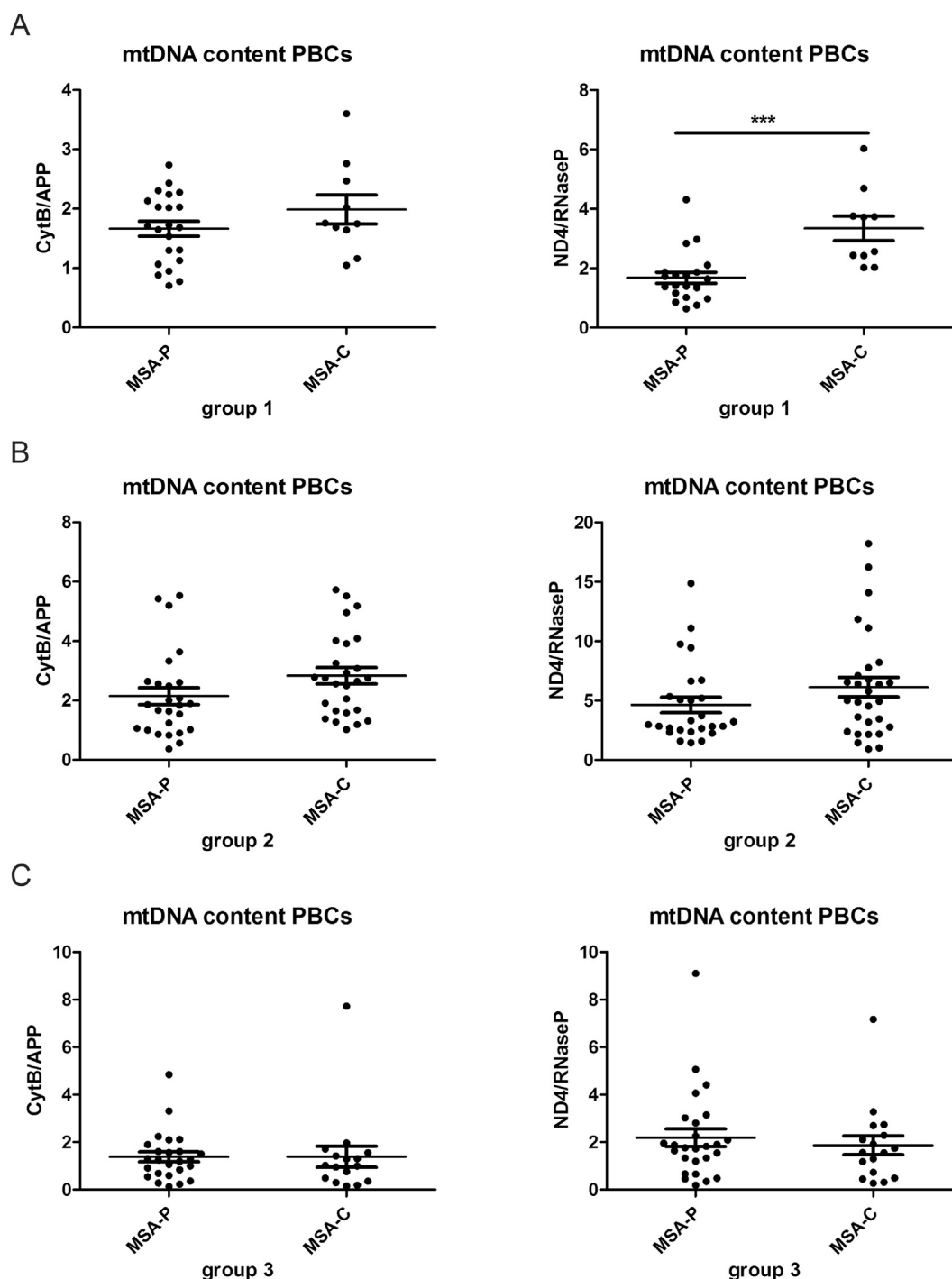


Fig. 3. Mitochondrial DNA content in peripheral blood cells. Mitochondrial DNA content, assessed by RT-PCR using two independent mitochondrial/nuclear probes combinations (MT-CYT B vs APP and MT-ND4 vs RNaseP) in peripheral blood cells of MSA-P and MSA-C patients from three independent Italian movement disorders outpatient services. *** = $p < 0.001$.

oxidative stress may be somehow involved, since complex II dysfunction has been associated with an increased ROS production [34] and CoQ10 also plays a crucial role as antioxidant [35].

Another emerging aspect is the potential mitophagic defect in MSA cells. This mechanism has been recently investigated in fibroblasts of patients affected with Parkinson's disease and a functional defect has been detected in patients carrying mutations in mitophagy-related genes (Parkin and PINK1) and even in sporadic cases [25]. Further analyses will evaluate whether the mild mitophagic impairment observed in MSA fibroblasts is due to a specific mitochondrial

involvement or to a generalized autophagic defect, the latter supported by the finding of LC3II reduction.

The increased amount of mtDNA in PBCs of MSA-C patients from two cohorts represents a novel observation that requires confirmation in larger patients' groups. In this view it could be hypothesized that the early cerebellar degeneration in MSA-C may induce a compensatory increase of mtDNA levels in blood. This compensation is not present in MSA-P, which displays a milder cerebellar involvement respect to MSA-C and in the late disease stage [36]. Other conditions, which are not primary mitochondriopathies, have been reported to show a similar

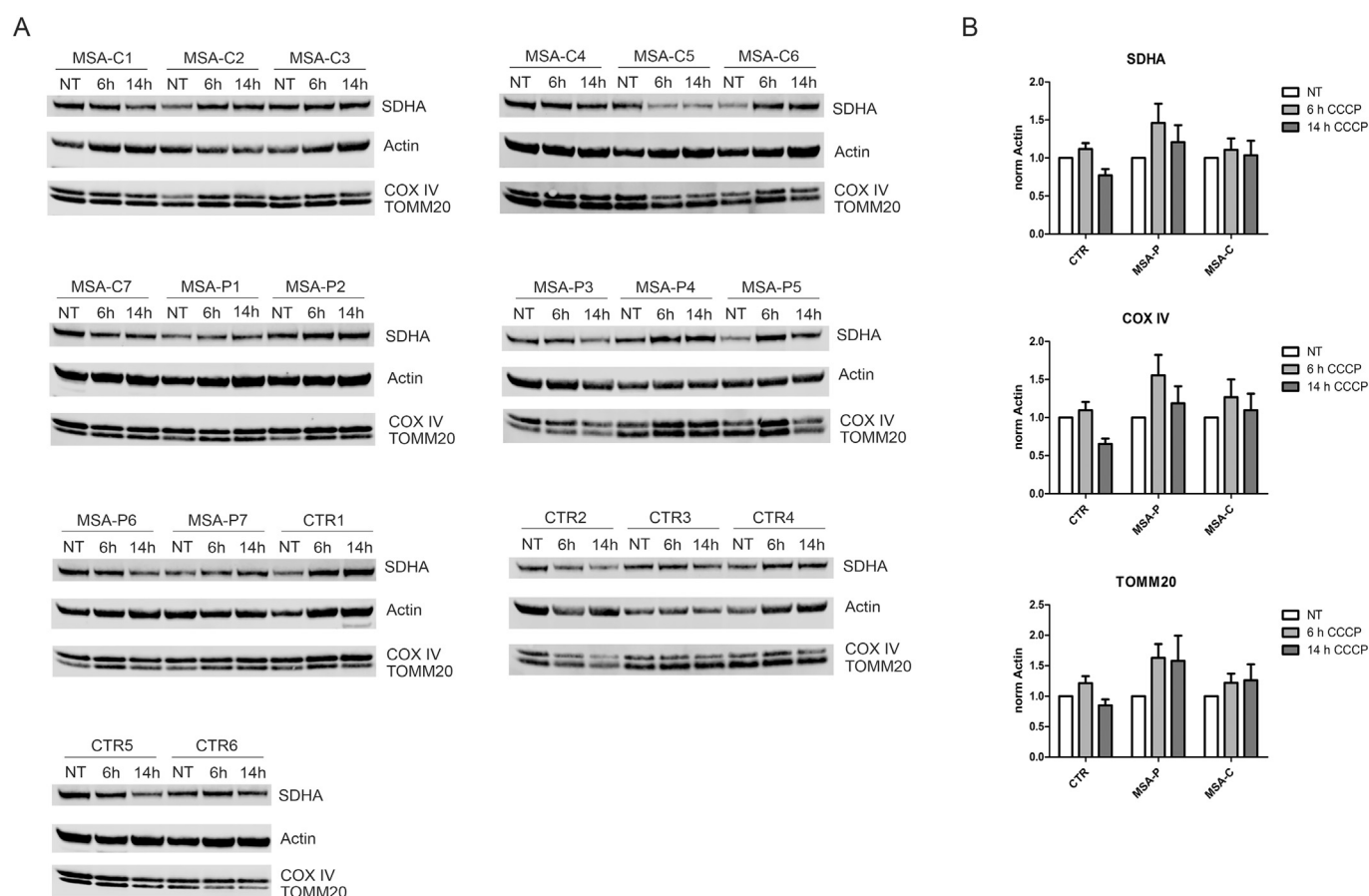


Fig. 4. Mitophagy in fibroblasts. A) WB assessing the amount of the mitochondrial markers SDHA, COX IV and TOMM20 and of Actin in control, MSA-P and MSA-C fibroblasts at basal level and after 6 and 14 h from the administration of the mitochondrial membrane uncoupler CCCP (40 μ M). B) Graphs showing quantification (normalized for Actin) of WB assessing the amount of the mitochondrial markers SDHA, COX IV and TOMM20 in control, MSA-P and MSA-C fibroblasts at basal level and after 6 and 14 h from CCCP administration (triplicate). Values are expressed as mean $\pm$ SEM.

compensatory response. For example, oxidative stress can lead to mitochondrial biogenesis paralleled by an increased mtDNA content [37]. Moreover, other neurodegenerative disorders, such as Parkinson's disease, Leber's hereditary optic neuropathy and Huntington's disease are characterized by peripheral alterations of mtDNA level [38–40], which further supports the hypothesis that central neurodegenerative changes may affect peripheral tissues, too.

Although MSA-P and MSA-C are usually considered as different manifestations of the same disease, the finding of discrepancies between the two groups suggests that different molecular pathways may be involved in the pathogenic mechanisms.

5. Conclusions

The present study provides several hints to better elucidate the pathogenic mechanisms of MSA. The rationale of using fibroblasts as a disease model is that these cells can be withdrawn and expanded easily and with little economic effort, which allows to analyze large cohorts of subjects. However, it must be acknowledged that this model displays some intrinsic defects. Indeed, fibroblasts do not show an evident phenotype in the disease and the defects observed in this study are limited to a molecular scenario which does not affect the clinical outcome. Therefore, the establishment of patient-specific models which recapitulate the pathogenic mechanisms of the disease in cellular subtypes which are primarily affected, namely iPSC-derived neurons and glia [41,42], is warranted.

Abbreviations

MSA	Multiple System Atrophy
GCI	glial cytoplasmic inclusions
CoQ10	Coenzyme Q10
mtDNA	mitochondrial DNA
3-NP acid	3-nitropropionic acid
SDH	succinate dehydrogenase
PBCs	peripheral blood cells

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Authors' contributions

Conceptualization and design of the study: GMC, ADF.
Acquisition and analysis of data: GMC, GK, AB, FF, DR, SS, CB, ADF.
Writing of the manuscript: GMC, ADF.
Blood samples collection: GMC, MTP, AV, FDS, AE, CR, BG, GM, AA,

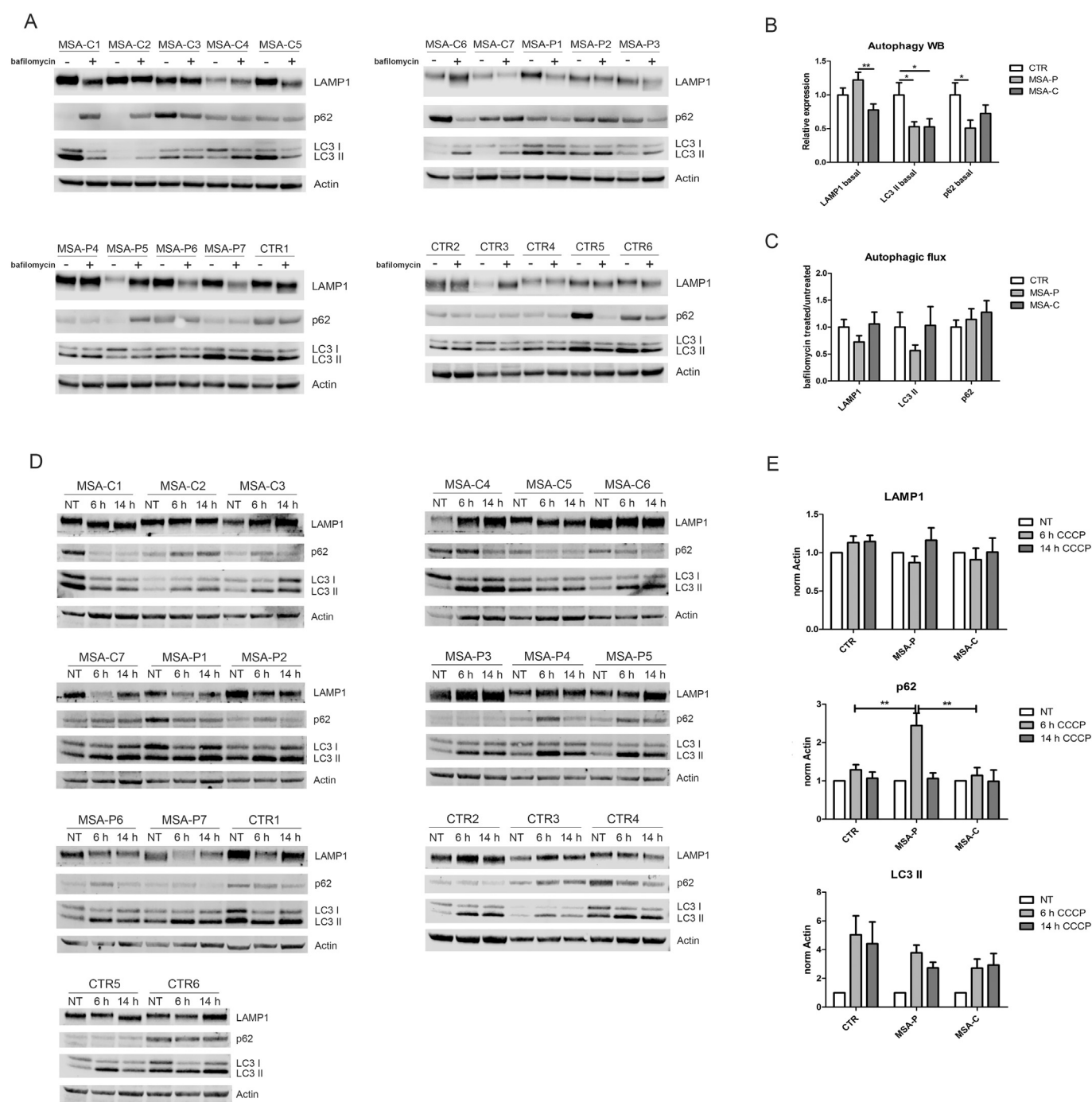


Fig. 5. Autophagy in fibroblasts. A) WB assessing the amount of the autophagy-related proteins LAMP1, p62 and LC3 and of Actin in control, MSA-P and MSA-C fibroblasts at basal level and after the administration of bafilomycin (200 nM, 24 h). B) Graph showing quantification (normalized for Actin) of the basal amount of the autophagy-related proteins LAMP1, p62 and LC3 II in control, MSA-P and MSA-C fibroblasts (triplicate). C) Graph showing the autophagic flux of the autophagy-related proteins LAMP1, p62 and LC3 II, measured as the ratio between the values of bafilomycin treated and untreated cells, in control, MSA-P and MSA-C fibroblasts (triplicate). D) WB assessing the amount of the autophagy-related proteins LAMP1, p62 and LC3 and of Actin in control, MSA-P and MSA-C fibroblasts at basal level and after 6 and 14 h from the administration of the mitochondrial membrane uncoupler CCCP (40 μ M). E) Graphs showing the flux of the autophagy-related proteins LAMP1, p62 and LC3 II, measured as the ratio between the values of CCCP treated and untreated cells (after 6 and 14 h from CCCP administration), in control, MSA-P and MSA-C fibroblasts (triplicate). Values are expressed as mean $\pm$ SEM. * = $p < 0.05$; ** = $p < 0.01$.

FC, GA, NB, SC, GPC, ADF.

Critical review of the manuscript: GMC, GK, AB, FF, DR, SS, MG, CC, IP, CB, RF, MTP, AV, FDS, AE, CR, BG, GM, AA, FC, GA, NB, SC, GPC, CMQ, ADF.

All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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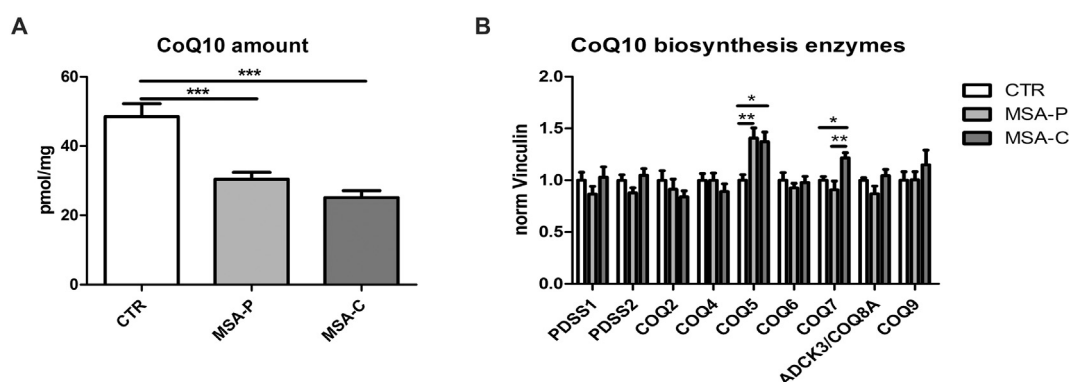


Fig. 6. Coenzyme Q10 pathway in fibroblasts. A) Graph showing CoQ10 amount, assessed by HPLC, in control, MSA-P and MSA-C fibroblasts. B) Graph showing quantification of WB assessing the amount of CoQ10 biosynthesis enzymes PDSS1, PDSS2, COQ2, COQ4, COQ5, COQ6, COQ7, ADCK3/COQ8A and COQ9 in control, MSA-P and MSA-C fibroblasts (triplicate). Values are expressed as mean $\pm$ SEM. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbdis.2018.09.018>.

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