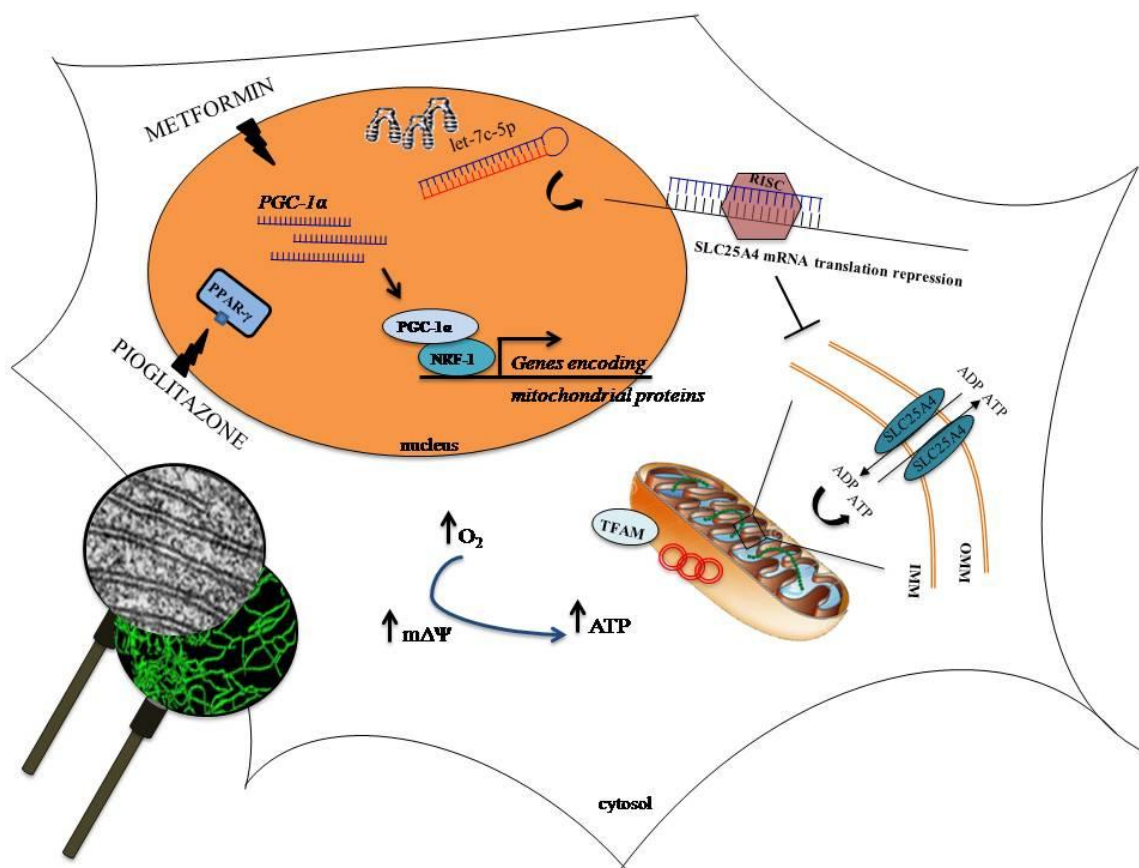


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Mitochondrial alterations in Down syndrome: molecular mechanisms and therapeutic opportunities



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ABSTRACT

Increasing evidence suggests that the mitochondrial dysfunction represents a hallmark in Down syndrome (DS) and that it can play a role in the pathogenesis of DS. Impaired expression/activity of PGC-1 α , a crucial modulator of mitochondrial biogenesis and functions, is emerging as a common underlying cause of mitochondrial dysfunction in several diseases. The analysis of molecular mechanisms responsible for the DS phenotype identified in the over-expression of the chromosome 21 repressor gene *NRIP1/RIP140* a cause of the decreased in the expression and activity of PGC-1 α and of several mitochondria-related genes. We demonstrated that by silencing *NRIP1* in trisomic cells it was possible to counteract the mitochondrial alterations observed in these cells, including irregular mitochondrial dynamics together with defects in the fission-fusion machinery.

On this basis we tested the hypothesis that the induction of PGC-1 α and of its downstream pathways might be able to reverse the mitochondrial dysfunction in DS. To this end we induced PGC-1 α in trisomic fetal fibroblasts using two strategies: i) we used metformin, which stimulates PGC-1 α through AMPK and SIRT1 activity; ii) we activated PGC-1 α through the PPAR agonist pioglitazone. We demonstrated that both drugs were able to induce the *PGC-1 α* pathway in trisomic cells. Both strategies stimulated respiratory capacity and increased ATP levels, while ROS production decreased. Most interestingly, both drugs affected mitochondrial dynamics promoting the formation of a mitochondrial network with branched and elongated tubular morphology. Accordingly, the expression of genes involved in the fission-fusion machinery, namely OPA1, Mitofusins and DRP-1, was modulated by either treatment, though the two drugs elicited different changes.

These results indicate that the induction of PGC-1 α counteracts the mitochondrial dysfunction in DS cells stimulating the mitochondrial biogenesis and promoting the formation of a mitochondrial network.

We also investigated the role of chromosome 21 miRNAs in promoting mitochondrial alterations in DS. We put special attention to let-7c-5p, miR-155-5p and miR-99a-5p which are up-regulated in human fetal hearts. The study of their targets down-regulated in DS fetal hearts and involved in mitochondrial function identified the ATP translocator the *SLC25A4/ANT1* as a let-7c-5p target candidate for mitochondrial anomalies.

Taken all together these results indicate that unraveling the molecular mechanisms that underlie mitochondrial dysfunction in DS paves the way to new therapeutic approaches to counteract DS phenotypic traits and/or to prevent DS associated pathologies.

1. INTRODUCTION

1.1 *The presence of three copies of chromosome 21 causes the Down syndrome*

Down syndrome (DS) is one of the more commonly occurring genetic disorders with an incidence of 1/800 live births. DS is caused by a complete or partial trisomy of chromosome 21 (Hsa21) (**Figure 1**) and is characterized by a highly complex and variable phenotype (1). Symptoms always found include intellectual disability, associated with some impairment in language, learning and memory (2) (3) and narrow slanted eyes, flat nose, muscle hypotonia and short stature. DS is a major cause of congenital heart disease and the most frequent known cause of atrioventricular septal defects (4). In addition, there are other health problems affecting different organs and tissues such as immune and endocrine systems defects or nutritional disorders, such as obesity, diabetes and hypothyroidism (5).

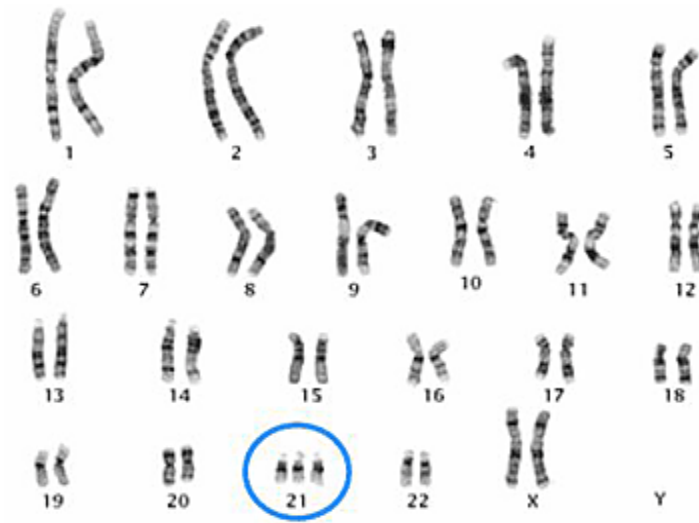


Figure 1. DS karyotype. This karyotype shows the classic appearance of trisomy 21.

The phenotype of DS is thought to result from the dosage imbalance of multiple genes. Hsa21 encodes a number of transcription factors, co-regulators and post-translational modifiers, whose dysregulation might contribute to the DS phenotype by perturbing also gene expression of target genes mapping to different chromosomes (6). This hypothesis is supported by many studies, which demonstrate that three copies of Hsa21 may cause a 50% increase in the expression most of trisomic genes as primary dosage effect and an altered expression of disomic genes as secondary effects. Both primary and secondary effects will finally result in developmental defects and phenotypic alterations. High-throughput technologies have allowed the measurement of steady-state RNA levels for thousands of transcripts in human DS cells (7) (8) and in tissues from mouse models of DS (9) (10) (11) (12). All of them reported over-expression of triplicated genes, in a dosage-dependent way. These studies highlighted also that only a subset of Hsa21 genes is consistently over-expressed in comparison to euploid controls, and the increase in expression may differ from the expected~1.5-fold (7) (13) (8). Moreover, the set of over-expressed Hsa21 genes might differ across the trisomic cell types and developmental stages (14). These findings suggest that, in addition to gene dosage, epigenetic factors may affect gene expression (15).

1.1.1 *Experimental models to study Down syndrome pathogenesis*

Given the complexity of the DS phenotype and of molecular mechanism involved, it is difficult to find adequate experimental models to study this pathology. For this reason many mouse models and cell culture models have been proposed for simulate DS condition.

- ***Mouse models***

Based on the homology (synteny) of Hsa21 traits with the murine chromosomes Mmu16, Mmu17 and Mmu10, several mouse models of DS have been developed. The first attempt to generate a mouse model of DS was the Ts16, a mouse trisomic for the entire Mmu16 (16). However, this model differs from the DS phenotype because Mmu16 presents syntenic regions with other humans chromosomes causing other phenotypic traits. Furthermore, Ts16 embryos die *in utero*, making it impossible to study the phenotypes of young and adult mice. The next approach consisted in generating mouse models with trisomies of sets of Mmu16 genes, orthologous to Hsa21 genes (17). In 1993, Davisson et al. (18) generated the Ts65Dn mouse, the most popular and widely used model of DS (19). Unfortunately it also presents a trisomy of a trait of Mmu17 containing non homologous to Hsa21 genes (20). Thus also this model does not show perfect matching with DS condition. Despite this bug, the Ts65Dn mouse is currently the model that better than others simulates DS phenotypic features such as learning and memory deficits, and impairments in tasks involving the hippocampus.

Another mouse model, Ts2Cje, carries a rearrangement of the Ts65Dn genome with a Robertsonian translocation of the marker chromosome to chromosome 12 (21). This model is trisomic for a syntenic region smaller than that of Ts65Dn and does not contain other trisomies. Sago and co-workers generated two mouse models with the triplication of two different regions of Mmu16: the Ts1Cje mouse and the Ms1Ts65 mouse. Ts1Cje mouse, which presents a trisomy of 81 genes localized in the region of Mmu16 that extend from Sod1 to Znf295; the Ms1Ts65 mouse, which has a partial trisomy of 33 genes mapped in the region of Hsa21 that extend from App to Sod1 (22). These mice showed distinct learning and behavioral deficits, possible caused by abnormalities of the hippocampus. In addition Olson et al. (23) produced the Ts1Rhr mouse, a model that is trisomic for another Mmu16 region which contains 33 genes. Finally, Li et al. (24) generated a mouse trisomic for the complete Hsa21 syntenic region on Mmu16 containing 110 orthologous genes, the Dp(16)1Yey/+ mouse (17). Other two mouse models have been created trisomic for Hsa21 orthologous genes located in Mmu17: the Ts1Yah mouse, trisomic for 12 genes in the Mmu17 region (25) and the Dep(17)1Yey/+ mouse which is trisomic for the entire Hsa21 syntenic region on Mmu17 that contains 19 orthologous genes (17) (26).

The “transchromosomal” mouse line, Tc1, is a model of trisomy 21 that carries an almost complete human chromosome 21 (27). This strain summarizes features seen in DS patients including phenotypic alterations in behavior, synaptic plasticity, cerebellar neuronal number, heart development, and mandible size (27) (28). However, the Tc1 mouse presents variable levels of mosaicism of the extra chromosome in different tissues, because of an instability of the extra human chromosome during the development. After the three partial trisomic models, Yu et al. (26) generated a mouse model trisomic for all three Hsa21 syntenic regions. This mouse model, named Dp(10)1Yey/+;Dp(16)1Yey/+;Dp(17)1Yey/+, seems to be the best opportunity for modeling human trisomy 21 and it shows the DS related abnormalities in the central nervous system, including impairments in learning and hippocampal LTP as well as hydrocephalus. This is a promising new model with excellent construct and face validities but unfortunately is difficult to obtain it (26).

Finally, to study the role of particular genes in the DS phenotypes, a number of mouse models with the triplication of single genes in euploid models and trisomic mice, in which the expression of one trisomic gene has been normalized, have been created (29).

In conclusion, many mouse models of DS have been generated, but none of them turns out to be a perfect model of DS mainly due to the lack of 3 copies of some Hsa21 orthologues and/or the trisomy of non-Hsa21 genes.

- ***Human cell culture models***

Starting from 2005 we have collected primary cultures of DS human fetal fibroblasts (DS-HFFs) because they are well characterized and have successfully been used in previous studies (30). Several DS-HFFs can be obtained from the "Bank of Fetal Biological Samples" at Federico II University of Naples.

Stem cell models have been successfully used to study neuronal alterations that occur in DS. Induced pluripotent stem cells from monozygotic twins discordant for trisomy 21 were used to investigate the anomalies occurring when they were induced to differentiate into neural progenitor cells (NPCs) and neurons (31). Studies on fetal brain derived neurospheres indicate that DS is associated with neurite and synapse formation defects (32), overrepresentation of glial lineages (33) and increased propensity for neuronal apoptosis (34) (35) (36). Finally, neurons derived from iPSCs of patients with DS demonstrated to reproduce early stages of Alzheimer's disease type pathology in vitro (37).

All these data indicate that iPSCs from DS subjects are a good model to recapitulate neurodevelopmental stages in the study of DS pathogenesis.

1.2 Mitochondrial defects are a phenotypic hallmark in DS

Growing evidence suggests that the mitochondrial dysfunction, well documented in cellular models, animal models and human subjects, represents a phenotypic hallmark in DS.

In DS-HFFs we found a global decrease of mitochondrial gene expression associated with the loss of mitochondrial bioenergetic capacities and abnormalities in mitochondrial structure. Trisomic fibroblasts showed a decrease of mitochondrial respiratory activity, inhibition of complex I, enhanced reactive oxygen species (ROS) production, decrease of mtDNA (**Figure 2**) and increased levels of intra-mitochondrial calcium (30). Interestingly, fibroblasts from trisomic fetuses with cardiopathy showed a more pronounced mitochondrial dysfunction if compared with those without cardiopathy. These data suggest that an altered bioenergetic background in DS fetuses might be among the factors responsible for a more severe phenotype (30).

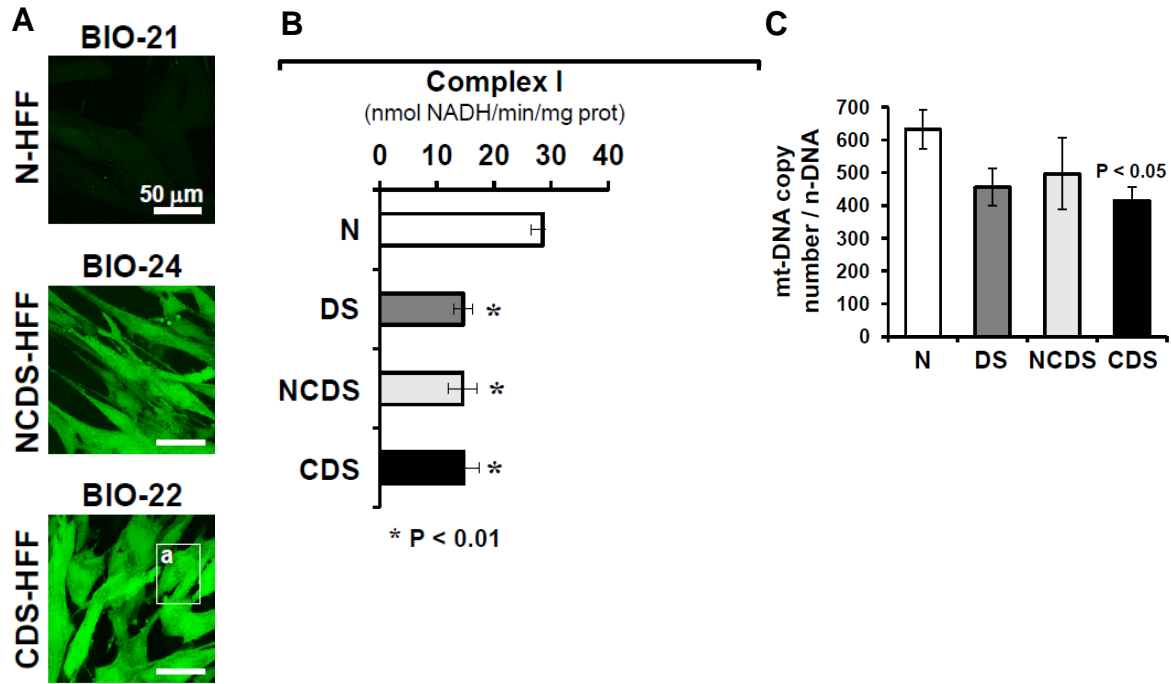


Figure 2. Chronic pro-oxidative state and mitochondrial dysfunctions are more pronounced in fibroblasts from DS fetuses with congenital heart defects. (A) Confocal microscopy analysis of ROS production in DS live fibroblasts. Representative LSCM imaging of the DCF-related fluorescence of euploid (N-HFF) and DS non-cardiopathic (NCDS-HFF) and cardiopathic (CDS-HFF) fibroblasts. Statistical analysis of the fluorescence intensity per cell as resulting from the averaged values \pm SEM of about 100 randomly selected different cells for each sample from at least in-duplicate experiments was performed. (B) The specific activities of complex I (NADH dehydrogenase) was measured in cell lysates. The values are means \pm SEM of the clustered N-, NCDS-, CDS-HFF. (C) Absolute qRT-PCR analysis of mtDNA. The bars are means \pm SEM from five euploid and eight DS fibroblast samples (30).

Likewise, a deficit in the system of mitochondrial energy production, due to the impairment of mitochondrial respiratory chain complex I, ATP synthase, ADP/ATP translocator and adenylate kinase activities was reported in both fetal and adult fibroblasts with trisomy 21 (38).

1.3 Molecular basis of mitochondrial dysfunction in Down syndrome

1.3.1 PGC-1 α is a crucial modulator of mitochondrial biogenesis and function

The molecular machinery responsible for mitochondrial energetic functions requires the regulation of many transcription factors and cofactors. The peroxisome proliferator-activated receptor γ co-activator 1 α (PGC-1 α /PPARGC1A) has emerged as a master regulator of mitochondrial biogenesis and function. PGC-1 α regulates several aspects of oxidative metabolism enhancing the expression and activity of some transcription factors, such as the nuclear respiratory factor NRF-1 and nuclear receptors, such as PPAR- γ (39) (40) (41) (Figure 3).

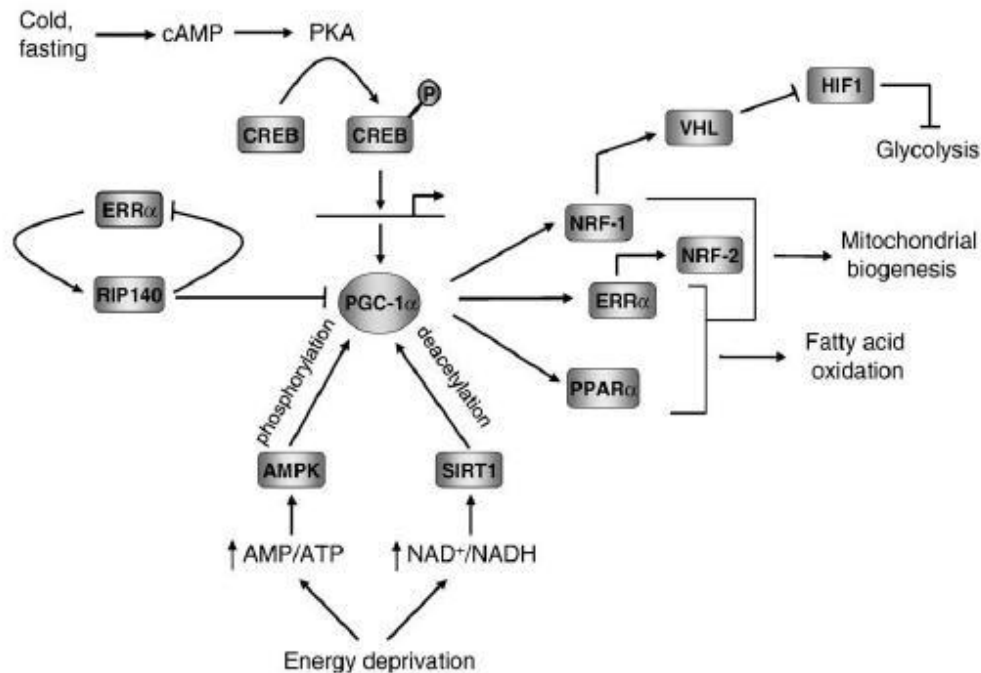


Figure 3. Regulatory network governing mitochondrial functions orchestrated by PGC-1 α . Interactions among some key participants in the transcriptional network regulating mitochondrial biogenesis are depicted schematically. The diagram summarizes the regulation of PGC-1 α by transcriptional and post transcriptional pathways and its interactions with some of its target transcription factors involved in metabolic regulation. Potential suppression of glycolysis through NRF-1 control of VHL expression and negative is also shown (42).

Forced over-expression studies performed in cultured adipocyte lines (43), cardiac myocytes (44) and in conditional, tissue specific, transgenic mice (45), have shown that PGC-1 α is capable of driving virtually all aspects of mitochondrial biogenesis and functions, including activation of respiratory complexes, increased mitochondrial number, and improved mitochondrial respiratory capacity. Indeed PGC-1 α knockout mice manifest a reduction of mitochondrial number and of respiratory capacity in skeletal muscle (41).

It is interesting to note that PGC-1 α expression is decreased in cell types from DS patients (**Figure 4**) and animal models (30) (8) (46).

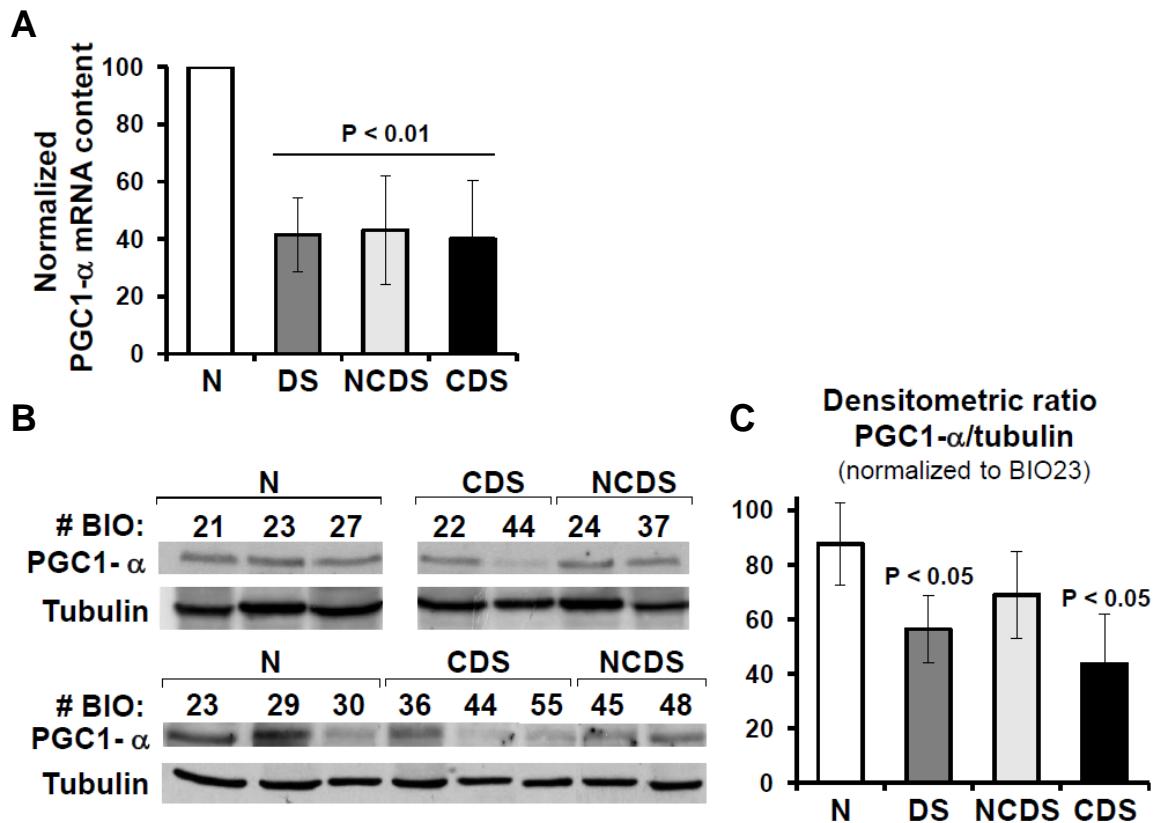


Figure 4. PGC-1 α expression is decreased in trisomic fetal fibroblasts. (A) Expression analysis of the PGC-1 α expression by qRT-PCR. The bars are means \pm SEM from five euploid and eight DS fibroblast samples; a distinction between non-cardiopathic (NCDS, n = 4) and cardiopathic (CDS, n = 4) fetuses-derived fibroblasts is also reported. The values of trisomic samples are normalized to that of the euploid ones. (B) Analysis of the PGC-1 α protein. Western blotting of PGC-1 α on total cellular protein extracts from euploid and trisomic samples (representative of two to three different analyses carried out for each sample). (C) Densitometric analysis of the PGC-1 α -related immunodetected bands (means \pm SEM of two to three assays) (30).

PGC-1 α is indirectly involved in controlling the expression of mtDNA transcription by increasing the expression of mitochondrial transcription factor A (TFAM), which in turn is positively regulated by NRF-1 (42) (45). Gene expression analyses in trisomic cells and tissues and mouse models of DS revealed a disruption of the PGC-1 α regulatory pathway in this condition. Analysis of the expression levels of the PGC-1 α target genes, NRF-1 and TFAM, revealed a decrease of both transcription factors in DS cells and tissues when compared with controls (46) (30) (8). Consistently, a strong decrease in relative mtDNA content was found in DS human fibroblasts and in the Ts65Dn mouse model (46) (30). Interestingly, NRF-1 is significantly down-regulated in trisomic fetal hearts and fibroblasts, and 40% of the mitochondria-related genes down-regulated in DS hearts have consensus DNA binding sites for the nuclear respiratory factor NRF-1 (8) (30).

Activation of the cAMP signaling pathway is a major mechanism underlying the induction of PGC-1 α in several different cell types. In fact, the proximal promoter of PGC-1 α contains a functional CREB binding site that is required for cAMP response (47). In this regard a decrease in PKA (protein kinase A) activity due to reduced basal levels of cAMP has been found in human fetal skin fibroblasts with trisomy 21 (48). Furthermore a decrease in basal levels of cAMP has also been reported in the hippocampus of the mouse model Ts65Dn, due to the altered basal activity of adenylate cyclase (49) (50). In addition PGC-1 α expression and post-translational levels are regulated through sirtuin 1-dependent deacetylation (SIRT1) (51).

1.3.2 Which Hsa21 genes are candidate for mitochondrial dysfunction in Down syndrome?

A major task in the study of the molecular pathology of DS is to establish genotype-phenotype correlations, i.e. the identification of Hsa21 genes, or other genomic functional elements, that contribute to specific features of the DS phenotype. In this respect, many genes were identified as potential candidates in determining the mitochondrial dysfunction present in trisomic cells, as are discussed below.

• *NRIP1/RIP140*

We have previously demonstrated that NRIP1 is a good Hsa21 candidate gene for the mitochondrial dysfunction observed in DS (52).

NRIP1 acts as a scaffold protein recruiting regulatory factors, such as histone deacetylases (53), COOH terminal binding protein (54) and histone methyltransferases (55), to exert its co-repressive function. NRIP1 is known to affect oxidative metabolism and mitochondrial biogenesis (56) (57) by negatively controlling the mitochondrial pathways regulated by *PGC-1 α* (58). Indeed, in *Pgc-1 α* null mice (41) (59), as well as in knock-in *Nrip1* mice (60), mitochondria-related gene expression decreased. NRIP1 transcript is up-regulated by 1.5-to 4-fold in trisomic fetal hearts (8) and fibroblasts (30). NRIP1 protein is increased in the hippocampal tissue from DS subjects (6). Interestingly, NRIP1 emerged as the main dysregulator of mitochondrial gene expression from the analysis of an experiment of modulation of Hsa21 genes (61) (52). To test the hypothesis that NRIP1 over-expression perturbs mitochondrial function, we silenced *NRIP1* gene in trisomic human fibroblasts. We found an increase in the levels of *PGC-1 α* (**Figure 5**) and mitochondria-related genes together with an improvement of mitochondrial function, demonstrated by the increase of ATP production and mitochondrial activity and by the decrease of the ROS production (52).

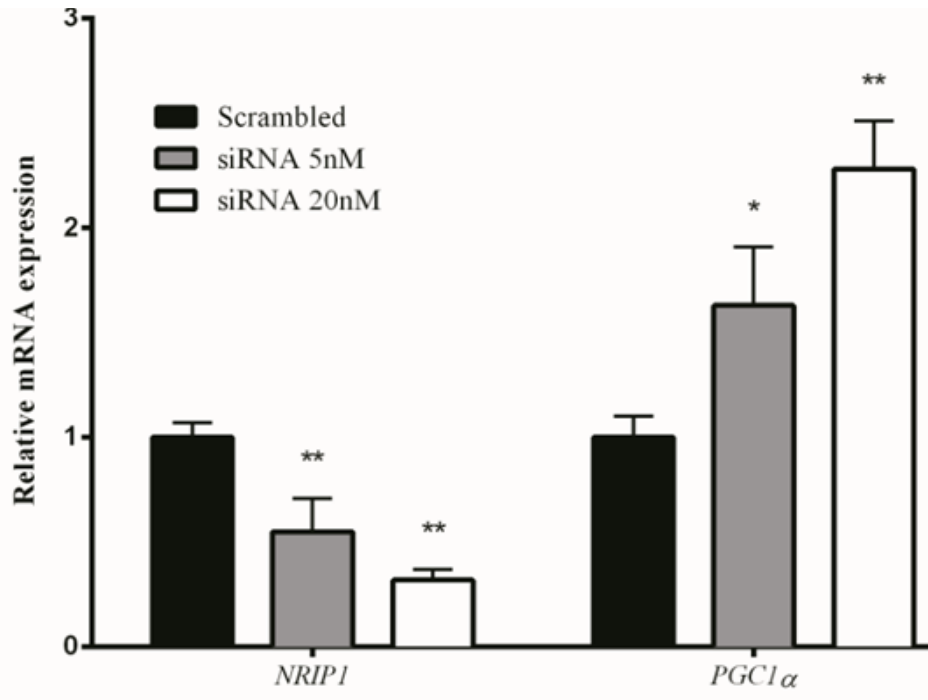


Figure 5. NRIP1 silencing increases *PGC-1α* expression. *NRIP1* and *PGC-1α* expression levels in trisomic cells transfected with a scrambled siRNA and with a NRIP1-specific SMART pool of siRNAs. Values represent the average determination \pm SEM for eight *NRIP1*-silenced DS-HFFs carried out in triplicate (52).

* $P < 0.05$; ** $P < 0.01$.

• *SUMO3*

NRIP1 repressive activity is modulated by SUMOylation (62). *SUMO3* is a gene mapping to chromosome 21 which is over-expressed in trisomic tissues due to gene dosage effect (6). The over-expression of *SUMO3* might further increase NRIP1 repressive activity. SUMOylation also attenuates the transcriptional activity of *PGC-1α*, possibly by enhancing the interaction between *PGC-1α* and the co-repressor NRIP1 (63). Therefore *SUMO3* over-expression in DS could contribute for a concurrent improvement of NRIP1 function and decrease of *PGC-1α* activity.

• *DSCR1/RCAN1 and DYRK1A*

Two other genes mapping to chromosome 21, *DSCR1/RCAN1* and *DYRK1A*, involved in the calcineurin/NFAT pathway may affect DS mitochondrial phenotype. These two genes control *PGC-1α* activity through the binding of *NFATc* genes to the *PGC-1α* promoter (64) (65). Accordingly, in DS fetal hearts and fibroblasts, it has been observed that whereas *NFATc3* and *NFATc4* were down-regulated, *DYRK1A* and *RCAN1* were up-regulated (8) (30).

RCAN1 has been found chronically over-expressed in the brain of DS patients (66). It regulates mitochondrial function and increases susceptibility to oxidative stress in mammalian cells (67). *RCAN1* over-expression is strictly related to calcium overloading, as it affects mitochondrial permeability transition pore leading to several consequences like swelling of mitochondria and rupture of the outer membrane (68). In agreement with these data, mitochondrial Ca^{2+} concentration was found higher in fibroblasts from DS fetuses (30) and in neutrophils derived from trisomic patients (69).

The over-expression of the brain-specific *RCAN1.1S* isoform in mice promotes dysregulation of dynamin-related protein 1 (DRP1) activity, a protein that promotes mitochondrial fission (70). Moreover, *RCAN1* was found to induce mitochondrial autophagy in cardiomyocytes

(71). In addition the over-expression of RCAN1.1S isoform remarkably up-regulated the expression of adenine nucleotide translocator (SLC25A4/ANT1) by stabilizing its mRNA (72).

- ***PKNOX1/PREP1***

The Hsa21 gene PKNOX1/PREP1 is an inhibitor of the oxidative phosphorylation that negatively regulates respiratory chain subunits and mitochondrial activity. PKNOX1/PREP1 gene codes for a homeodomain transcription factor which regulates multiple aspects of embryonic development through the homeobox protein Pbx activity (73). DS human fibroblasts, which show an increase of PREP1 expression, are more sensitive to genotoxic stress. Moreover, it has been demonstrated that PREP1 binds to the promoter region of genes encoding mitochondrial proteins and controls the stability of p160 Myb-Binding Protein, a powerful negative regulator of PGC-1 α activity (74) (75). In the muscle of Prep1 ablated mice, Pgc-1 α expression was increased with consequent increase in mitochondrial oxidative capacity together with an increase of expression of the mitochondrial fusion proteins Opal and Mfn2 (74).

1.3.3 Hsa21 genes affecting other mitochondrial related functions: oxidative stress and mitochondrial death pathway

- ***SOD1***

Overexpression of some of the genes present on chromosome 21 is responsible for the oxidative stress occurring in DS. One of the most probable inducer of oxidative stress is copper-zinc superoxide dismutase SOD1, a metalloenzyme catalyzing the conversion of superoxide anion to hydrogen peroxide (H₂O₂) (76). Levels of SOD1 in cells from DS patients are about 50% higher vs euploid ones (77).

- ***APP***

The amyloid β precursor protein APP, another gene consistently overexpressed in DS has been proposed to contribute to oxidative stress. Overexpression of APP induces mitochondrial oxidative stress and activates the intrinsic apoptotic cascade (78). In addition, amyloid- β fragments, particularly A β 42, exert direct toxic effects in the cells, including mitochondrial dysfunction, induction of oxidative stress and Ca²⁺ dysregulation (79) (80). APP protein progressively accumulates in the mitochondrial matrix increasing free radicals and impairing mitochondrial metabolism (81).

- ***S100B***

This gene codes for the β subunit of S100 proteins, a large family of calcium-binding proteins. Elevated brain S100B expression occurs in various disease states, including DS and AD. In vitro studies of DS fetal human neural precursors demonstrated that S100B is constitutively over-expressed in these cells and that over-expression leads to increased ROS formation, activation of stress response kinases and increased programmed cell death (82).

- ***ETS-2***

Transgenic mice studies show that ETS-2 over-expression induces apoptosis of thymus, spleen, and brain cells (83). Furthermore ETS-2 promotes the activation of a mitochondrial death pathway in DS neurons and induces cytochrome c cytoplasmic translocation and apoptotic features in normal human cortical neurons (35).

- ***ITSN1***

ITSN1 is a Hsa21 gene encoding a multidomain scaffold protein that functions in endocytosis and regulates the mitochondrial apoptotic pathway in endothelial cells (84). ITSN1 expression is elevated in DS brain (85).

1.3.4 Chromosome 21 miRNAs potentially involved in mitochondrial phenotype

A recent annotation of miRNA database (miRBase, release 22) indicates that the Hsa21 harbors at least 30 miRNAs. Five of these miRNAs (let-7c-5p, miR-99a, miR-125b-2, miR-155 and miR-802) were initially identified. They were found over-expressed in DS human fetal brain and cardiac tissue (86) (87) (87). We determined the expression level of these miRNAs in the heart tissue from 6 DS fetuses at about 20 weeks of gestational age. We found that let-7c-5p, miR-125b-2, miR-155-5p and miR-99a-5p were over-expressed in trisomic hearts when compared with euploid controls (**Figure 6**). MiR802 was not expressed.

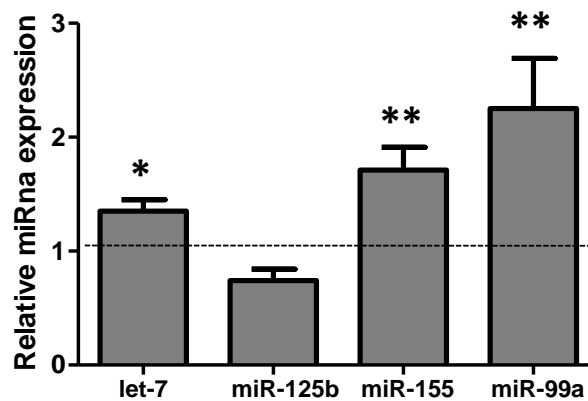


Figure 6. Chromosome 21 miRNA overexpressed in DS hearts. Hsa21 miRNA expression evaluated by qRT-PCR in fetal hearts. Results are expressed as relative mean values \pm SEM of 6 DS samples, compared with control hearts set equal to 1 (88).

*P < 0.05; **P < 0.01.

We focused on the over-expressed Hsa21 miRNAs let-7c-5p, miR-155-5p and miR-99a-5p. MiR-155-5p, a multifunctional microRNA, influences mitochondrial biogenesis by targeting TFAM (89), which is downregulated in trisomic hearts (*as above described*).

The overexpression of let-7c-5p in human induced neuronal cells as well as embryonic stem cells leads to morphological as well as functional deficits including impaired neuronal morphologic development, synapse formation, synaptic strength, and a marked reduction of neuronal excitability (90). Finally the forced expression of miR-99a-5p inhibits the feedback of AKT survival pathway and enhances the induction of apoptosis in cancer cells (91).

To investigate the role of these Hsa21 miRNAs, predicted targets from different databases were obtained and cross-validated using the gene expression profiling data set of human fetal hearts (88). For each miRNA, we compared predicted target genes, obtained by at least 2 different softwares, with the list of genes down-regulated in trisomic hearts. Eighty-five predicted target genes of let-7c, 33 of miR-155-5p, and 10 of miR-99a-5p were expressed in fetal heart and downregulated in trisomic hearts. Bioinformatics analysis indicated that let-7c has several predicted target genes that were down-regulated in trisomic fetal hearts and involved in mitochondrial function. The let-7c predicted target SLC25A4/ANT1, an ATP translocator, was identified among these genes and investigated during my PhD training (see Results).

1.4 Mitochondrial alterations might affect neuronal development and neurodegeneration in Down syndrome

1.4.1 Mitochondria as major regulators of neuronal phenotype

As mitochondria are critical for energy production, calcium buffering, and cell survival pathways, it is not surprising that impaired mitochondrial activity might be linked to neuronal

dysfunction and neurological disorders (92) (93). The structure of the mitochondrial compartment is highly dynamic in neurons and there is a fine-tuned coupling between neuronal activity and mitochondrial function (94).

Mitochondrial dynamics plays an important role in neuronal function. It is essential for neuronal processes such as synaptogenesis, Ca^{2+} buffering, axonal transport, and bioenergetics (95). The neurons critically depend on mitochondrial function to establish membrane excitability and to execute the complex processes of neurotransmission and plasticity. Specifically, the neurons display a complex morphology, highly compartmentalized in long neuronal segments according to their cell type and brain region, thus demanding a proper mitochondrial trafficking network. Many neural developmental processes rely on mitochondrial regulation, including self-renewal and differentiation of neural stem cells, neurogenesis, axonal and dendritic growth, and synaptic formation and reorganization (94).

Functionally and structurally damaged mitochondria do not synthesize sufficient ATP and are more prone to produce pro-apoptotic factors and ROS (96), which could represent an early step in the neurodegenerative process (97). Furthermore, alterations of mitochondrial dynamics are associated with neurodegenerative diseases such as Alzheimer's (AD), Parkinson's (PD) and Huntington's diseases (97) (98) (99) (100) (101).

1.4.2 Mitochondria as major regulators of neuronal development and neurodegeneration in Down syndrome

Although the phenotype of DS is complex, intellectual disability and an early development of AD neuropathology occur in almost all the individuals affected by DS (102). Increasing evidence suggests that mitochondrial dysfunction, consistently observed in DS, contributes to generating these phenotypic traits, as mitochondrial dynamics and metabolism play an important role in both fetal and adult neurogenesis (103), (104), (105).

Significant mitochondrial alterations have been well documented in DS fetal neurons showing an increase of ROS production, reduced membrane potential and increased fragmentation (34) (106) (107) (108). Evidence for mitochondrial damages was found also in the brain of the DS mouse model Ts1Cje (22) (109). Moreover, proteomic approach demonstrated a significant reduction of mitochondrial respiratory enzymes in different brain regions from DS patients (110).

Furthermore, Busciglio et al. (108) demonstrated that there is a marked alteration in A β PP processing and A β trafficking in cortical DS astrocytes and neurons. Development of the DS brain is associated with a decreased number of neurons, relative increase in the number of astrocytes and abnormal neuronal differentiation. The brain of DS fetuses show reduced weight (111) and a volume reduction was observed in various hippocampal structures (112) and in the cerebellum (113). Developmental defects in the dentate gyrus, altered dendritic spine morphology and reduced spine and synaptic density have also been described (114). These last features have been associated with the astrocyte-secreted thrombospondin 1, which is considered as a "spinotrophic" factor and is markedly reduced in DS astrocytes (115).

In addition to developmental defects, the DS phenotype is characterized by early neurodegenerative processes including dementia and AD. Clinical signs of AD are manifested in 75% of DS individuals starting from 40 years of age (116). This has been correlated with the increased expression of the amyloid precursor protein.

However also other factors must be involved in the development of the symptoms of AD since a mouse model in which *APP* is not trisomic is affected as well by neurodegenerative disorders (109). It is known that the calcineurin-*NFAT* signaling pathway, in which the Hsa21 genes *DYRK1A* and *RCAN1* are deeply involved, plays fundamental role in both neuronal development and degeneration. *DYRK1A* affects synaptic plasticity and memory consolidation. Its over-expression causes neurofibrillary degeneration and cell loss in the

hippocampus area (117).

It is interesting to note that alterations of mitochondrial dynamics described in DS are very similar to those observed in neurodegenerative diseases (118) (97) (98) (99) (100) (101). By comparing the list of mitochondria related genes down-regulated in DS fetal hearts and the list of genes belonging to AD (KEGG pathway hsa05010) we found a significant overlap of 20 genes, most of them being target of the NRIP1/PGC-1 α axis (52). This suggest that mitochondrial dysfunction might be a common denominator.

1.5 Mitochondria as potential therapeutic targets in Down syndrome

Targeting of signaling pathways which control mitochondrial functions represents a great opportunity for therapeutic approaches aimed at counteracting mitochondrial dysfunction in DS. In the last years we and other authors focused on several drugs and biological compounds, which have demonstrated favorable effects on the mitochondrial phenotype.

Polyphenols

- ***Epigallocatechin gallate (EGCG)***

EGCG is a major flavonoid component of green tea. It inhibits cellular oxidation and prevents free radical damage. It also is a DYRK1A inhibitor. EGCG stimulates mitochondrial biogenesis and promotes oxidative phosphorylation through a cAMP/PKA and sirtuin dependent mechanism (119) inducing the phosphorylation of complex I subunits and deacetylation of PGC-1 α (120). It has been shown to be a multipotent therapeutic agent with beneficial neuroprotective effects (121). EGCG displays anti-apoptotic effects in many models of neurotoxicity (122). A therapeutic benefit on mitochondrial activity by EGCG has been demonstrated in cellular and DS murine model (119). Treatment with EGCG of neural progenitor cells, isolated from the hippocampus of Ts65Dn, reactivates mitochondria bioenergetics and biogenesis and promotes neural progenitor cell proliferation (46). On the other hand, in vivo studies demonstrated that EGCG counteract cognitive deficits in DS mouse models and in humans (123). Unfortunately even during this critical time window for hippocampal development, EGCG does not elicit enduring effects on the hippocampal physiology (124).

The prenatal EGCG treatment also demonstrated to improve craniofacial dysmorphology associated with DS in Ts65Dn embryos and mice. However, a long-lasting EGCG treatment at a lower dose (E0–E9) did not produce the same corrective effects failing to improve cognitive deficits. All these results suggest that timing and dosage of EGCG treatment are important and have to be optimized for DS patient treatment.

- ***Resveratrol (RSV)***

RSV is a stilbenoid, a type of natural phenol, found in red grape skin, blueberries and some other berries. RSV improves mitochondrial function and protects against oxidative stress by activating PGC-1 α /SIRT1/AMPK axis (125) (46) (126). Recently it has been reported that RSV improves postnatal hippocampal neurogenesis and induces brain derived neurotrophic factor in prenatally stressed rat (127). Interestingly RSV also downregulates the miR-155 (128), which controls TFAM-regulated mitochondrial biogenesis.

A major pitfall in the treatment with polyphenols is represented by the absence of correlation between the dose of polyphenols and their bioavailability in the human body, due to their rapid metabolism and extensive modifications occurring during absorption. Moreover, the polyphenols are present at low concentrations in food with respect to their effective dose as therapeutic agents in humans (129).

Antioxidants, CoQ10 and melatonin

Several clinical trials to test the effects of antioxidant nutrients or diets to scavenge oxygen-derived free radicals (130) (131) (132) (133) were performed in DS patients and DS mouse models.

A randomized, controlled trial of antioxidant supplementation (alpha-tocopherol, ascorbic acid and alpha-lipoic acid) showed neither an improvement in cognitive functions nor a stabilization of cognitive decline (130). To assess whether supplementation with antioxidants (selenium, zinc, vitamin A, vitamin E, and vitamin C) and folic acid, or both, improves the psychomotor and language development of DS children, Ellis et al., (132) performed a randomized, controlled trial in 156 infants, below 7 months of age. Children receiving antioxidant supplements attained similar developmental outcome to the untreated controls. Therefore this study provided no evidence to support the use of antioxidant or folic acid supplements in DS children.

In agreement with this study, vitamin E administration did not demonstrate to slow down the progression of cognitive deterioration in older individuals with DS (134).

Coenzyme Q10 (CoQ10), was discussed as possible therapeutic agent due to its ability to target mitochondrial dysfunction (131) (135). CoQ10 is a bioenergetic enzyme cofactor, central to mitochondrial OXPHOS apparatus, providing potent anti-oxidant protection; it is a potent ROS scavenger (136). CoQ10 supplementation to 10 DS patients for 3 months improves only oxidative imbalance (133).

Melatonin, a hormone involved in the regulation of sleep and wake cycles, was found to be low in the serum of DS children with respect to controls (137). Even though melatonin treatment attenuated the oxidative damage and cellular senescence in the brain (138), pre- and post-natal melatonin administration partially regulated brain oxidative stress but did not demonstrate to improve cognitive or histological alterations in the Ts65Dn mice (139).

Overall, randomized trials based on standard antioxidant supplements or diets failed to produce uniform therapeutic effects. These results suggest that antioxidant supplementation is safe but it neither improve the cognitive performance nor counteracts dementia in DS patients.

2. AIMS OF THE STUDY

The overall rationale behind this project is that manipulating the molecular mechanisms underlying mitochondrial dysfunction in DS could allow to develop successful strategies for preventing and/or treating DS phenotypic alterations.

The basic assumption is that the mitochondrial dysfunction observed in DS plays a role in determining some aspects of congenital DS phenotype, such as neuronal development defects, along with other disorders, such as neurodegeneration, observed in postnatal life. Counteracting mitochondrial dysfunction might therefore represent a worthwhile approach to treat this as yet incurable genetic disease improving the neurological phenotypes and preventing DS associated pathologies.

First step of this project was to characterize the bioenergetic profile of human trisomic fetal fibroblasts (DS-HFFs) using the XF[®]96 Extracellular Flux Analyzer (Seahorse Bioscience), a very sensitive apparatus to study the mitochondrial respiration.

Based on the evidence that the dysregulation of a Hsa21 gene, the co-repressor *NRIP1*, plays a key role in DS mitochondrial dysfunction and that its activity is elicited by the inhibition of *PGC-1α*, a gene that plays a central role in regulating the cell respiratory function.

We have designed therapeutic strategies aimed at repairing DS mitochondrial dysfunction by restoring PGC-1α activity. To meet this objective we planned to pharmacologically modulate *PGC-1α* expression in DS-HFFs by using either metformin, a PGC-1α activator, or pioglitazone, a PPAR-γ agonist. We evaluated the impact of both treatments on mitochondria-related genes expression and on mitochondrial function and network. A further objective of my study was to investigate new molecular mechanisms underlying mitochondrial anomalies in targeting factors responsible for mitochondrial dysfunction in trisomic cells. We have investigated the role of overexpressed Hsa21 miRNAs. To this end *SLC25A4/ANT1*, a gene that encodes the adenine nucleotide translocator, which catalyzes the exchange of mitochondrial ATP for the cytosolic ADP, has been validated as target of the Hsa21 miRNA let-7c-5p.

The novelties of this study relies in: 1. the demonstration of anomalies in mitochondrial network and bioenergetic metabolism in trisomy 21 cells, and the identification of the molecular mechanism responsible for them; 2. the modulation of PGC-1α as a key factor in regulating structural and functional mitochondrial alterations in trisomic cells; 3. the demonstration that 2 drugs, already in use for different diseases, may counteract mitochondrial dysfunction in DS by affecting PGC-1α activity. If any of these drugs will demonstrate to be effective in counteracting DS features, it can be immediately introduced in human therapeutic protocols, maybe in association with specific therapies aimed at restoring the cognitive functions.

3. MATERIALS AND METHODS

Ethics statement

HFFs were obtained from the ‘Telethon Bank of Fetal Biological Samples’ at the University of Naples. All experimental protocols were approved by the local Institutional Ethics Committee.

Samples

Five skin biopsies explanted from human fetuses with trisomy of Hsa21 (DS-HFF) and four from euploid fetuses (N-HFF) after therapeutic abortion at 18-22 gestational weeks were used for this study. Fibroblasts from biopsies were cultured in T25 flasks (BD Falcon) with Chang medium B + C (Irvine Scientific) supplemented with 1% penicillin/streptomycin (Gibco) at 37°C in a 5% CO₂ atmosphere; all analyses described throughout this study were carried out at cell culture passages 4-5.

RNA extraction and quantitative RT-PCR

Total RNA from each sample was extracted using TRIzol reagent (Gibco/BRL Life Technologies, Inc., Gaithersburg, MD) and was reverse transcribed using the iScript cDNA Synthesis kit (BioRad Laboratories, Inc., Hercules, CA, USA). qRT-PCR was performed using SsoAdvanced universal SYBR Green supermix on a Bio-Rad iCycler CFX96 Touch Real-Time PCR Detection System according to the manufacturer’s protocols. Primer pairs (MWG Biotech, Ebersberg, Germany) were designed using the Primer 3 software (<http://bioinfo.ut.ee/primer3-0.4.0/primer3>; date last accessed 2015) to obtain amplicons ranging from 100 to 150 base pairs. In order to test primer efficiency, serial dilutions of cDNAs generated from selected samples, which expressed the target genes at a suitable level, were used to generate standard curves for each gene. qRT-PCR results are presented as relative mRNA levels normalized against reference control values. GAPDH or ABL housekeeping genes were chosen as reference genes.

For miRNA reverse transcription miScript II RT Kit (Qiagen) was used, and the mature miRNA let-7c-5p was quantified using miScript Primer Assay system and miScript SYBR Green PCR Kit (Qiagen). RNAUS and RNA5S were used as reference genes. For end point PCR, assays were performed using the PCR Master Mix (2X) Kit (ThermoFisher). Amplification products were visualized on 2% agarose gel and quantified using the Fiji software (<http://www.fiji.sc>) (140).

miRNA Mimic Transfection

For let-7c-5p up-regulation, a let-7c miRNA mimic (miScript miRNA Mimics, Qiagen) was transfected in 2 N-HFF lines. Cells were plated in a concentration of 70,000/well on 24 well plates (BD Falcon) and after 24 hrs were transfected with miRNA mimic using the INTERFERin transfection reagent (Polyplus-transfection). A fluorescent siRNA (AllStars Neg. siRNA AF 488, Qiagen) was used to monitor the efficiency of the chosen transfecting agent. Forty-eight hrs after the transfection cells were harvested and ANT1 and DICER expression were evaluated, cells treated with the INTERFERin transfection agent only were used as mock control for all experiments performed after transfection.

miRNA Inhibitor Transfection

For let-7c-5p down-regulation, an anti-hsa-let-7c miRNA (miScript miRNA Inhibitor, Qiagen) was transfected in 2 DS-HFF lines. Cells were plated in a concentration of 150,000/dish on 3.5 cm petri dishes (BD Falcon) and after 24 hrs were transfected with the inhibitor using the INTERFERin transfection reagent (Polyplus-transfection). Forty-eight hrs

after transfection cells were harvested for RNA collection while 72 hrs after transfection protein lysates were obtained.

Western blotting

Cells were washed twice with ice-cold phosphate-buffered saline (PBS, Hyclone Lab. INC) and lysed in radio immunoprecipitation assay buffer (1% Triton; 0,5% sodium deoxycholate; 0,1% sodium dodecyl sulfate; 0.15 M NaCl; 0.05 tris-HCl pH7.2) or JS buffer (Hepes, pH 7.5, 50 mM, NaCl 150 mM, glycerol 1%, Triton X-100 1%, MgCl₂ 1.5 mM, EGTA 5 mM) including protease inhibitor cocktail. Cell lysates were centrifuged at 10,000 g for 20 min at 4° C. Protein concentration was measured by the Lowry or Bradford procedure. Protein extracts were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were incubated with specific primary antibodies: anti-SLC25A4/ANT1 mAb (ab 110322, Abcam), anti-vinculin (N-19) (sc-7649, Santa Cruz), anti-PGC1 α (Calbiochem), anti-GAPDH (Cell Signaling), anti-luciferase (Invitrogen), anti-actin (Sigma), anti-MFN1 (Santa Cruz Biotechnology), anti-MFN2 (Santa Cruz Biotechnology), anti-OPA1 (Santa Cruz Biotechnology). Finally, primary antibodies were detected with the appropriate HRP conjugated secondary anti-bodies (Santa Cruz Biotechnology or GE-Healthcare) and revealed by chemiluminescence (Pierce) using digital imaging on a Bio-Rad ChemiDoc XRS apparatus or Fuji X-ray film.

Drug treatments

Metformin hydrochloride (Sigma-Aldrich) was dissolved in water to a stock solution of 100 mM and added to the cell growth medium at the final concentration of 0.05 mM or 0.5 mM. Fresh metformin solution was added every 24 hrs for 72 hrs. In control cells an equal volume of water was added.

Pioglitazone (*Cayman Chemical*) was dissolved in DMSO to a stock solution of 5 mM and added to the cell growth medium at the final concentration of 0.5 μ M or 2 μ M. Fresh pioglitazone solution was added every 24 hrs for 72 hrs. In control cells an equal volume of DMSO was added.

Mitochondrial bioenergetics measurements

Real-time measurements of oxygen consumption rate (OCR) were made using an XF^c-96 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA, USA). Cells were plated in XF^c-96 plates (Seahorse Bioscience) at the concentration of 25,000 cells/well. OCR was measured in XF^c media (non-buffered DMEM medium containing 10 mM glucose, 2 mM L-glutamine and 1mM sodium pyruvate) under basal conditions and in response to 5M Oligomycin, 1.5 μ M of carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) and 1 μ M of Antimycin-A and Rotenone (all from Sigma-Aldrich). The key parameters of mitochondrial function were measured. Each sample was plated at least in triplicate.

Luciferase measurements

The cells were plated on 13-mm diameter glass coverslips for single-sample luminescence measurements and allowed to grow to confluence. To measure total ATP content, the cells were infected with a VR1012-based construct encoding a mitochondrially targeted variant of the *Photinus pyralis* luciferase under the control of the CMV immediate early promoter (mtLuc). Cell luminescence was measured in a luminometer constantly perfused with a modified Krebs-Ringer buffer containing: 125 mM NaCl, 5 mM KCl, 1 mM Na₃PO₄, 1 mM MgSO₄, 1 mM CaCl₂ and 20 μ M luciferin. The light output of a coverslip of infected cells was in the range of 10,000–100,000 counts per second vs a background lower than 1000 counts per second.

Measurements of mitochondrial potential

Mitochondrial potential was measured by loading cells with 10 nM tetramethylrhodaminemethyl ester (TMRM; Life Technologies) for 20 min at 37° C, placed in a humidified chamber at 37° C and imaged with Nikon Swept Field Confocal microscope (Nikon Instruments Inc.) equipped with a CFI Plan Apo VC60XH objective and an Andor DU885 EM-CCD camera. TMRM excitation was performed at 560 nm and emission was collected through a 590 to 650 nm band-pass filter. TMRM fluorescence was analyzed using the NIS Elements software package (Nikon Instruments Inc.). FCCP (10 μ M) was added after eight acquisitions to completely collapse the electrical gradient established by the respiratory chain in order to obtain the basal fluorescence values. Fluorescence intensity was measured in regions drawn around mitochondria and normalized respect to baseline fluorescence recorded after FCCP.

Mitochondrial morphology analysis

The cells were seeded at a density of 50,000 cells per well onto 25-mm glass coverslips, allowed to grow for 24 hrs and then infected with mitochondria-targeted GFP inserted into an adenoviral vector (Ad-mtGFP Ex/Em: 495/515). Protein expression was then allowed for 72 hrs in the presence or absence of metformin. The efficiency of infection was comparable in non-trisomic and trisomic cells both in terms of percentage of GFP positive cells (about 80%) and of intensity of fluorescent GFP signal. Coverslips were placed in an incubated chamber with controlled temperature, CO₂ and humidity. Single cells were imaged, by using the same settings for non-trisomic and trisomic cells, with a Nikon Swept Field Confocal microscope (Nikon Instruments Inc.) equipped with a CFI Plan Apo VC60XH objective and an Andor DU885 EM-CCD camera, which was controlled by NIS Elements 3.2. Fifty-one-plane z-stacks were acquired with voxel dimensions of 133 x 133 x 200 nm (X x Y x Z). The mitochondrial network was then described in numbers of objects, total volume and object volume using the 3D object counter available in the software Fiji (<http://www.fiji.sc>; date last accessed 2015) (140) while 3D rendering was obtained with the 3D Viewer plugin.

Electron microscopy analysis

Cells were fixed in 1% glutaraldehyde dissolved in 0.2 M HEPES buffer (pH 7.4) for 30 min at room temperature and then post-fixed with a mixture of 2% OsO₄ and 100 mM phosphate buffer (pH 6.8) (1 part 2% OsO₄ plus 1 part 100 mM phosphate buffer) for 25-30 min on ice. Next, the cells were washed three times with water and incubated with 1% thiocarbonylhydrazide diluted in H₂O for 5 min. Finally, the cells were incubated in a mixture of 2% OsO₄ and 3% potassium ferrocyanide (1 part 2% OsO₄ plus 1 part 3% potassium ferrocyanide) for 25 min on ice and overnight at 4° C in 0.5% uranyl acetate diluted in H₂O. After dehydration in a graded series of ethanol, the cells were embedded in epoxy resin and polymerized at 60° C for 72 hrs. Thin 60-nm sections were cut with a Leica EM UC7 microtome. EM images were acquired from thin sections using an FEI Tecnai-12 electron microscope equipped with a VELETTA CCD digital camera (FEI, Eindhoven, The Netherlands). The EM analysis was carried out on two euploids and three trisomic cell lines under control conditions and after metformin treatment. The number of damaged mitochondria including any type of ultrastructural changes (partially or completely damaged mitochondria, reduced cristae, broken, shorter or high swollen cristae, giant mitochondria) was calculated as the % of total mitochondria (n > 60 per cell line). Similarly, the number of giant mitochondria were counted and expressed as the % of total mitochondria (n > 60 per cell line). The maximal width of cristae was quantified using the ImageJ software line tool (National Institutes of Health, Bethesda, MD, USA) following a previous protocol (141). A line was drawn across the opening between membranes, not including membrane density. Mitochondria were analysed

using the same magnification within a 25 μm square field of view. The width was calculated by averaging the width of 2-4 measurable cristae for each mitochondrion, then averaging the mean value obtained for all mitochondria ($n > 20$) in each sample and in each condition (untreated and treated).

Statistical procedures

Unless otherwise indicated, all assays were performed independently and in duplicate. Statistical analysis was performed using GraphPad Prism software vers.5.0 (GraphPad Software, La Jolla California USA, <http://www.graphpad.com>; date last accessed 2010). The ANOVA test, with Bonferroni post hoc correction in case of multiple comparisons, was applied to evaluate the statistical significance of differences measured throughout the data sets presented. The threshold for statistical significance (p-value) was set at 0.05.

4. RESULTS

4.1 The whole energy status of trisomic cells is significantly impaired

- Respiratory activity in trisomic cells

Previous studies in our lab indicated that trisomic cells, along with a decrease of mitochondrial gene expression, exhibit a deficit in cellular energy production (30). For a better characterization of mitochondrial metabolism, mitochondrial respiration was investigated in trisomic human fetal fibroblasts (DS-HFFs) versus euploid ones (N-HFFs) by determining directly their bioenergetic profile (118). Overall the indices of mitochondrial respiratory functions were drastically reduced by about half in trisomic cells when compared with euploid ones. Basal OCR, ATP-linked respiration, and maximal respiration were assessed using the XF^e-96 Extracellular Flux Analyzer (Seahorse Bioscience) (**Figure 8A**). The comparison showed that Basal OCR was reduced by 55% in DS-HFFs (**Figure 8B**, “Basal”). The OCR related to ATP production was decreased in DS-HFFs by 58% (**Figure 8B**, “ATP-linked”). The maximal respiratory capacity, (**Figure 8B**, “Maximal”) was also reduced by 58% in trisomic cells.

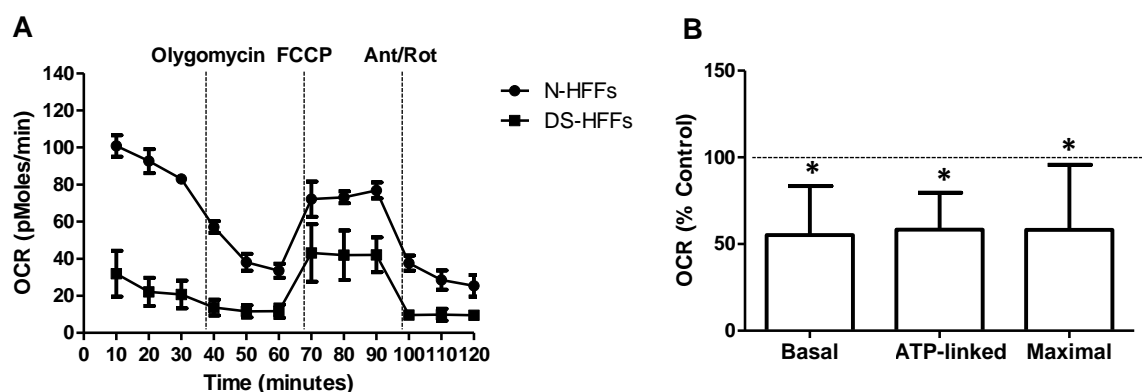


Figure 8. DS-HFFs manifest a reduced respiratory activity.(A) Representative curves of OCR in N-HFFs and DS-HFFs cells (single cell culture in triplicate) obtained in basal condition and after consecutive addition of Oligomycin 5 μ M, FCCP 1.5 μ M and Antimycin-A/Rotenon 1 μ M (Ant/Rot) (left panel). (B) The bars show the mean values of three respiratory parameters (basal OCR, ATP-linked and Maximal OCR) \pm SEM of four DS cell samples compared with four N-HFFs (set equal to 100).

*P < 0.05.

- *ATP basal level in trisomic cells*

In addition to the indirect measure of ATP OCR-related the measurement of the basal cellular ATP levels was carried also using a chimera of the ATP sensitive photoprotein luciferase, as previously described (52) (118). This analysis revealed that the rate of basal ATP content was significantly lower in trisomic cells when compared with euploid ones (**Figure 9A**). This is consistent with the findings from the respiratory assay where OCR related to ATP production was indeed decreased. Considering that basal ATP content is dependent on the amount of transfected luciferase, we determined by western blot analysis that the levels of luciferase protein expressed in DS-HFFs were comparable with those detected in euploid cells (**Figure 9B**).

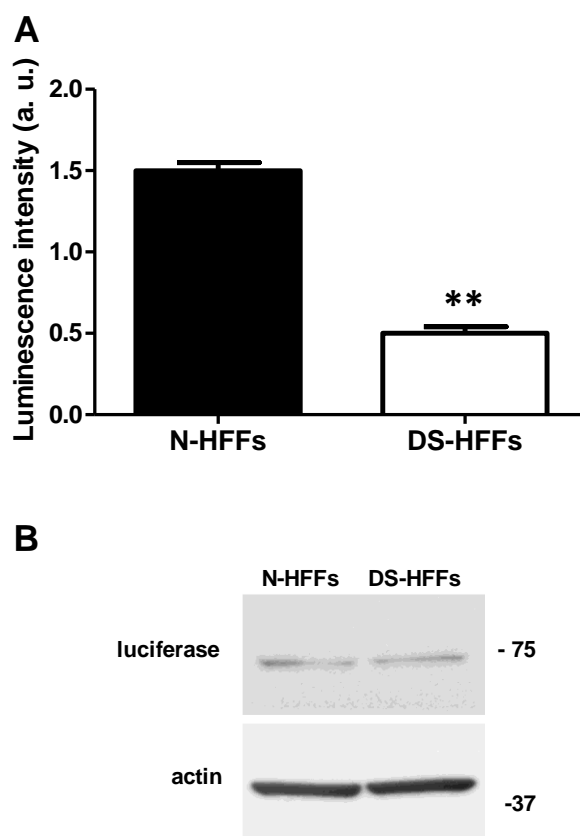


Figure 9. The amount of ATP content is decreased in DS-HFFs. (A) Basal ATP in DS-HFFs versus N-HFFs, measured by a luciferase probe. The bars show the mean values \pm SEM of a comparative analysis between three euploid and three trisomic samples. (B) Representative immunoblot of luciferase protein in N-HFFs and DS-HFFs transfected with a luciferase probe and cultured in complete medium for 72 hrs. Actin was used as loading control.

**P < 0.01; a.u.: arbitrary units.

- ***Mitochondrial membrane potential in trisomic cells***

To further analyze the energetic status of DS-HFFs, the mitochondrial membrane potential was determined by confocal microscopy using the specific mitotropic probe tetramethylrhodamine methylester (TMRM), a red fluorescent dye sensitive to membrane potential changes (118). Fluorescence intensity was measured and normalized to basal fluorescence intensity levels obtained exposing cells to FCCP (see also Materials and Methods). Using this methodological approach, DS-HFFs showed a significant 50% decrease in TMRM fluorescence intensity when compared with N-HFFs (**Figure 10A-B**).

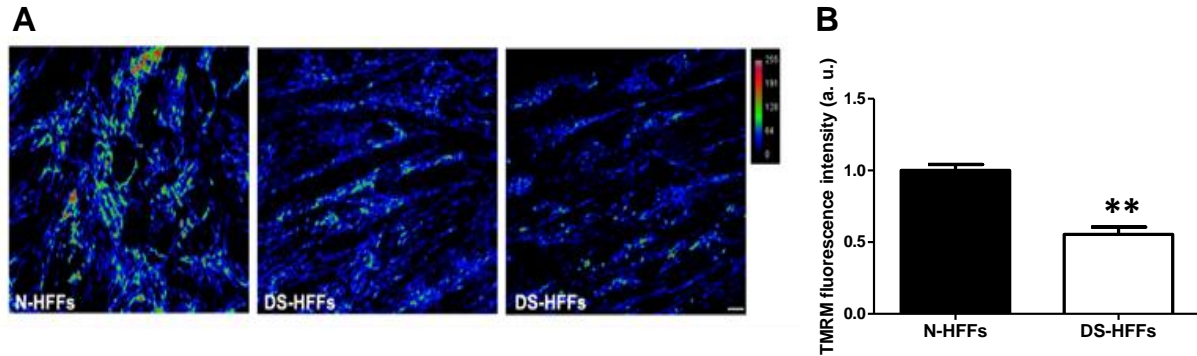


Figure 10. DS-HFFs show a significant decrease in TMRM fluorescence. (A) Representative confocal microscopy live cell imaging of TMRM fluorescence before FCCP in N-HFFs and DS-HFFs. (B) The bars show relative mean values \pm SEM of fluorescence intensity per cell in four trisomic cell lines compared with four non-trisomic ones (set equal to 1). Fifty randomly selected cells for each sample/experimental condition were analyzed.

**P < 0.01; a.u.: arbitrary units.

4.2 Trisomic cells exhibit abnormalities in mitochondrial architecture

- Ultrastructure of mitochondria in trisomic cells

Altered mitochondrial function is often associated with abnormalities in mitochondrial structure we performed morphological analysis of mitochondria in trisomic fibroblasts versus euploid unraveling mitochondrial alterations and remodeling of mitochondrial membrane structures (118).

Mitochondria of DS-HFFs exhibited short and/or damaged cristae (**Figure 11A**, arrow heads), and some of them appeared with an enlarged size and swelling (**Figure 11A**, arrows). The number of damaged mitochondria in DS-HFFs was 3,5 fold higher when compared with N-HFFs (90% in DS-HFFs vs 25% in N-HFFs) (**Figure 11B**) and 40% of DS-HFFs mitochondria displayed intra-edema with respect to 6% in non-trisomic cells (**Figure 11C**). Since cristae shape determines the assembly and stability of respiratory chain complexes and hence mitochondrial respiratory efficiency (142, 143), we evaluated the maximal width of cristae (141). In DS-HFFs the mitochondrial membranes appeared more widely spaced when compared with euploid cells where they were closely juxtaposed at the level of the cristae (**Figure 11A**). In detail trisomic cells showed mitochondria with significantly wider cristae (2-fold) vs non-trisomic cells (**Figure 11D**). Furthermore, whereas in N-HFFs only 15% of mitochondria exhibited swollen cristae (mean maximal width > 30 a.u.), in DS-HFFs about 60-90% of mitochondria showed this characteristic (**Figure 11E**).

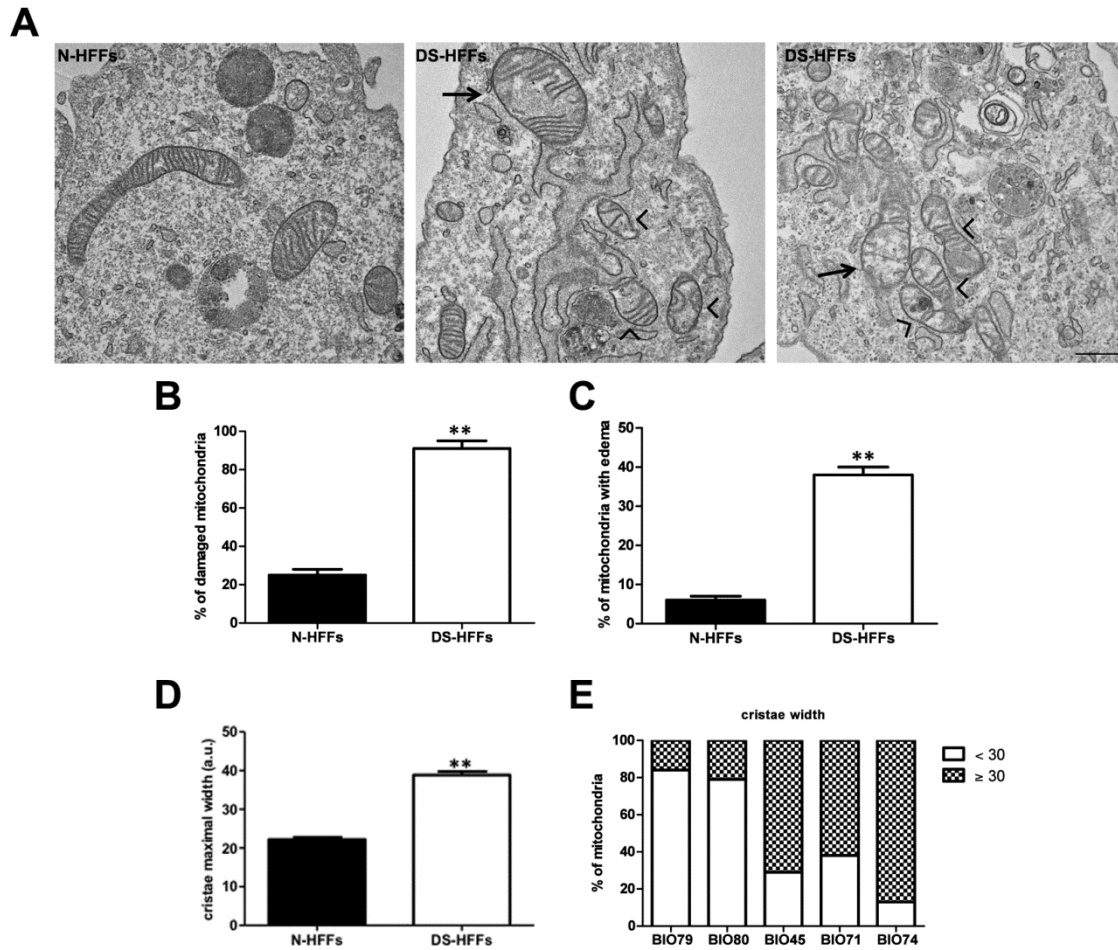


Figure 11. Mitochondria of DS-HFFs show morphological abnormalities. (A) Representative ultrastructural images of N-HFFs vs DS-HFFs. The arrow heads indicate damaged mitochondria. (B) The percentage of damaged mitochondria in trisomic cells compared with non-trisomic cells and (C) the percentage of mitochondria with intra-edema. The bars show mean values \pm SEM of two non-trisomic cells and three trisomic lines; $n > 80$ per cell line. (D) Mean cristae maximal width in N-HFFs versus DS-HFFs (see Methods). (E) The percentage of mitochondria with enlarged cristae (> 30 a.u.) in DS-HFFs in comparison with N-HFFs. In D and E the bars show mean values \pm SEM of two euploid and three trisomic ones; $n > 20$ per sample. Scale bar $1 \mu\text{m}$. ** $P < 0.01$; a.u.: arbitrary units.

4.3 Mitochondrial network and fission-fusion machinery genes in trisomic cells

Studies on the correlation between the modulation of the energetic state of the cell and mitochondrial shape and network (143) (144) have demonstrated that mitochondria can switch from an elongated and interconnected network to a fragmented state via fusion and fission events during the so-called ‘mitochondrial life cycle’ (145). We examined the overall organization of the mitochondrial network at the confocal microscope by looking at the distribution of a green fluorescent protein targeted to mitochondria (mtGFP) (118) in order to investigate the fission-fusion phenotype in trisomic fibroblasts.

DS-HFFs appeared fragmented with many small and short mitochondria when compared with N-HFF mitochondrial network which exhibited a branched, tubular morphology (**Figure 12A**). The mitochondrial number was higher in DS-HFFs (**Figure 12B**), whereas their average volume was significantly decreased (**Figure 12D**). Total mitochondrial volume was not significantly changed (**Figure 12C**).

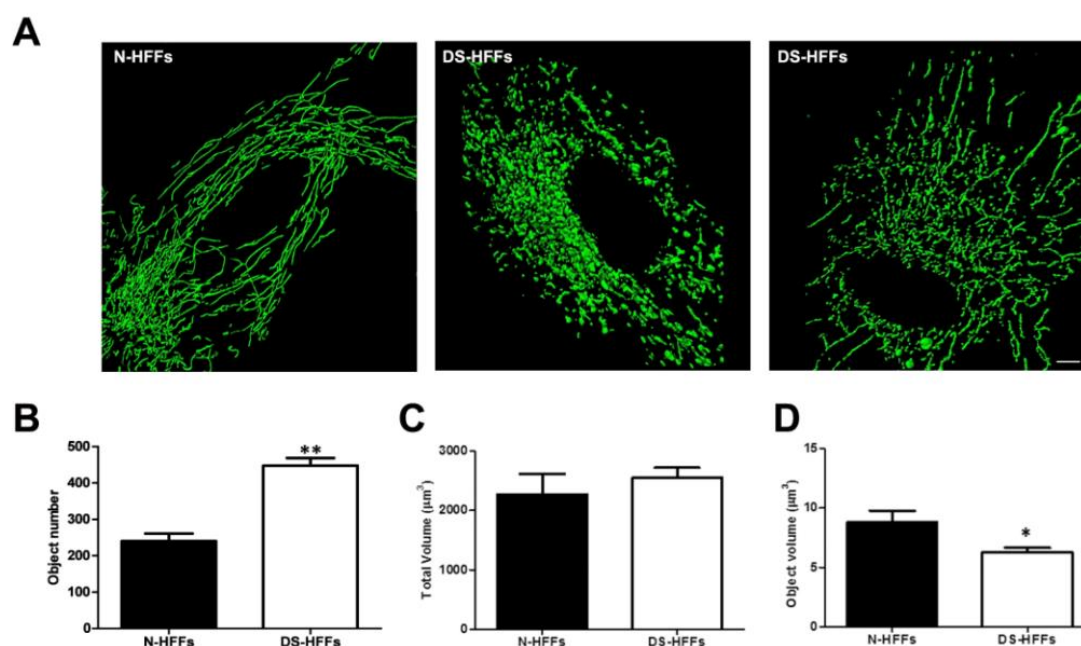


Figure 12. DS-HFFs mitochondrial network is highly fragmented (A) Representative images showing the mitochondrial network in N-HFFs versus DS-HFFs. (B) The number of mitochondria, (C) the total volume and (D) the mitochondrial volume were compared in trisomic vs non trisomic cells. The bars show mean values \pm SEM of two euploid and five trisomic cell cultures. Fifty randomly selected cells for each sample/experimental condition were analyzed.

* $P < 0.05$; ** $P < 0.01$.

To understand the molecular bases of the differences in mitochondrial network organization between trisomic and non-trisomic cells, we focused on some proteins involved in mitochondrial fission-fusion machinery that play a role in the organization of mitochondrial network (118). We determined mRNA and protein levels of OPA1, MFN1 and MFN2 in trisomic and non trisomic cells. We found that OPA1 and MFN2 mRNA and protein expression was significantly reduced in DS-HFFs vs N-HFFs (**Figure 13**). This correlates well with the extensive fragmentation of mitochondria observed in trisomic cells (**Figure 12**).

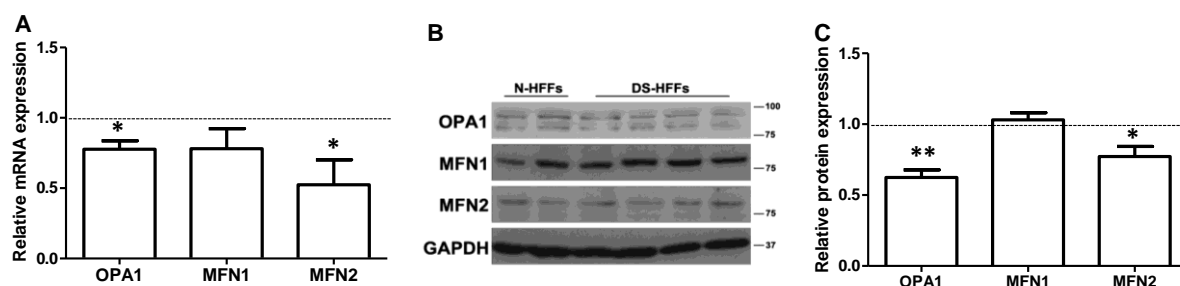


Figure 13. Mitochondrial fusion gene expression is diminished in DS-HFFs. (A) *OPA1*, *MFN1* and *MFN2* mRNA expression measured by qRT-PCR upon normalization to a reference gene (*ABL*). (B) Representative immunoblotting of *OPA1*, *MFN1* and *MFN2* and densitometric analysis of three different experiments are shown. *GAPDH* was measured as a loading control. (C) The bars show the relative mean values \pm SEM of three DS cell cultures compared with N-HFFs (set equal to 1).

* $P < 0.05$; ** $P < 0.01$.

4.4 Metformin and pioglitazone activate PGC-1 α in trisomic cells

We tested the hypothesis that the induction of the PGC-1 α , might restore the mitochondrial activity in trisomic cells. Among the drugs which demonstrated to affect PGC-1 α activity we chose the biguanide, metformin (MTF) and a thiazolidinedione, namely pioglitazone (PG). We treated DS-HFFs with these two drugs. MTF stimulates PGC-1 α through AMPK and SIRT1 activity and PG is a PPAR γ agonist. To determine the effects of drug administration on the PGC-1 α signaling pathway, based on literature data (146) and unreported preliminary experiments, we exposed DS-HFFs to different concentrations of the drugs for 72 hrs. For MTF 0.05 and 0.5 mM concentrations were chosen.

Trisomic fibroblasts treated with MTF for 72 hrs showed a significant increase in PGC-1 α expression, at both the mRNA (Figure 14A) and protein level (Figure 14B-C), when compared with untreated control cells. To understand if MTF activated the PGC-1 α signaling pathway, we measured the mRNA levels of two transcription factors dependent upon PGC-1 α activity: NRF-1 and TFAM, which are both downregulated in trisomic cells (30). Furthermore NRF-1 is induced by PGC-1 α in a dose dependent way (147) while TFAM is positively regulated by NRF-1 expression (40) and plays a central role in expression, maintenance, and organization of the mitochondrial genome (148).

MTF at both concentrations induced an increase of both *NRF-1* and *TFAM* mRNA expression in DS-HFFs (Figure 14D-E). Also the amount of mtDNA, measured as the ratio of *D-LOOP/ACTIN* mRNA expression, was found increased in trisomic cells after MTF treatment (Figure 14F).

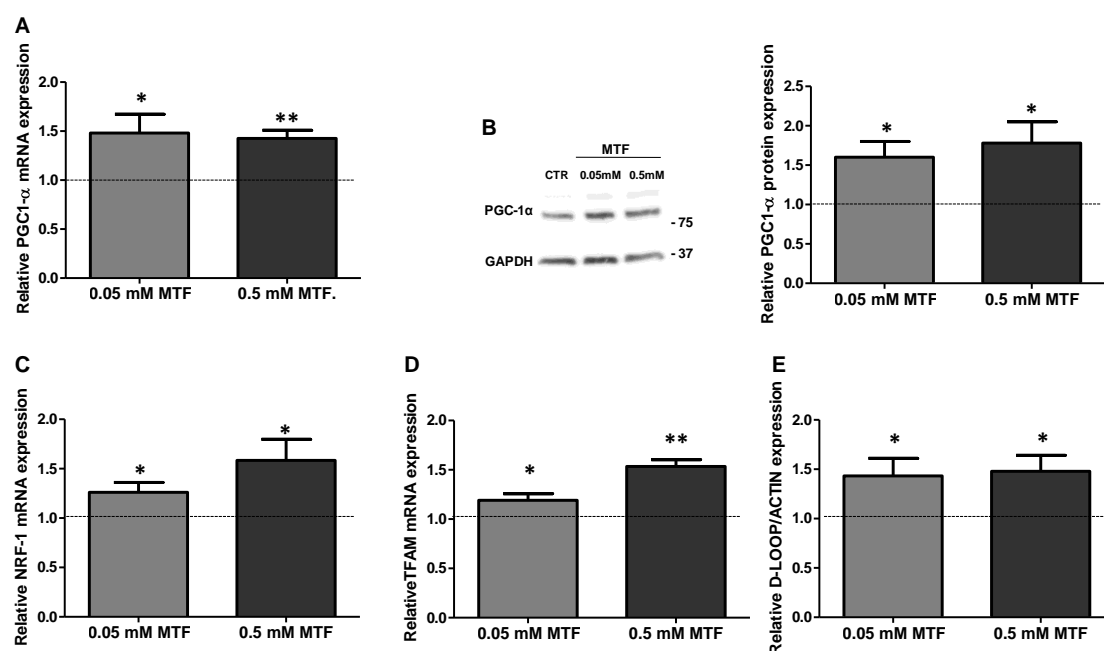


Figure 14. The PGC-1 α signaling pathway is a MTF target in DS-HFFs. DS-HFFs were treated with different MTF doses for 72 hrs. **(A)** *PGC-1 α* mRNA expression by qRT-PCR upon normalization to a reference gene *GAPDH*. **(B)** *PGC-1 α* protein detected by immunoblotting in total protein fractions. Representative immunoblotting of *PGC-1 α* and **(C)** densitometric analysis of three different experiments is shown. *GAPDH* was used as a loading control. **(D)** *NRF-1* and **(E)** *TFAM* mRNA expression. **(F)** Ratio between the mtDNA marker *D-LOOP* and the nuclear DNA marker *ACTIN* by qRT-PCR upon normalization to a reference gene *GAPDH*. Results are expressed as relative mean values \pm SEM of three DS cell cultures treated with MTF, compared with untreated cells (set equal to 1). * $P < 0.05$; ** $P < 0.01$.

PG treatments were performed with four increasing concentrations (0.25 μ M; 0.5 μ M; 1 μ M and 2 μ M) taking into account the PG half maximal effective concentration (EC_{50} \approx 500-600 nM for both human and murine *PPAR γ* activation) (149) (150) and previous reports in which the effects of thiazolidinediones *PGC-1 α* were investigated (146). We did not analyze higher doses since it has been previously demonstrated that they inhibit the mitochondrial respiratory chain (151). We found that PG significantly increases the *PGC-1 α* protein level starting from the lowest tested concentration of 0.25 μ M (**Figure 15A**).

We evaluated the potential toxicity of the PG four concentrations in trisomic cells by MTT assay. All the subsequent experiments were carried out at 0.5 and 2 μ M PG concentrations. Consistent with the increase of *PGC-1 α* protein expression, *PGC-1 α* mRNA levels were significantly increased in trisomic cells after PG treatment at both concentrations (**Figure 15B**). Furthermore a significant increase of the expression levels of *PPAR- γ* mRNA was observed, primarily at the 0.5 μ M dose (**Figure 15C**).

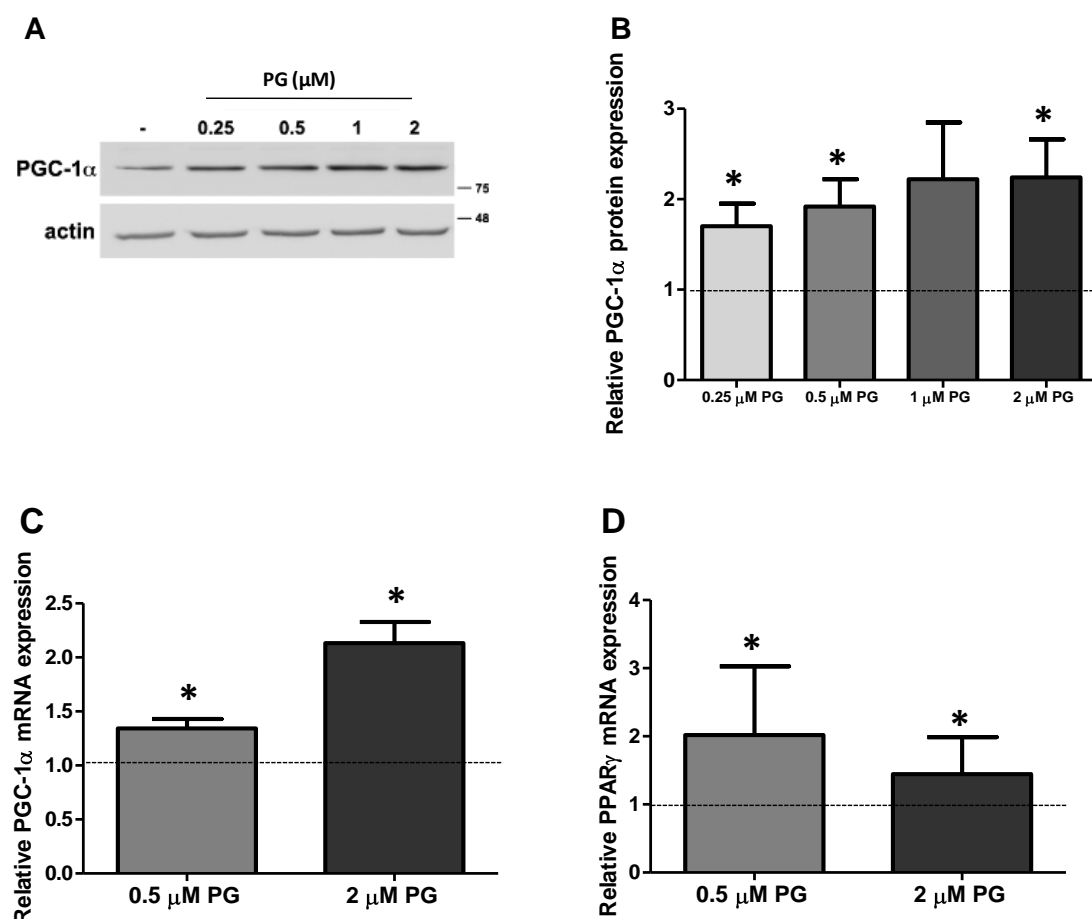


Figure 15. PG induces PGC-1 α expression in DS-HFFs. DS-HFFs were treated with different PG concentrations for 72 hrs. (A) PGC-1 α protein expression in total protein fractions. Representative immunoblotting of PGC-1 α and (B) densitometric analysis of three different experiments is shown. ACTIN was used as a loading control. Results are expressed as relative mean values \pm SEM of three DS cell cultures treated with PG, compared with untreated controls (set equal to 1). (C) PGC-1 α mRNA expression. (D) PPAR γ mRNA expression. The mRNA levels were measured by qRT-PCR upon normalization to a reference gene (*GAPDH*). For each sample, values represent the average determination for 2 qRT-PCR experiments. Results are expressed as relative mean values \pm SEM of four DS cell cultures treated with PG, compared with untreated controls (set equal to 1).

* $p \leq 0.05$; ** $p \leq 0.01$.

4.5 PGC-1 α induction improve the bioenergetic profile of trisomic cells

To assess whether the increase of PGC-1 α expression was adequate to ameliorate mitochondrial activity, we determined the bioenergetic profiles of DS-HFFs treated either with MTF (118) or with PG.

MTF treatment at both doses was associated to a significant increase in basal OCR, OCR related to ATP production and maximal OCR (**Figure 16A-B**). PG treatment was associated to the increase in each of the three bioenergetic parameters at the lowest concentration (0.5 μ M). No significant variation in OCR was found after PG treatment at the concentration of 2 μ M.

Overall, both drugs induced a recovery of mitochondrial respiration associated with the induction of PGC-1 α activity.

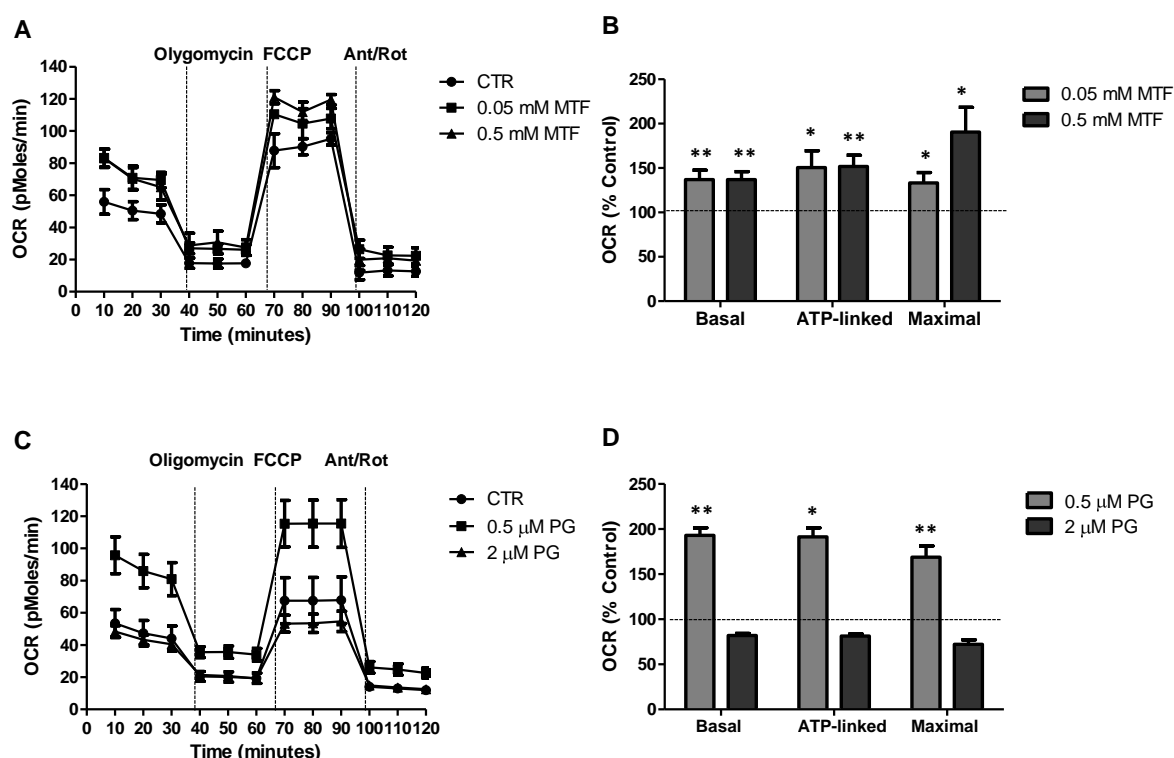


Figure 16. MTF and PG induce a gain of respiratory function in DS-HFFs. (A) Representative curves of OCR in MTF-treated DS-HFFs for 72 hrs (single cell culture in triplicate) (left panel). (B) The bars show the relative mean values of three respiratory parameters (basal OCR, ATP-linked and Maximal OCR) \pm SEM of four trisomic cell lines treated with MTF, compared with untreated cells (set equal to 100). (C) OCR measurement after 72 hrs of exposure to PG compared with untreated controls. OCR merged curves of the mean value in 4 trisomic samples. (D) The bars show the relative mean values of three respiratory parameters \pm SEM of four trisomic cell lines treated with PG, compared with untreated cells (set equal to 100).

* $p \leq 0.05$; ** $p \leq 0.01$.

We performed a direct measurement of basal cellular ATP content in trisomic cells after drug treatments using the luciferase protein assay (see above). A significant increase in basal ATP content was detected in trisomic cells treated for 72 hrs with MTF (**Figure 17A**) or PG at all the tested dosages (**Figure 17B**).

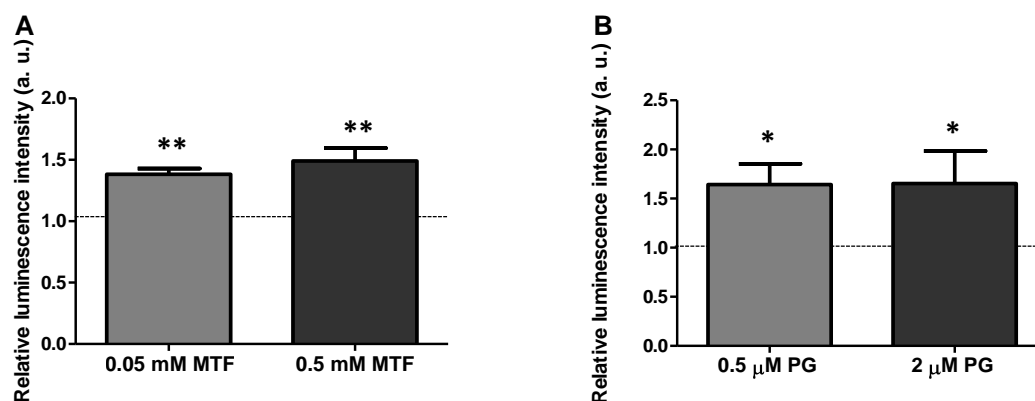


Figure 17. MTF and PG treatments induce an increase of ATP production in DS cells. ATP measurement in trisomic cells after 72h of MTF or PG treatment. **(A)** The bars show relative mean values \pm SEM for four DS cell cultures treated with MTF, compared with untreated cells (set equal to 1). **(B)** The bars show relative mean values \pm SEM for three DS cell cultures treated with PG, compared with untreated controls (set equal to 1). * $p \leq 0.05$; ** $p \leq 0.01$; a.u.: arbitrary units.

Metformin increases mitochondrial membrane potential

To obtain additional evidence that MTF improves mitochondrial activity, we incubated MTF-treated trisomic cells with TMRM to measure mitochondrial membrane potential. A significant difference in TMRM-related fluorescence was observed in MTF-treated cells at a concentration of 0.5 mM. The increase in fluorescence at the 0.05 mM concentration was not significant (**Figure 18D**).

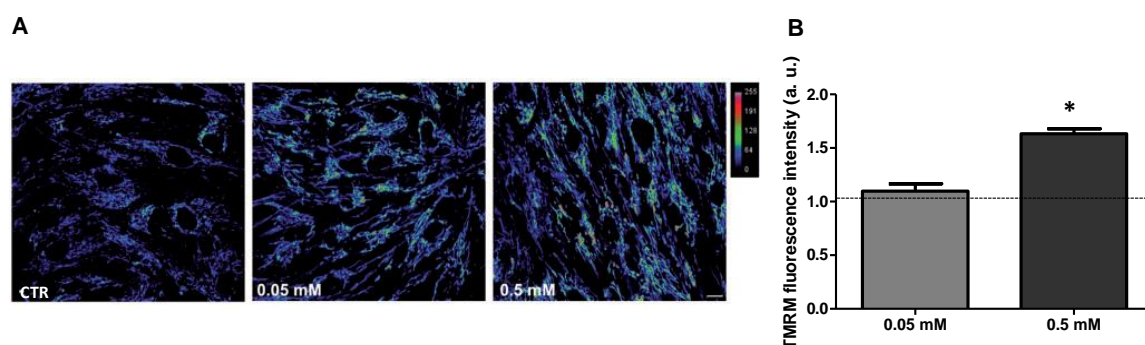


Figure 18. Membrane potential increases in MTF-treated DS-HFFs. **(A)** Representative confocal microscopy live cell imaging of TMRM fluorescence in untreated (CTR) DS-HFFs and those treated with MTF. **(B)** The bars show relative mean values \pm SEM of fluorescence intensity per cell in four trisomic cell cultures treated with MTF relative to untreated cells (set equal to 1). Fifty randomly selected cells for each sample/experimental condition were analyzed.

* $p \leq 0.05$; a.u.: arbitrary units.

4.6 ROS production is decreased in trisomic cells treated with metformin and pioglitazone

We previously demonstrated that trisomic cells display an increased ROS production, with a larger redox imbalance when compared with euploid cells (30). MTF and PG are known to normalize hyperglycemia induced intracellular ROS and mtROS production (152) (153). Based on these premises, we used the redox-sensitive fluorescent probe 2', 7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA) to investigate the effects of 0.5 mM MTF (118) and 0.5 μ M or 2 μ M PG treatments on intracellular ROS in trisomic cells. H₂DCF-DA-associated fluorescence was significantly decreased in trisomic cells after treatments with both drugs (**Figure 19**). ROS production was affected by PG in a dose dependent manner.

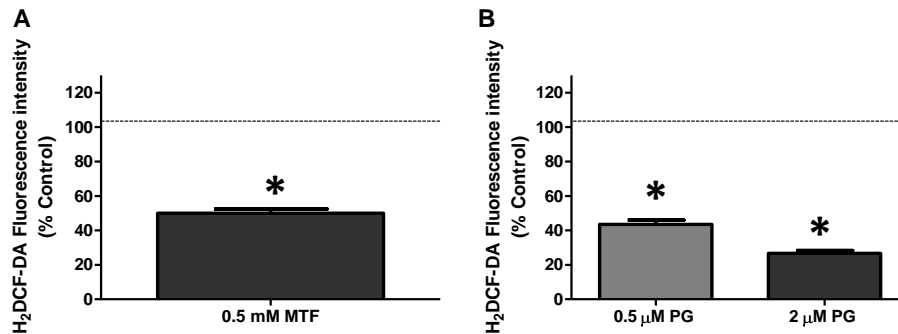


Figure 19. ROS production are decreased in DS-HFFs after MTF and PG treatments. DS-HFFs were treated with MTF or PG for 72 hrs. (A) Trisomic cells stained with H₂DCF-DA probe after MTF treatment and (B) PG respect to untreated-cells (set equal to 100). Data are expressed as arbitrary units of H₂DCF-DA fluorescence and reported as mean \pm SEM from four independent experimental sessions.

* $p \leq 0.05$; ** $p \leq 0.01$.

4.7 Metformin and pioglitazone affect mitochondrial dynamics by modulating the expression of fission/fusion machinery genes

To determine if MTF and PG affect mitochondrial dynamics in trisomic cells, we infected DS-HFFs with a mitochondria-targeted GFP, which allowed to measure mitochondrial number and volume in treated vs untreated cells (118).

The confocal microscopy analysis demonstrated that both drugs affect mitochondrial network. A full rescue of the tubular morphology of mitochondria was observed in MTF-treated DS-HFFs (**Figure 20A**) that showed a network closely resembling that of euploid cells (**Figure 12**). The mitochondrial number was decreased (**Figure 20B**) while the total volume as well as the individual mitochondrial volume were increased after MTF treatment (**Figure 20C-D**).

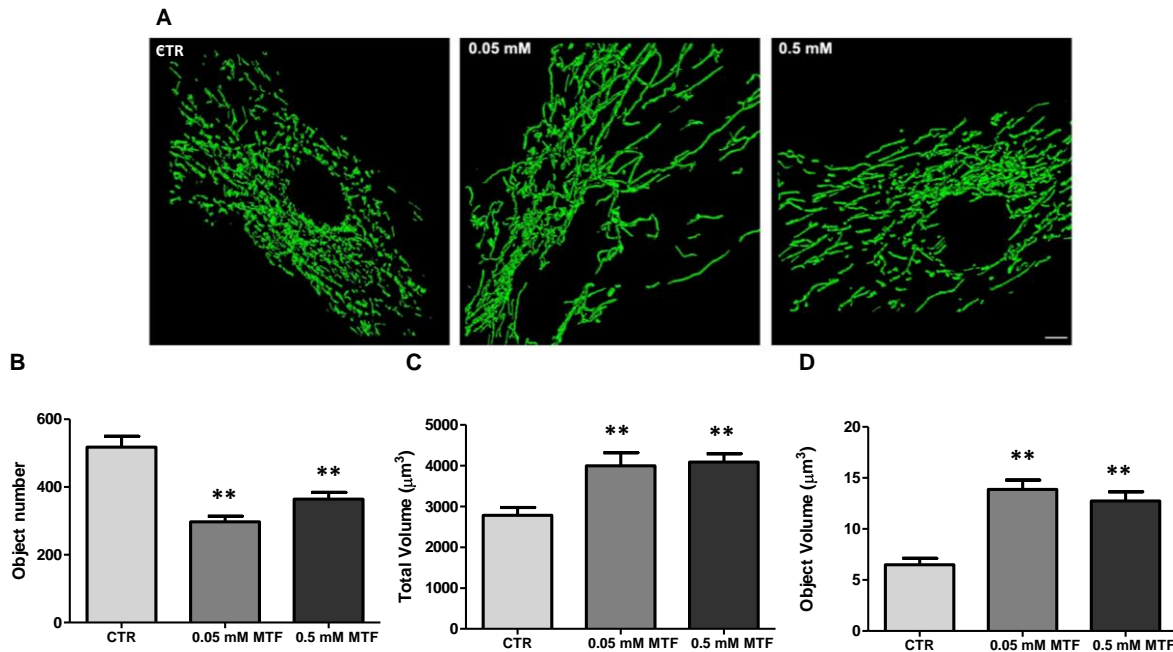


Figure 20. Mitochondrial fragmentation is decreased in MTF-treated DS-HFFs. (A) Representative images of the mitochondrial network in untreated DS-HFFs (CTR) and those treated with MTF for 72 hrs. (B) The number of mitochondria (C) the total volume and (D) the mitochondrial volume were compared in MTF-treated trisomic cells with untreated cells. The bars show mean values \pm SEM of five trisomic cell cultures. Fifty randomly selected cells for each sample/experimental condition were analyzed.

* $p \leq 0.05$; ** $p \leq 0.01$.

PG showed a similar trend after both the concentrations. DS cells exhibited a less fragmented mitochondrial network, with elongated and branched mitochondria, similar to that observed in non-trisomic cells (**Figure 21A**). An average a lower number of mitochondria (**Figure 21B**) and an increase of individual mitochondrial volume (**Figure 21C**) were observed. Total volume was significantly increased only at 2 μ M PG dose (**Figure 21D**).

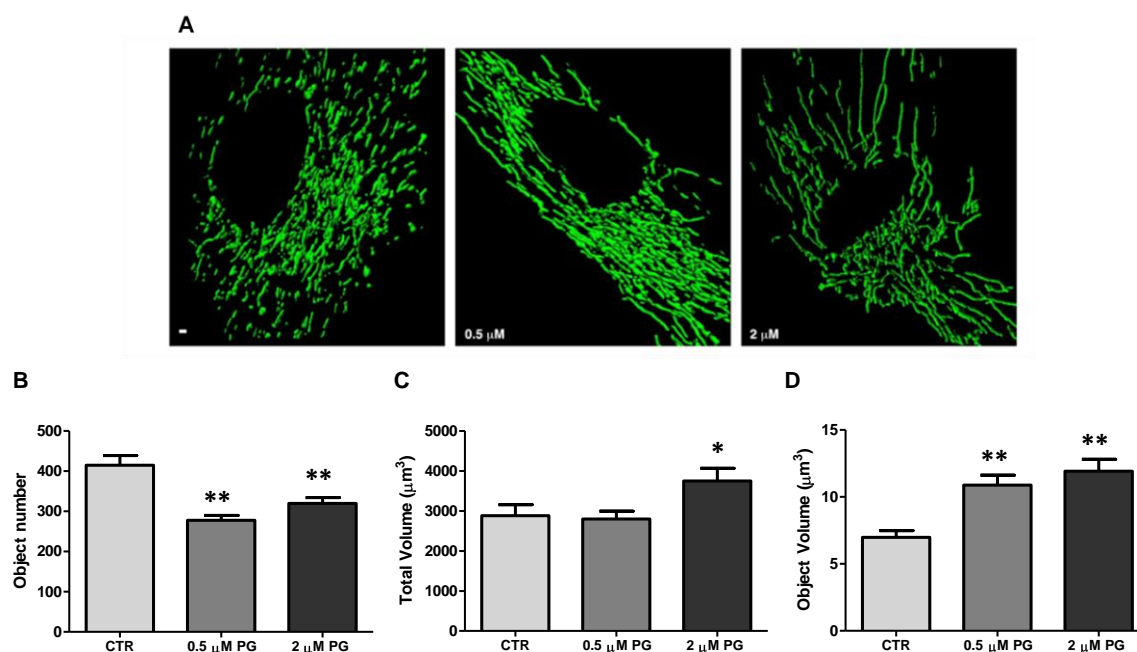


Figure 21. Mitochondrial fragmentation is decreased in DS-HFFs treated with PG. (A) Representative images of the mitochondrial network in untreated DS-HFFs (CTR) and those treated with PG for 72 hrs. (B) The number of mitochondria, (C) the total volume and (D) the mitochondrial volume were compared in PG-treated trisomic cells with untreated cells. The bars show mean values \pm SEM of four trisomic cell cultures. Fifty randomly selected cells for each sample/experimental condition were analyzed.

* $p \leq 0.05$; ** $p \leq 0.01$.

In order to explore if the restoration of the mitochondrial network was associated with changes in the expression of mitochondrial fission-fusion genes we analyzed their expression in trisomic cells, before and after drug treatments (118). mRNA and protein levels of OPA1 were significantly increased following exposure to both drugs. At any concentration both mRNA and protein expression of MFN2 were increased only upon MTF treatment. MFN1 expression was upregulated by the highest doses of both drugs but protein levels were affected to a lesser extent. mRNA expression level of the mitochondrial fission *DRP-1* gene was decreased in a dose dependent manner after PG treatment (**Figure 22**) while it was not significantly affected by MTF treatment (data not shown). Protein levels were too variable in both treatments to obtain a statistical significance. Our results suggest that MTF and PG treatments restore mitochondrial network architecture and that this result is reached by modulating the expression of mitochondrial fission/fusion genes, with a consistent effect particularly on OPA1 gene.

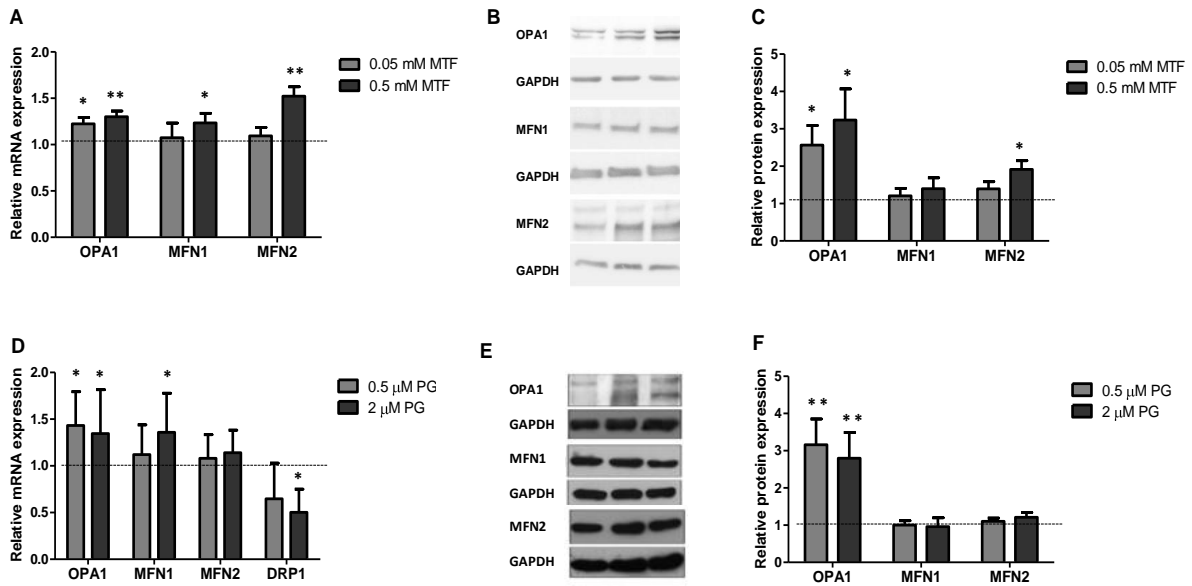


Figure 22. MTF and PG modulate mitochondrial fission-fusion gene expression. (A) *OPA1*, *MFN1* and *MFN2* mRNA expression after 72 hrs of treatment with MTF and (B) representative immunoblotting and (C) densitometric analysis of their protein levels. GAPDH was measured as a loading control. (D) *OPA1*, *MFN1*, *MFN2* and *DRP-1* mRNA expression after 72 hrs of treatment with PG and (E) representative immunoblotting and (F) densitometric analysis of their protein levels. GAPDH was measured as a loading control.

All these results are expressed as mean values \pm SEM of three trisomic cell cultures compared with untreated cells (set equal to 1) in three independent experiments.

* $p \leq 0.05$; ** $p \leq 0.01$.

4.8 Mitochondrial ultrastructure abnormalities are restored by metformin

As OPA1 modulation may affect mitochondrial cristae, we investigated the effects of MTF treatment on the mitochondrial morphological phenotype (**Figure 23**). Notably, with respect to untreated cells, DS-HFFs treated with 0.5 mM MTF for 72 hrs presented significantly fewer damaged mitochondria (40%) as well as mitochondria with intra-edema (5%) and narrower cristae than untreated-cells (**Figure 23**) with width comparable to N-HFFs cells (**Figure 11**).

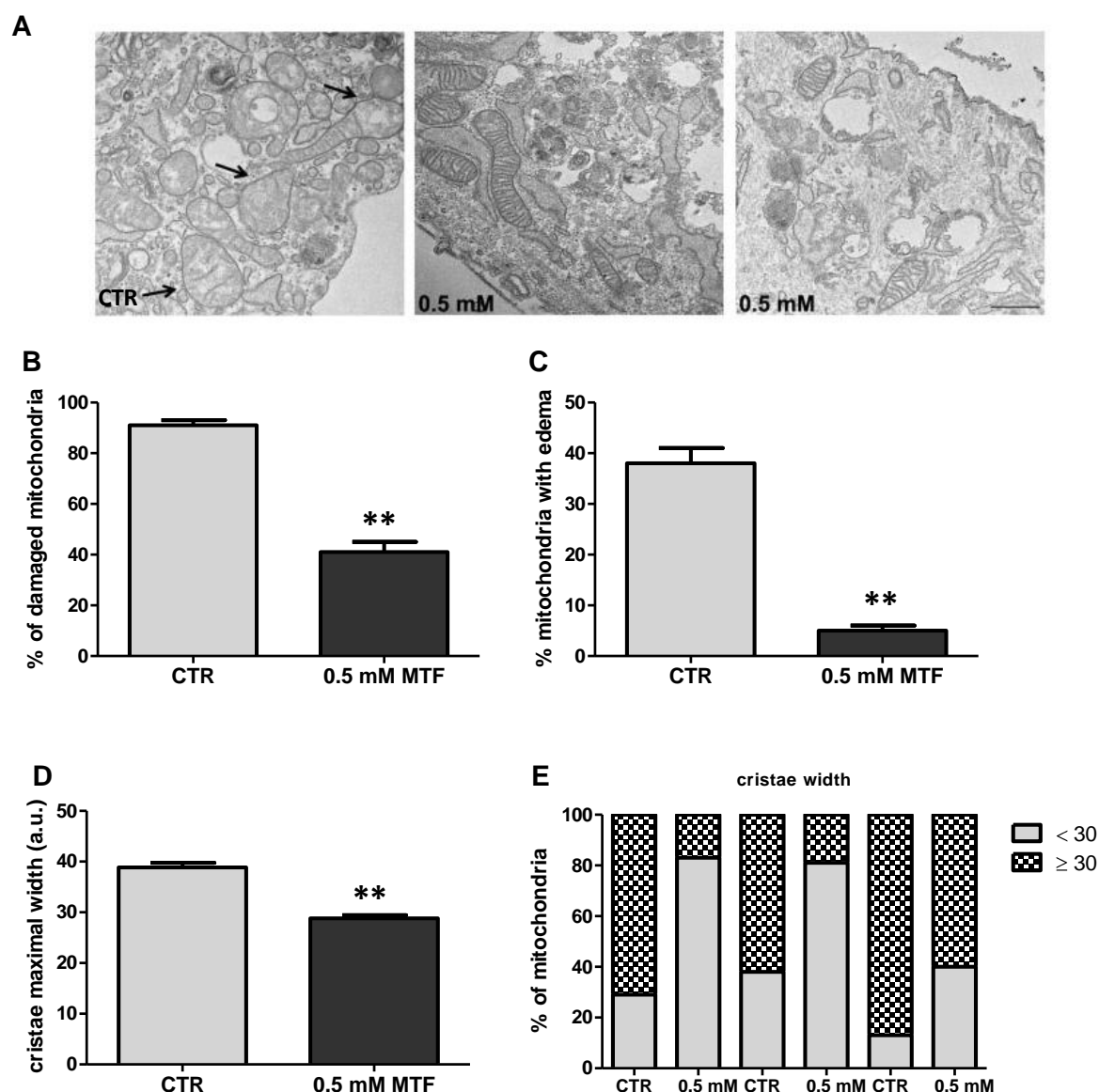


Figure 23. Mitochondrial ultrastructure abnormalities are reversed upon MTF treatment. (A) Representative images of untreated and treated trisomic cells. The arrow heads indicate damaged mitochondria. (B) The percentage of damaged mitochondria in treated trisomic cells compared with untreated cells and (C) the percentage of mitochondria with intra-edema. The bars show mean values \pm SEM of three trisomic lines; $n > 80$ per cell line. (D) Mean cristae maximal width in treated trisomic cells versus controls (see Methods). (E) The percentage of mitochondria with enlarged cristae (> 30 a.u.) in MTF treated cells in comparison with untreated cells. In D and E the bars show mean values \pm SEM of three trisomic ones; $n > 20$ per sample. Scale bar 1 μ m. ** $P < 0.01$; a.u.: arbitrary units.

4.9 Chromosome 21 miRNAs overexpression might contribute to mitochondrial dysfunction in trisomic cells

- *SLC25A4/ANT1* is a predicted *let-7c-5p* target

The amount of phosphorylated adenosine nucleotides, including the main energy carrier ATP, defines the cellular energy status and depends on mitochondrial activity (154). ANT isoforms are mitochondrial proteins encoded by nuclear DNA that catalyze the exchange of ATP generated in the mitochondria for ADP produced in the cytosol (155). ANT isoforms were found to be defective in trisomic human fibroblasts (38) (our unpublished data) while their expression can be induced by *PGC-1 α* over-expression (156). Looking for molecular mechanisms by which trisomy of Hsa21 may cause down-regulation of ANT genes in DS, we found that the *SLC25A4/ANT1* is a predicted target of *let-7c-5p*, a Hsa21 miRNA up-regulated in trisomic hearts (88) (see Introduction). In order to understand if the *SLC25A4/ANT1* down-regulation could be due to the specific interaction of its mRNA and *let-7c-5p*, at the seed sequence, we up-regulated this miRNA transfecting a *let-7c-5p* mimic miRNA in euploid fibroblasts (88). The expression of *DICER*, a *let-7c-5p* validated target (157), was evaluated as control. Fibroblasts were transfected for 48 hrs with 10, 25 and 50 nM miRNA mimic and then *SLC25A4/ANT1*, *DICER*, and *GAPDH* expression was evaluated by end point PCR. As shown in **Figure 24 (A-B-C)**, at 25 nM mimic miRNA concentration

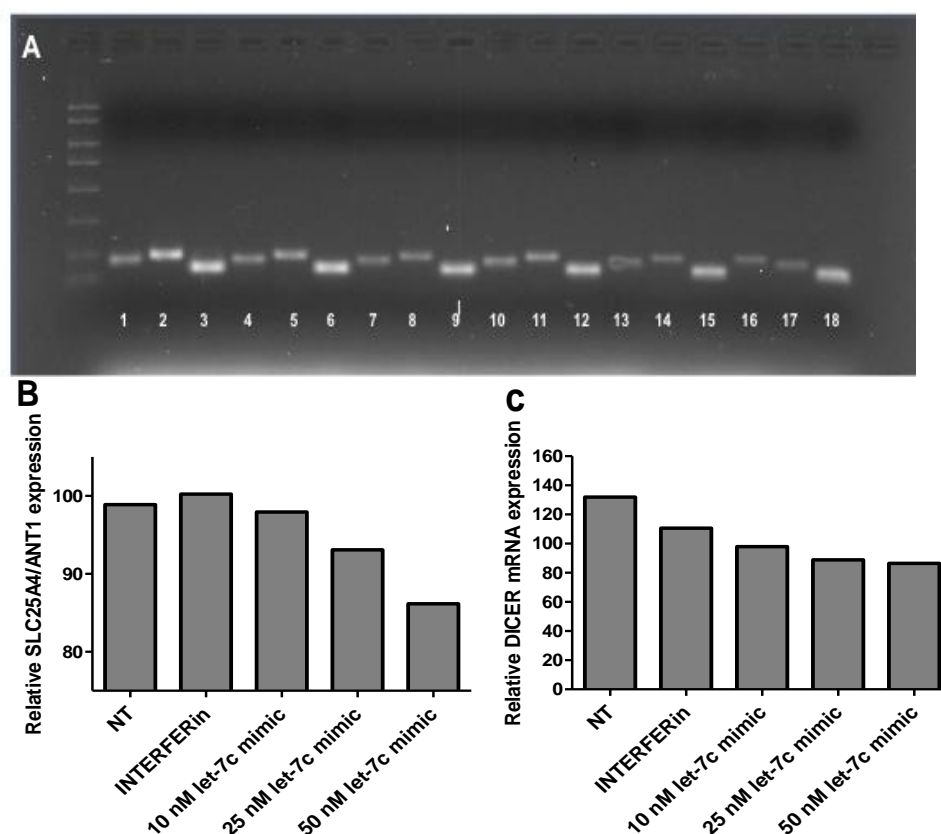


Figure 24. *let-7c-5p* mimic causes a reduction of *SLC25A4/ANT1* and *DICER* expression. (A) Agarose gel of *SLC25A4/ANT1*, *DICER*, and *GAPDH* amplification in: not transfected cells (lanes 1-3); cells + INTERFERin (lanes 4-6); cells + 5 nM miRNA mimic (lanes 7-9); cells + 10 nM miRNA mimic (lanes 10-12); cells + 25 nM miRNA mimic (lanes 13-15); cells + 50 nM miRNA mimic (lanes 16-18). Amplification products of *SLC25A4/ANT1*: lanes 1, 4, 7, 10, 13, and 16; amplification products of *DICER*: lanes 2, 5, 8, 11, 14, and 17; amplification products of *GAPDH*: lanes 3, 6, 9, 12, 15, and 18. (B) Densitometric analysis of *SLC25A4/ANT1* and (C) *DICER* amplification products relative to *GAPDH*.

(lanes 13-14), a decrease of target amplification was already detectable versus non transfected cells (lanes 1-2) and cells transfected with transfection agent alone (lanes 4-5). No variation in *GAPDH* amplification was found.

SLC25A4/ANT1 and *DICER* expression were also evaluated by qRT-PCR. The transfection with let-7c-5p mimic, at a dose of 25 nM, caused a significant decrease of *SLC25A4/ANT1* and *DICER* expression if compared with controls (**Figure 25**).

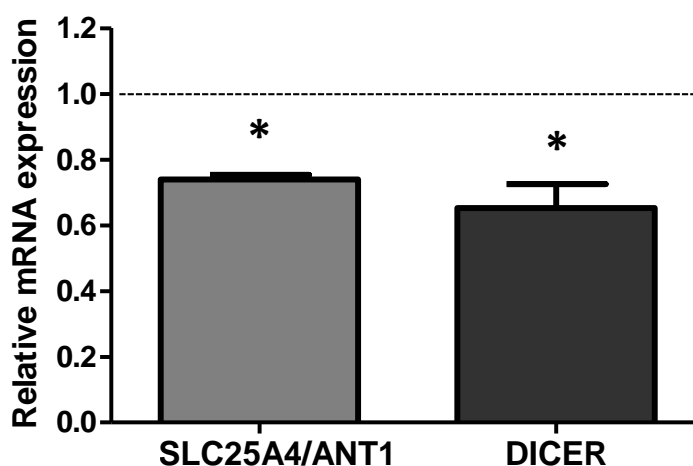


Figure 25. Let-7c-5p mimic leads to a decrease of *SLC25A4/ANT1* and *DICER* expression. *SLC25A4/ANT1* and *DICER* mRNA expression measured by qRT-PCR upon normalization to a reference gene (*GAPDH*). Results are expressed as mean values \pm SEM of three different determination, compared with cells treated with transfecting agent alone (set equal to 1).

* $P < 0.05$.

To validate these results we performed the reciprocal assay by treating DS-HFFs with a let-7c-5p inhibitor. Treatment with 25 nM inhibitor, dose which reduced let-7c-5p expression by 35%, caused a significant increase of *SLC25A4/ANT1* both at mRNA (**Figure 26A**) and protein (**Figure 26B-C**) level as determined by qRT-PCR and western blot analysis, respectively.

These results demonstrate that *ANT1* expression is modulated by the abundance of let-7c-5p. A final validation must be performed using a reporter to demonstrate a direct interaction between *ANT-1* mRNA and the seed sequence and the miRNA.

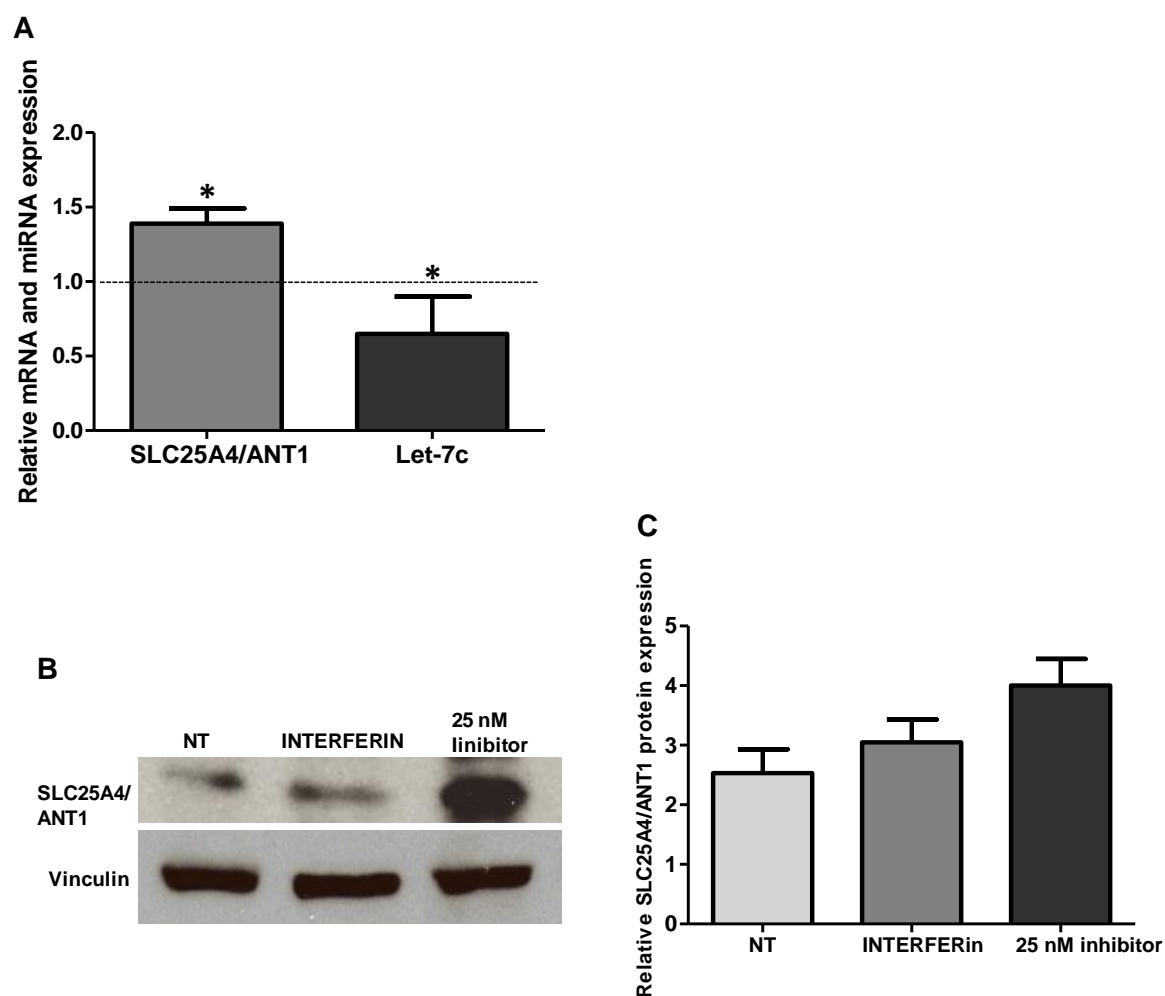


Figure 26. Let-7c-5p inhibitor induces an increase of SLC25A4/ANT1 expression. (A) *SLC25A4/ANT1* mRNA and *let-7c-5p* expression measured by qRT-PCR upon normalization to respectively *GAPDH* and the *snRNA U6*. The bars show relative mean values \pm SEM of three different determination, compared with cells treated with transfecting agent alone (set equal to 1). (B) Representative immunoblotting of SLC25A4/ANT1 and (C) densitometric analysis of two DS-HFFs in duplicate. Vinculin was measured as a loading control.

NT: not transfected cells; INTERFERIN: cells transfected only with transfecting agent; 25 nM Inhibitor: cells transfected with 25 nM let-7c-5p inhibitor.

* $P < 0.05$.

5. DISCUSSION

Down syndrome is the most frequent cause of intellectual disability and early development of AD neuropathology.

Increasing studies consistently demonstrated that there is a mitochondrial dysfunction in DS (30) (108) (110) (106) and that mitochondrial dysfunction plays an important primary role in both altered neurodevelopment and neurodegeneration (103) (104) (105) (158). These evidences strongly support the hypothesis that a mitochondria targeted therapy might attenuate cognitive disability in DS.

Impaired *PGC-1 α* activity is emerging as a common underlying cause of mitochondrial dysfunction in DS and also in some neurodegenerative diseases. Indeed *PGC-1 α* activity is decreased in cells and tissues of DS individuals (8) (30) (52), as well as in brain tissues from AD patients (159) indicating a similarity in the molecular pathways involved in DS and AD neurodegeneration (160) (161) (162). We found that the dosage related over-expression of the corepressor *NRIP1* mapping to the chromosome 21 inhibits the activity of the transcriptional activator *PGC-1 α* (52). Therapeutic approaches against mitochondrial dysfunction in DS, based either on *PGC-1 α* activators or on PPAR agonists, have been successfully tested in mouse models for other diseases (163) (164) (125) (165) (166) (167) (168) (169).

On these premises we have examined two different strategies to pharmacologically activating *PGC-1 α* pathway. Specifically the former therapeutic approach was based on a *PGC-1 α* activator, the biguanide metformin (MTF) which stimulates *PGC-1 α* through AMPK and SIRT1 activity whereas the latter was based on a PPAR agonist, the pioglitazone (PG).

MTF is a drug commonly used in clinical practice in type 2 diabetes, in polycystic ovary syndrome and insulin resistance. Its administration demonstrated to improve cognition in patients with mild cognitive impairment and AD (170) (171), to promote neurogenesis in rodent and human neural precursors and to enhance spatial memory formation in normal adult mouse (172). On the other side other authors associate MTF administration to an increased risk of developing AD (173) (174). These controversial results on MTF effects may be ascribed by a closely dose-dependent effect.

Among the PPAR agonists, PG, belonging to thiazolidinediones, selectively stimulates PPAR- γ (175). This drug attenuates mitochondrial dysfunction in animal models of central nervous system injury, improving mitochondrial ATP production and oxygen consumption (176) (166) (167). Furthermore PG restores brain ATP levels (177) and increases mitochondrial DNA content, oxygen consumption, *PGC-1 α* and TFAM expression in human adipose tissue and in the neuronal-NT2 cell line (178) (179).

PG plays a neuroprotective role in dopaminergic neurons in both *in vitro* and *in vivo* PD models preventing the loss of dopaminergic neurons (180) and reducing iNOS induction and oxidative stress (181) (182). This neuroprotective effect was associated also with *PGC-1 α* activation and the regulation of proteins involved in mitochondrial function, such as *NRF1*, *NRF2*, *FIS1*, and *MFN2* (183).

We are interested in using the smallest effective dose of the drugs because both MTF and PG, besides their slight side effects, are known to inhibit respiratory complex I when used at high concentrations (151) (184). This is the reason why we are testing more than one drug with similar effects either to identify the best one or to formulate a synergistic cocktail with similar efficiency at lower doses.

Metformin and pioglitazone improve the whole energy status of trisomic cells by activating *PGC-1 α*

We first analyzed the effects of two MTF treatments at 0.05 and 0.5 mM concentration. Analyzing *PGC-1 α* mRNA transcript and protein levels in treated vs untreated cells, we found that MTF enhances *PGC-1 α* expression and activity in trisomic cells in a dose-dependent

manner. Crosstalk between the nuclear and mitochondrial genomes is required to provide building blocks for new mitochondria (185) (186). *NRF-1* and *TFAM*, two downstream targets of PGC-1 α , essential in this regard (187), were significantly increased after MTF treatment, as well induced a significant increase of *mtDNA* in treated trisomic cells.

Several experiments demonstrate and support that mitochondrial function is a key factor in the neuronal phenotype. Cheng et al., (188) demonstrated an important role of PGC-1 α and of its targets in the formation and maintenance of hippocampal dendritic spines and synapses. Indeed, in cultured neurons from E17 rat hippocampus, PGC-1 α over expression increased dendritic spines and enhanced the molecular differentiation of synapses, whereas knockdown of PGC-1 α inhibited spinogenesis and synaptogenesis in vitro and reduced the density of dendritic spines in hippocampal dentate granule neurons in vivo (188). In addition, recent experiments demonstrated that mitochondrial dysfunction, induced by the loss of the mitochondrial transcription factor TFAM, resulted in the inability of activated neuronal progenitor cells to undergo proper differentiation into neurons (105).

The functional purpose of mitochondrial biogenesis is to maintain mitochondrial quality and secure sufficient oxygen and ATP production. We and other authors previously demonstrated that bioenergetic deficit of trisomic cells involves both respiration and ATP synthesis due to a defect in the respiratory complex activity (30) (38). In the present study to assess mitochondrial respiration in live trisomic cells we used the XF^e-Analyzer, a very sensitive approach, for measuring respiratory parameters in real-time. An almost complete recovery of mitochondrial respiration was achieved in trisomic cells 72 hrs after MTF administration as demonstrated by the increase in both OCR, ATP production and a decrease of ROS production.

Mitochondrial energy is exported to the cytosol through the exchange of ADP and ATP molecules mediated by ANT translocator. In human there are 3 ANT isoforms (ANT1, 2, 3) which are encoded by nuclear DNA embedded in the inner mitochondrial membrane (156) (155). All ANT isoforms were found to be all down-regulated in trisomic cells (30).

PGC-1 α and NRIP1 regulate genes encoding ANT isoforms through different transcription factors, depending on cell type (156) thus increasing production and cytoplasmic transfer of ATP. It is therefore conceivable that PGC-1 α activation promotes not only a better production but also a better transfer of ATP molecules. SLC25A4/ANT1 is one of the 37 mitochondria related genes down-regulated after NRIP1 overexpression in the GEO GSE 19836 experiment (61). Accordingly after NRIP1 silencing in trisomic cells (52), SLC25A4/ANT1 expression increased, thus allowing a more efficient exchange of ATP and an improvement of mitochondrial activity in DS samples.

As a possible consequence of the improvement in energy status, mitochondrial membrane potential, which was decreased in DS fetal fibroblasts, showed a dose-dependent increase after MTF treatment.

Even though MTF is a very promising molecule, we experimented different approaches, which affect same targets by different ways. This possibly could allow a dose reduction when the drugs are administered together, with lower side effects. For this reason we have tested drugs that may regulate mitochondrial function by activating PGC-1 α pathway stimulating of PPARs. PPAR- γ has proven to be an attractive target for treatment of central nervous system diseases as well as agonists of this receptor have been shown to be effective in ameliorating disease-related symptomology and pathology in a number of animal models (189). PGC-1 α activators and PPAR- γ agonists share the function to regulate the expression of several target genes involved in neuronal survival and neuroprotection by inhibiting mitochondrial dysfunction, oxidative stress, proteosomal dysfunction, autophagy, neuroinflammation and apoptosis (175) (190).

In the present study we have tested in DS fetal fibroblasts the effects of PG at two

concentrations 0.5 μ M and 2 μ M. We were interested in using the smallest effective dose of the drugs because both MTF and PG, besides their slight side effects, are known to inhibit respiratory complex I when used at high concentrations (151) (184). We assessed whether PG would enhance expression and activity of PGC-1 α in trisomic cells by analyzing *PGC-1 α* gene and protein expression, and mRNA abundance of its regulator *PPAR- γ* . The increase of expression of these genes in trisomic cells treated with PG is compatible with the hypothesis that PGC-1 α induction by PG is PPAR γ -dependent. PG treatment demonstrated to induce a quite complete recovery of mitochondrial respiration in trisomic cells at 0.5 μ M concentration, while no significant variation in OCR was found after PG treatment at the concentration of 2 μ M. PG treatment at both concentrations was associated to a significant increase of the basal ATP content in treated trisomic cells. All these data together with a significant 50% to 75% decrease of ROS production were in agreement with a global improvement in bioenergetic metabolism mediated by PG.

Metformin and pioglitazone promote mitochondrial network organization modulating fission-fusion machinery genes

Mitochondria are dynamic organelles, which readily adapt to changes in cellular energy demands through network remodeling by continuous fusion and fission processes (191) (192). Mitochondrial fusion involves the mitofusins MFN-1 and MFN-2, anchoring outer mitochondrial membranes, and OPA1 (Optic atrophy-1) anchoring the inner mitochondrial membranes (193) (194). Mitochondrial fusion results in the formation of an interconnected mitochondrial network that allows the mixing and redistribution of proteins and mtDNA (195) (196) (197). In contrast, mitochondrial fission, induced by DRP1 and FIS1 (193) (194), leads to mitochondria with a fragmented morphology, which facilitate the segregation of damaged mitochondria. We observed that the mitochondrial network in trisomic fetal fibroblasts was highly fragmented, with an increased number of shorter mitochondria and a decreased average mitochondrial volume. Detailed analysis of fusion-inducing genes demonstrated that MFN2 and OPA1 were down-regulated in trisomic fibroblasts. While fission inducing DRP1 gene was found increased in of murine DS models (198).

In this study mitochondrial network and fission-fusion genes were analyzed in trisomic fibroblasts after treatments with MTF or PG.

Overall both treatments promoted a near complete rescue of the mitochondrial network (118). Confocal images showed that the mitochondrial network in trisomic cells was much less fragmented after both drug treatments with a preponderance of elongated mitochondria in treated cells, whereas puncta like and globular mitochondria were more represented in untreated control cells.

Both MTF and PG treatments decreased the number of mitochondria and increased their individual volume. This effect appeared more significant for MTF treatment. MTF administration induced also an increment of total mitochondrial volume in comparison with non treated-cells. This was not observed in PG treatments.

The analysis of fission-fusion machinery genes demonstrated that in OPA1 mRNA and protein levels were consistently increased after both treatment at any dosage. Mitofusins were induced by PG and only by the higher dosage of MTF. Lower MTF doses were still able to induce mitochondrial fusion without significantly affecting MFNs expression, possibly due to regulatory mechanisms activating these proteins (199) independently of their expression.

On the other hand after PG administration the fusion inducing genes were increased while *DRP1* gene expression was decreased in a dose dependent fashion. Protein expression of this gene was too variable to draw a statistical significance indicating that post-transcriptional regulating events may occur.

A relationship between *PGC-1 α* expression and mitochondrial fusion genes has been reported

(200) (201). The silencing of *PGC-1 α* in smooth muscle cells is known to increase mitochondrial fragmentation and decrease the MFN2 levels in smooth muscle cells. Conversely, MFN2 silencing decreases PGC-1 α levels. Furthermore, MFN2 over-expression causes an increase in PGC-1 α levels, identifying a bidirectional relationship between MFN2 and PGC-1 α expression. Silencing of both genes increases mitochondrial fragmentation in the same cells (202).

Recent experiments in *Drosophila* also demonstrated a transcriptional relationship between PGC-1 α and OPA1 genes. In indirect flight muscles of *Drosophila*, the nuclear-encoded mitochondrial inner membrane fusion gene, Opa1-like, is regulated in a spatiotemporal fashion by the transcription factor/co-activator Erect wing (Ewg), the *Drosophila* homolog of human NRF-1 (201). In addition to, and independently of its role in mitochondrial fusion, OPA1 regulates cristae remodelling by forming oligomers that participate in the cristae junction formation and maintenance (203) (204). Mechanistically, OPA1-dependent stabilization of mitochondrial cristae increases mitochondrial respiratory efficiency and blunts mitochondrial dysfunction, cytochrome c release and ROS production. In agreement with these considerations, a higher number of altered mitochondria with abnormal cristae was observed in trisomic fibroblasts by electron microscopy. These alterations were fully reversed by MTF treatment.

Regulating the equilibrium between mitochondrial fusion and fission is essential to maintain integrity of the organelle. In living cells, mitochondrial content depends on the cell-controlled balance between mitochondrial biogenesis and degradation. These processes are intricately linked to changes in network morphology and spatio-temporal positioning of mitochondria and are mechanistically linked to their function (205). This means that proper quantification of mitochondrial morphology and content is of primary importance in understanding bioenergetics processes in health and disease (206).

The chromosome 21 miRNA let-7c-5p regulates ANT1 expression

The over-expression of Hsa21 miRNAs can result in down-regulation of specific target genes possibly involved in DS phenotype. To select relevant miRNA targets, which may affect mitochondrial function, we have intersected information deriving from:

- Hsa21 miRNA expression in fetal DS hearts;
- bioinformatics prediction of miRNA targets;
- gene expression in heart samples from DS fetuses;
- genes classified in Gene ontology as mitochondria related;

The analysis pointed out SLC25A4/ANT1 among let-7c-5p targets. SLC25A4/ANT1 functions as a gated pore that translocates ADP and ATP between cytoplasm and mitochondria, regulating the intracellular energetic balance. We found that the dosage related over-expression of a miRNA, mapping to Hsa21, namely *let7c-5p* exerted a negative effect over the regulation of an ATP translocator, the ANT1/SLC25A4 gene. It is down-regulated in DS fetal heart and fibroblasts in which *let-7c-5p* is up-regulated. qRT-PCR after let-7c-5p over-expression showed an inverse relationship with SLC25A4/ANT1, which was down-regulated. These results may be considered a first validation of our hypothesis but further experiments will be necessary to definitely validate that SLC25A4/ANT1 is a let-7c-5p target.

6. CONCLUSIONS

The complex neurological function is the result of many molecular, cellular, and environmental events that occur and must be coordinated precisely at the right time (207). In mammals, many of these processes are initiated and sometimes completed before birth. This suggests that the overall impact of the neurological deficits in DS may be lessened if the initial pathologic changes in the brain are prevented from occurring. Permanent brain alterations of DS originate during fetal life (208). Early prenatal diagnosis offers a temporal window to exert a positive impact on brain development and to improve postnatal cognitive outcome in affected individuals. Therefore, the first window of opportunity for cognitive improvement occurs well before birth. Only a few approaches (EGCG, NAP/SAL, fluoxetine, and apigenin) have been used to treat mice *in utero*; some of these showed therapeutic effects that persisted to adulthood (209).

Mitochondrial dysfunction, observed in DS since the prenatal life, plays an important role in neurodevelopment. The therapeutic goal will be to counteract this dysfunction *in utero*.

Recent evidence indicates that mitochondrial morphology is crucial for cellular physiology, as changes in mitochondrial shape have been linked to neurodegeneration, lifespan and cell death (210). This highlights the importance of targeting therapeutically mitochondrial dynamics.

Overall both MTF and PG demonstrated to improve the whole energy status and to promote mitochondrial network organization with similar efficacy. These drugs have been used and are well tolerated during pregnancy.

Next step will be to test a combination of the drugs at the lowest effective concentrations to reduce side effects and complex I inhibition.

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9. LIST OF PUBLICATIONS

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4. Izzo A, Manco R, de Cristofaro T, Bonfiglio F, Cicatiello R, **Mollo N**, De Martino M, Genesio R, Zannini M, Conti A, and Nitsch L. **Overexpression of Chromosome 21 miRNAs May Affect Mitochondrial Function in the Hearts of Down Syndrome Fetuses.** International Journal of Genomics. Volume 2017, Article ID 8737649, 10 pages <https://doi.org/10.1155/2017/8737649>. Published: 5 September 2017.
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