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SYNERGISTIC ROLES OF THE PROTEASOME AND MITOCHONDRIA IN ALPHA-SYNUCLEIN OLIGOMERIZATION: IMPLICATIONS IN PARKINSON'S DISEASE

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TRABALHO REALIZADO SOB A ORIENTAÇÃO DE:
SANDRA MORAIS CARDOSO, PHD
MARIA CRISTINA JANUÁRIO SANTOS, MD, PHD

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SYNERGISTIC ROLES OF THE PROTEASOME AND MITOCHONDRIA IN ALPHA-SYNUCLEIN OLIGOMERIZATION: IMPLICATIONS IN PARKINSON'S DISEASE

Martins-Branco D[1,4], Esteves AR[1], Arduino DM[1], Swerdlow RH[2], Januario C[3,4], Oliveira CR[1,4], Cardoso SM[1,4*]

[1]CNC–Center for Neuroscience and Cell Biology, University of Coimbra;

[2]Departments of Neurology, Biochemistry and Molecular Biology, and Molecular and Integrative Physiology, University of Kansas Medical Center , Kansas City, Kansas;

[3]Neurology Department, Coimbra University Hospital;

[4]Faculty of Medicine, University of Coimbra.

*Corresponding author: Sandra Morais Cardoso, Center for Neuroscience and Cell Biology, University of Coimbra, Largo Marquês de Pombal. 3004-517 Coimbra, Portugal

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Email: diogo.mbranco@gmail.com

Abstract

Parkinson's disease (PD) is the most common neurodegenerative movement disorder, characterized by selective loss of nigrostriatal dopaminergic neurons and presence of intracellular insoluble proteinaceous inclusions, known as Lewy Bodies. Although PD etiopathogenesis remains elusive, the leading hypothesis establishes that mitochondrial dysfunction, protein quality control system deficiency, and protein oligomerization are major events that act synergistically to cause this devastating disease.

The main goal of this work is to study ubiquitin-proteasome system (UPS) and ubiquitin dependent alpha-synuclein (aSN) clearance in different PD cellular models with mitochondrial deregulation.

We used three different PD cellular models: SH-SY5Y ndufa2 knock-down (KD) cells, PD cybrids and peripheral blood mononuclear cells (PBMC) of patients with diagnostic of PD.
For each model we studied proteasome activity, using fluorimetric analysis, and quantified cellular ubiquitination and aSN aggregation by Western Blot. We used lactacystin as negative control of proteasomal function. In PBMC of PD patients population we proceed to evaluate aSN secretion to plasma by Dot Blot and the influence of several demographic characteristics in the above mentioned determinations.

SH-SY5Y ndufa2 KD cells shown a proteasome activity up-regulation with increased levels of total ubiquitination, ubiquitin monomers and aSN oligomers as compared with SH-SY5Y parental cells.
PD Cybrids did not show differences concerning proteasome enzymatic activity.

PBMC of patients do not exhibit statistical significant differences in proteasome activity compared to age-matched controls. However, there is a negative correlation of both chymotrypsin-like activity and total ubiquitin content with age in control and LOPD groups. Despite there are no statistical significant differences in ubiquitin levels between patients and controls, total ubiquitin content increases and is positively correlated with chymotrypsin-like activity and with aSN oligomers levels. aSN levels in plasma are slightly increased in LOPD and significantly increased in EOPD.

Thus, we conclude that in SH-SY5Y ndufa2 KD cells there is an up-regulation of proteasomal enzymatic activity that could mean an interesting cell rescue attempt. Moreover, although ubiquitinated proteins content are increased in the other two models, proteasome activity is not significantly altered, what is compatible with an over request of UPS due to high rate of protein misfolding or somehow a compromise in the UPS pathway, upstream the enzymatic core. We also conclude that in PBMC of both patients and control individuals, there is a decrease of UPS activity with age. In addition, aSN oligomers are ubiquitinated and we identified an ubiquitin-dependent clearance insufficiency. Secretion of aSN in PBMC seems to be a cell mechanism to prevent its cellular accumulation.

Keywords

Parkinson's disease, Ubiquitin-proteasome system, Mitochondria, Alpha-synuclein, Ubiquitin, SH-SY5Y ndufa2 knock-down cells, PD Cybrids and PD Peripheral blood cells.

Resumo

A Doença de Parkinson (PD) é a doença neurodegenerativa do movimento mais comum sendo caracterizada pela perda selectiva de neurónios dopaminérgicos nigro-estriatais e pela presença de inclusões proteicas intracelulares insolúveis, os Corpos de Lewy. Apesar de a etiopatogenia não estar completamente esclarecida, existem evidências que apontam para que a disfunção mitocondrial, um ineficiente sistema de controlo de qualidade proteica e a oligomerização proteica sejam eventos fundamentais que actuam de forma sinérgica causando esta doença.

O principal objectivo deste trabalho é estudar o sistema ubiquitina-proteassoma (UPS) e a degradação de alfa-sinucleina (aSN) dependente de ubiquitina em diferentes modelos de PD com disfunção mitocondrial.

Foram usados três modelos de PD: células SH-SY5Y ndufa2 knock-down (KD), PD Cybrids e células mononucleadas do sangue periférico (PBMC) de indivíduos com diagnóstico de PD. Para cada modelo, estudámos a actividade do proteassoma, usando análise fluorimétrica. Por Western Blot, quantificámos a ubiquitinação e agregação de aSN. Os dois primeiros modelos foram incubados com lactacistina, condição que desempenha o papel de controlo negativo para a função do proteassoma. Nas PBMC dos doentes quantificámos a secreção de aSN para o plasma através de Dot Blot e procurámos influências demográficas nas determinações acima mencionadas.

As células SH-SY5Y ndufa2 KD apresentam aumento da actividade do proteassoma com níveis aumentados de ubiquitinação, monómeros de ubiquitina e oligomeros de aSN comparando com a linha parental SH-SY5Y.
Os PD Cybrids não mostram diferenças no que respeita a actividade do proteassoma.

As PBMC dos doentes não têm diferenças estatisticamente significativas na actividade do proteassoma comparadas com as de controlos de idade aproximada. Contudo, existe uma correlação negativa com a idade nos grupos controlo e LOPD, quer da actividade "chymotrysin-like" do proteassoma, quer do conteúdo total de ubiquitina. Apesar de não existirem diferenças estatisticamente significativas nos níveis de ubiquitina entre doentes e controlos, o conteúdo total de ubiquitina está aumentado e está positivamente correlacionado com a actividade "chymotrysin-like" bem como com os níveis de oligomeros de aSN. Os níveis de aSN no plasma estão discretamente aumentados nos doentes LOPD e de forma significativa nos EOPD.

Assim, concluímos que nas células SH-SY5Y ndufa2 KD existe um mecanismo de activação do proteassoma que pode significar uma tentativa interessante de sobrevivência celular. Além disso, apesar do conteúdo de proteínas ubiquitinadas também estar aumentado nos outros dois modelos, o mesmo não se pode afirmar face à actividade enzimática do proteassoma, o que pode corresponder a um aumento da função do UPS devido a uma concentração aumentada de proteínas disfuncionais ou de certa forma um compromisso da via do UPS, acima do complexo enzimático central. Concluímos também que existe diminuição da actividade do UPS com a idade nas PBMC, quer dos doentes quer dos indivíduos controlo. A aSN oligomerizada está ubiquitinada possivelmente devido a uma insuficiência da degradação dependente de ubiquitina. Por último, a secreção de aSN nas PBMC parece ser um mecanismo para prevenção da sua acumulação intracelular.

Palavras-chave

Doença de Parkinson, Sistema Ubiquitina-proteasoma, Mitocondria, Alfa-sinucleina, Ubiquitina, Células SH-SY5Y ndufa2 knock-down, PD Cybrids e Células Mononucledas do Sangue Periférico.

Contents

Abbreviations List

CNS: Central Nervous System

CXI: Complex I

DA: Dopamine

EOPD: Early On-set Parkinson's Disease

ETC: Mitochondrial Electron Transport Chain

FBS: Fetal Bovine Serum

IP: Immunoprecipitation

KD: Knock-down

LBs: Lewy Bodies

LOPD: Late On-set Parkinson's Disease

MD: Mitochondrial Disorder

MMSE: Mini-Mental State Examination

mtDNA: Mitochondrial DNA

PBMC: Peripheral Blood Mononuclear Cells

PBS: Phosphate-Buffered Saline

PD: Parkinson's Disease

PGPH-like: Peptidyl-glutamyl peptide hydrolytic-like

SNpc: Substantia Nigra pars compacta

TBS: Tris-buffered Saline

UPDRS: Unified Parkinson's Disease Rating Scale

UPS: Ubiquitin-Proteasome System

WB: Western Blot

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Chapter 1
Introduction

Parkinson´s disease (PD) is a neurodegenerative disorder of the central nervous system (CNS) and is the commonest movement disorder. PD is characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) in the ventral midbrain, affecting the nigroestriatal pathway. Severe depletion of dopamine (DA) in the striatum results in the imbalance of acetylcholine, glutamate and gama-aminobutyric acid in subthalamic nucleus, thalamus and cortex, underlying the clinical symptomatology of the disorder (Wichmann and DeLong, 2003). The loss of non-DAergic neurons in other basal nuclei have been observed and are also involved in the pathophysiology of PD (Jellinger, 1999).

PD clinical features are bradykinesia, tremor at rest, rigidity, postural instability, gait alterations and dysarthria. These symptoms are mainly explained by deficiency of DA in striatum, whereas other symptoms such as autonomic dysfunction, depression and cognitive impairment may be associated with pathological changes in non-DArgic systems. The therapeutic approach to the disorder is symptomatic. L-dopa is the standard drug, although the disease will still progress. So the ultimate therapeutic goal has to be restorative and protective. The commonest type of PD is the sporadic or late-onset (LOPD) form which affects about 1-2% of individuals older than 65years (de Lau and Breteler, 2006). However, 5% of PD cases, manifesting before 50 years, have been referred as familial or early-onset PD (EOPD) (Hatano et al., 2009). Some authors, divide EOPD in another subgroup of young-onset PD (YOPD), when first symptoms start between 21-39 (Golbe, 1991).

In the last decade linkage studies revealed 15 PD-related genetic loci (PARK1-15) (Hatano et al., 2009), and in a posterior report a new locus, PARK16, was identified (Satake et al., 2009). Mutations described in these loci, include autosomal dominant and recessive mutations like those in lrrk2 gene (PARK8) (Funayama et al., 2002) and parkin gene (PARK2) (Kitada et al., 1998; Mizuno et al., 2008), respectively, the identified familial forms in this study. Additionally, mutations in mitochondrial DNA (mtDNA) codifying for two complex I (CXI)

subunits were found in fibroblasts of a patient (Piccoli et al., 2008). Moreover, previous studies revealed disease-causing mutations in several CXI nuclear structural genes (revised by Hoefs et al., 2008). Accordingly, cells with ndufa2 gene knock-down (KD) is one of our cellular models. This gene is nuclear encoded and is located in a homozygous region on chromosome 5, that codify a protein localized in the peripheral arm of CXI. This gene mutation is reported to Leigh Disease (Hoefs et al., 2008).

Identification of single genes linked to the disease has yielded crucial insights into possible mechanisms of PD pathogenesis, giving strong evidences of the involvement of mitochondria and intracellular degradation pathways as ubiquitin-proteasome system (UPS) and autophagy in the pathophysiology of PD (revised in Arduino et al., 2010).

Protein aggregation leading to Lewy bodies (LBs) formation is also a central feature of PD pathophysiology and a histopathological hallmark of the disorder. LBs are eosinophilic intracytoplasmatic aggregates of several proteins such as alpha-synuclein (aSN) and ubiquitin (Forno, 1996). LBs are also typical features of other aSNopathies with different distribution through the CNS, like Dementia with LBs and Multiple System Atrophy. Mitochondrial dysfunction, oxidative stress and/or UPS impairment, were shown to potentiate aSN aggregation in sporadic PD models (revised by Arduino et al., 2010).

Evidence exists supporting the notion that oxidative stress and impaired mitochondrial function may trigger the etiopathogenesis of the disorder. Thus, in this work we propose to focus on the role of UPS as a protein quality control system and evaluate how mitochondrial dysfunction potentiates aSN aggregation through direct study of proteasome activity and ubiquitin-dependent aSN clearance.

Chapter 2
Materials & Methods

2.1. NDUFA2 KD and cell culture

The sequence for NDUFA2 siRNA (forward (5'→3') ATCCGCCAAGAGACGAATGT CCCTTTGAATTCAAGAGATTCAAAGGGACATTCGTCTCTTGGC, reverse (5'→3') AAAAGCCAAGAGACGAATGTCCCTTTGAATCTCTTGAATTCAAAGGGACATTCGT CTCTTGGC) was purchased from Invitrogen Online Ordering. The sequence was then cloned into lentiviral vector for siRNA pGreenPuro (System Biosciences) according to manufacturer's instructions. The resultant siRNA lentivector construct was then purified. The siRNA construct is packaged into pseudoviral particles tranduced into SH-SY5Y cells. Because infected cells stably express copGFP as well as the shRNA cloned into the pGreenPuro they can be selected for green fluorescent protein (GFP) positive cells by FACS.

SH-SY5Y human neuroblastoma cells (ATCC-CRL-2266) were cultured in DMEM F12 medium supplemented with 10% nondialyzed fetal bovine serum (FBS), 1.2g/L $NaHCO_3$, 10ml/L penstrep. SH-SY5Y human neuroblastoma ndufa2 KD cells were cultured in DMEM F12 medium supplemented with 10% nondialyzed FBS, 1.2g/L $NaHCO_3$, 10ml/L penstrep, 100mM sodium pyruvate and 75mg/ml Uridine. Both cell lines were maintained at 37°C in a humidified incubator containing 95% air and 5% CO_2. Cells were plated at 0.25×10^6cells/ml for measurement of proteasome activity and WB analysis.

2.2. Creation of cybrid cell-lines and cell culture

Subject participation was approved through the Institutional Review Board of the University Hospital of Coimbra. The three sporadic PD patients, without any nuclear DNA mutation known to be relevant to PD, meeting diagnostic criteria (Hughes et al., 1992) and three healthy, age-matched control subjects provided 10 ml blood samples following written informed consent. Blood was drawn directly into tubes containing acid-citrate-dextrose.

Creation of cybrid cell lines and cell culture was performed accordingly to previously described by Esteves and Colleagues (Esteves et al., 2010a). (See Appendix section).

2.3. Lactacystin Incubation

Twenty-four hours after seeding the cells, the medium was aspirated and replaced with similar medium containing 2μM of lactacystin ($C_{15}H_{24}N_2O_7S$) (Sigma Aldrich, St. Louis, MO, USA). Incubations were performed for 6h for proteasome activity assay and for 12h to WB analysis. For all conditions tested, control experiments were performed in which lactacystin was not added; all other incubation parameters were unchanged.

2.4. MTT cell proliferation assay

Cell proliferation was determined by the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Mosmann, 1983). In viable cells, the cellular dehydrogenases metabolize MTT into a formazan that absorbs light at 570nm.

2.5. Separation of mononuclear cells (PBMC) from Peripheral Human Blood Samples

Subject participation was approved through the Institutional Review Board of the University Hospital of Coimbra. Twenty-six PD patients, meeting diagnostic criteria (Hughes et al., 1992), followed by the Movement Disorders Consulting of Neurology department of the University Hospital of Coimbra and ten healthy, age-matched, volunteer individuals provided 10 ml blood samples after written informed consent, under the following exclusion criteria: Hepatic, Renal or Heart Failure, Severe Hypertension, Other Neurological Disease, Mini-Mental State Examination (MMSE) lower than 24, Cranial trauma in less than 6 months and anti-inflammatory, anti-neoplasic or immunosupressor drugs administration during the study. Blood was collected from the PD patients and from control individuals and drawn into a tube containing anticoagulant. PD patients samples were divided in three groups: (a) LOPD group where age of onset was >50years, (b) EOPD group where age of onset was <50years and (c) Special cases of identified mutations, where are included three cases of PARK2, two of PARK8 and one of specific identified Mitochondrial Disorder (MD). Then, no later than 2h

after drawing, 10ml of blood were carefully laid with Pasteur pipette over 8ml of histopaque (Sigma Aldrich, St. Louis, MO, USA) in a 50ml Falcon tube, avoiding mixing of blood and separation. The Falcon tube was centrifuged at 2500rpm, 20min at 18ºC in a swing-out rotor, without brake. After centrifugation, the mononuclear cells form a distinct band at the sample/medium interface and were removed without the upper layer of serum, using a Pasteur pipette. The harvested fraction was diluted in 45ml of phosphate-buffered saline (PBS) in a 50ml Falcon tube and centrifuged for 10min at 1500rpm at 18ºC. The supernatant was removed and the pellet resuspended in respective lysis buffers and further treated as cell culture extractions described in the two next topics.

The serum was collected after the first centrifugation into aliquots and centrifuged at 4000rpm for 15minutes in order to sediment the platelets. Then, the plasma (supernatant) was collected and stored at -80ºC and the platelets (pellet) were washed with 300µl of PBS. The centrifugation was repeated at 4000rpm for 15min and the pellet was resuspended in 125µl of lysis buffer (0,25M Sacarose, 5mM Hepes, pH 7,4) and stored at -80ºC.

2.6. Mitochondrial respiratory chain NADH-ubiquinone oxidoreductase assay

ETC CXI activity assay was done as previously described by Esteves and Colleagues (Esteves et al., 2008). (See Appendix section).

2.7. Fluorimetric proteasomal activity analysis

Proteasome activity analysis was done as previously described by Domingues and Colleagues (Domingues et al., 2008). (See Appendix section).

2.8. Immunoblotting

Immunoblotting procedure was performed as previously described with modifications (Esteves et al., 2010a). (See Appendix section).

2.9. Immunoprecipitation (IP)

Cells were scraped in buffer containing 20mM Tris, 100mM NaCl, 2mM EDTA, 2mM EGTA (pH 7.0), protease inhibitors (200mM PMSF and a commercial protease inhibitor cocktail), 0.1% SDS and 1% Triton X-100. Cell suspensions were centrifuged at 20000 g for 10 min at 4ºC. Supernatants were removed and stored at -80ºC. The protein concentration of each sample was determined by the Bradford method. 500 μg of cell lysate protein was incubated with 2μg of primary antibody (anti-aSN antibody (211) sc-12767 from Santa Cruz Biotechnology, Inc.) overnight at 4ºC and with gentle agitation. Lysates were then incubated with 100 μl of protein-A beads for 2 hours at 4ºC and with gentle agitation. After completing this incubation lysate tubes were centrifuged at 65g for 5 min at 4ºC, the supernatant was removed, and the beads were washed in the previously described buffer seven times (each time centrifuging at 4°C and removing the supernatant). For the first two washes the buffer was supplemented with 1% Triton X-100. For the next three washes the buffer was supplemented with 1% Triton X-100 and 500mM NaCl. The final two washes were performed using unsupplemented buffer. After removing the last supernatant 25 μl of 2x sample buffer were added. The sample was boiled at 95-100°C for 5 minutes to denature protein and separate it from the protein-A/G beads. The boiled proteins were centrifuged at 20,000 g for 5 min at room temperature and the supernatants collected. The resulting co-immunoprecipitated proteins were subjected to SDS-PAGE using anti-aSN antibody.

2.10. Dot Blot assay

Dot Blot assay was done as previously described with modifications (Domingues et al., 2008). (See Appendix section).

2.11. Data analysis

Each experimental endpoint for each sample was run in duplicate. Experimental results were analyzed by Kolmogorov-Smirnov normality test and depending on the result p values were calculated by parametric or non-parametric distribution tests. One-way ANOVA or Kruskal-Wallis test, followed by a post hoc Bonferroni's or Dunnet's t test, respectively, were used to compare multiple conditions studies. To punctual comparison of two isolated conditions, Paired t test or Mann-Whitney test were performed. Correlation studies were done using Pearson Correlation or Spearman Correlation test when appropriate.

Chapter 3
Results

3.1. Lactacystin effect on cell proliferation

The concentration of lactacystin used did not affected cell viability in both SH-SY5Y and cybrid cells.

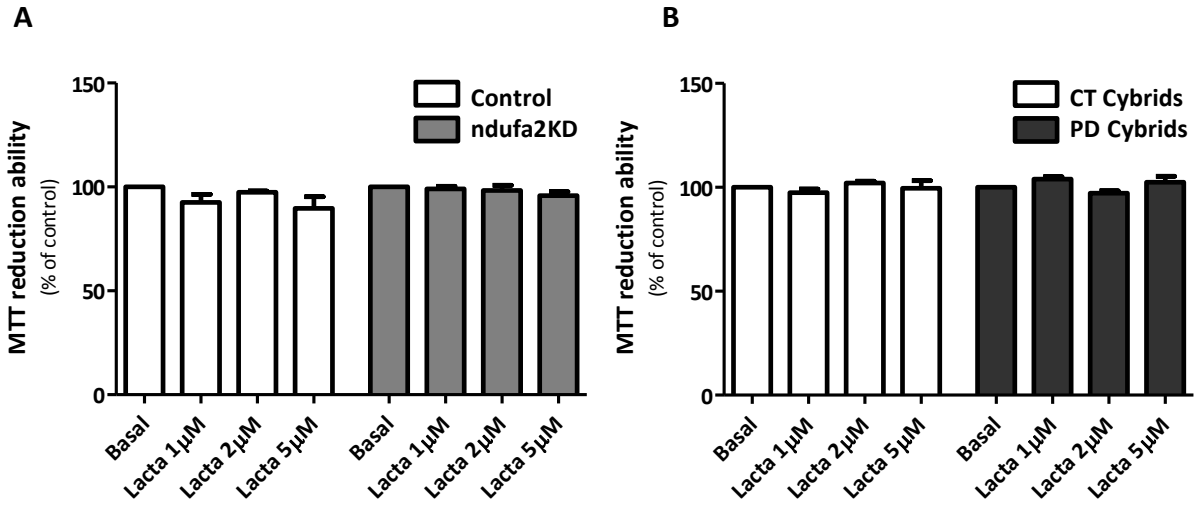

Figure 1. Effect of lactacystin on MTT reduction. (A) SH-SY5Y cells; (B) Cybrid cells; Lactacystin concentration used does not affect viability in both cell-line models. N=3

3.2. SH-SY5Y ndufa2 KD cells characterization

WB analysis revealed a decrease in ndufa2 expression in SH-SY5Y cells.

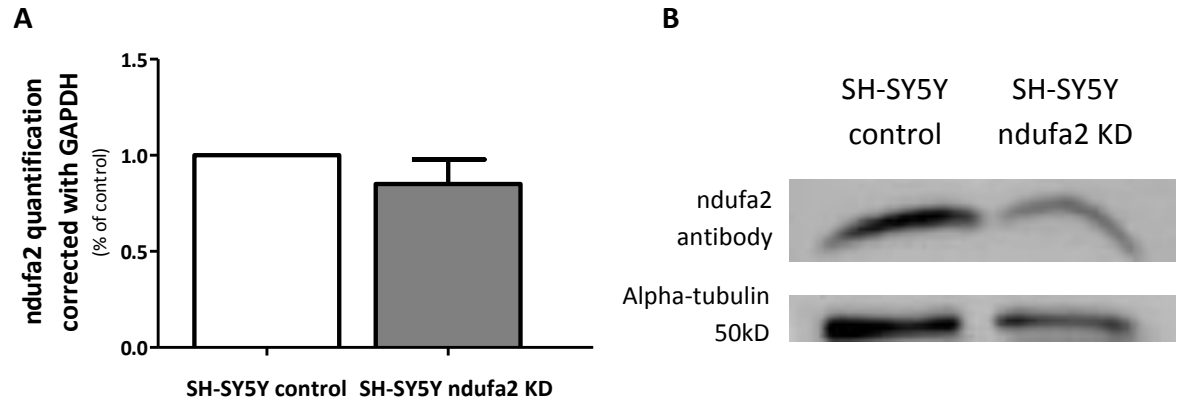

Figure 2. ndufa2 KD in SH-SY5Y ndufa2 KD cells. (A) SH-SY5Y ndufa2 KD cells show reduced amount of ndufa2 protein as expected. N=5 (B) WB of NDUFA2 protein in SH-SY5Y control and ndufa2 KD.

3.3. Mitochondrial function in PD cellular models

Esteves and coworkers have previously shown significant reduction of CXI activity in both platelets of PD patients and PD Cybrids compared with the respective controls (Esteves et al., 2008). ETC CXI activity is also reduced in SH-SY5Y ndufa2 KD (Figure 3).

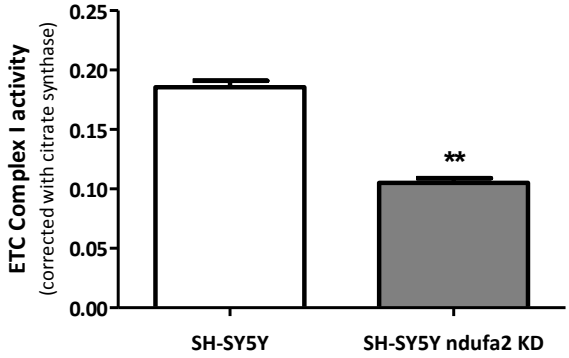

Figure 3. ETC CXI activity in SH-SY5Y ndufa2 KD. There is a reduction in CXI activity in SH-SY5Y ndufa2 KD cells. N=2, **P<0,01.

3.4. UPS function in PD cellular models

3.4.1. UPS function in SH-SY5Y ndufa2 KD cells

Significant differences were found between basal 26S chymotrypsin-like activity of both SH-SY5Y control and ndufa2 KD cells when compared with 20S chymotrypsin-like activity in SH-SY5Y ndufa2 KD cells. Isolated comparison between 20S chymotrypsin-like activity in SH-SY5Y ndufa2 KD basal condition and the same activity in SH-SY5Y control basal condition reveal a significant increase (*P*=0,0308) (Figure 4A). Moreover, an increase in 26S peptidyl-glutamyl peptide hydrolytic-like (PGPH-like) activity was observed in both cell lines when compared to 20S activity (*P*=0,0068). Comparisons between, 26S and 20S PGPH-like activity of SH-SY5Y control and SH-SY5Y ndufa2 KD basal conditions revealed a significant increase (*P*=0,0092) (Figure 4B).

Total ubiquitination levels were increased in ndufa2 KD cell lines, besides we can also see an increase in ubiquitin monomer levels, despite there is no significant difference (Figure 4C and D). As expected, lactacystin treatment worked as a negative control of proteasome activity, resulting in accumulation of both ubiquitinated species and ubiquitin monomer.

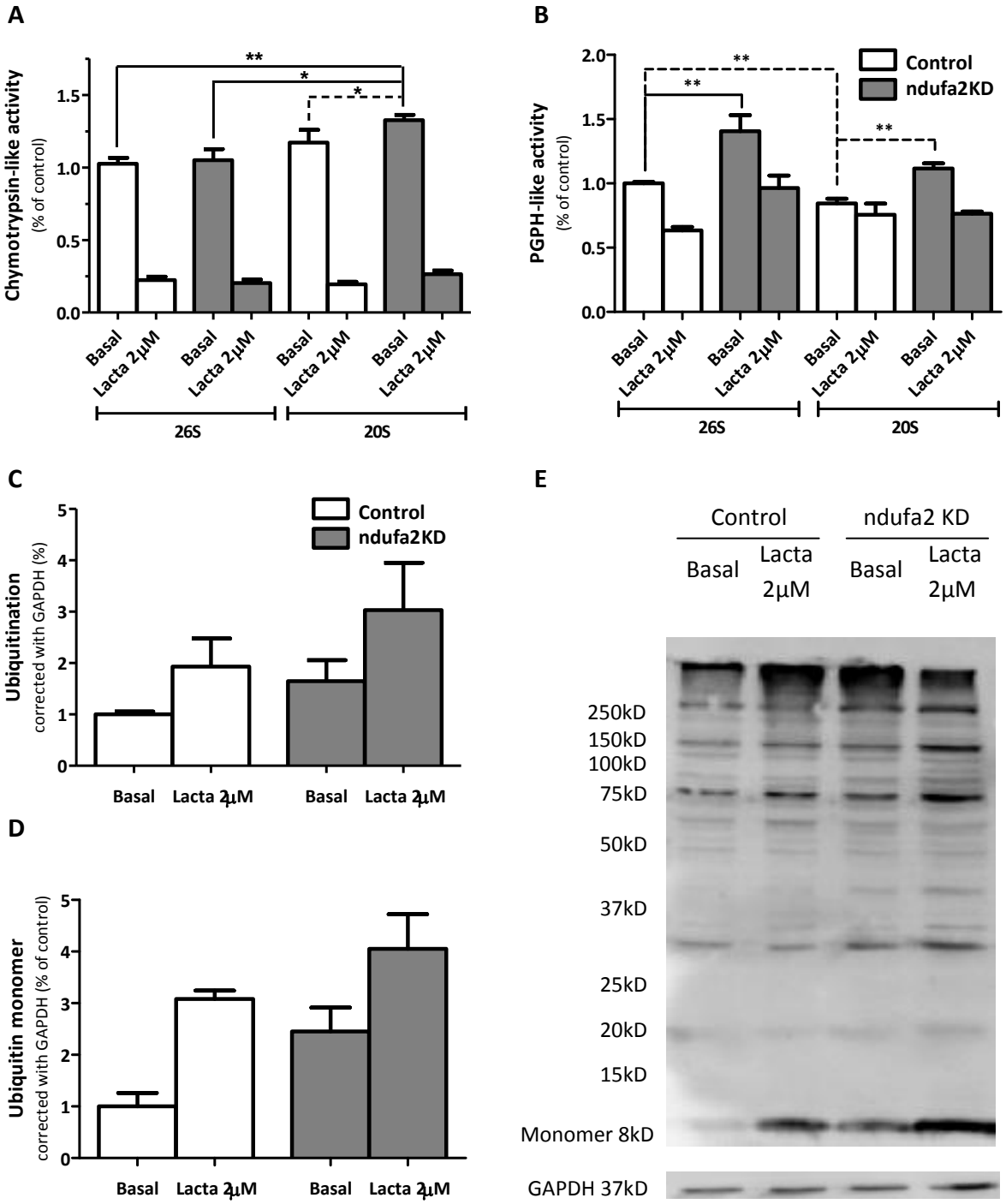

Figure 4. UPS function in SH-SY5Y ndufa2 KD cells. (A) Proteasome 26S and 20S chymotrypsin-like activity. *N*=4, ***P*<0,01, **P*<0,05 (B) Proteasome 26S and 20S PGPH-like activity. *N*=4, ***P*<0,01 (C) Densitometry analysis of total ubiquitinated protein content *N*=3, *P*=0,0534 (D) Densitometry analysis of ubiquitin monomer. *N*=3, *P*=0,0499. (E) Representative WB of ubiquitin in SH-SY5Y control and ndufa2 KD cell lines, basal and lactacystin treatment conditions.

3.4.2. UPS function in PD Cybrids

There are no major differences concerning to proteasome activity in PD Cybrids. Thus, increased ubiquitination in PD Cybrids, previously shown by our group (Esteves et al., 2010b), seems to be not related with proteasome activity compromise.

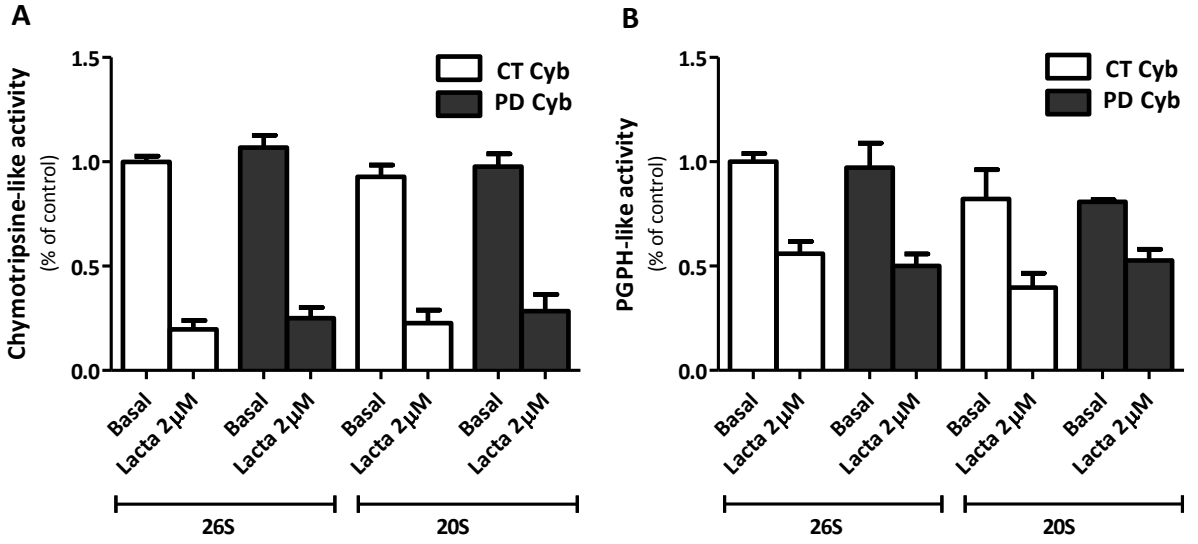

Figure 5. Proteasome function in PD Cybrids. (A) Proteasome 26S and 20S chymotrysin-like activity. *N*=3 (B) Proteasome 26S and 20S PGPH-like activity. *N*=1; There are no statistical differences between similar conditions in both activities.

3.4.3. UPS function in PBMC of PD patients

Although post-hoc multiple comparison test has not shown any difference between columns, there is significant difference between means (*P*=0,0438) and we can detect an increase of proteasome activities in younger groups, both control and PD, compared to respective older conditions, mainly in 26S chymotrypsin-like activity. Preliminary results of 20S PGPH-like activity show evident differences between controls and patients (Figure 6A and 6B).

Relatively to ubiquitin levels, despite there is a great variability, we can observe an increased mean of ubiquitination levels in both disease groups when compared with controls similarly to what we observed in our in vitro cellular models. There is also more protein ubiquitination in younger individuals (Figure 6C). Ubiquitination levels are positively correlated with 20S chymotrypsin-like activity in LOPD group (Figure 6D).

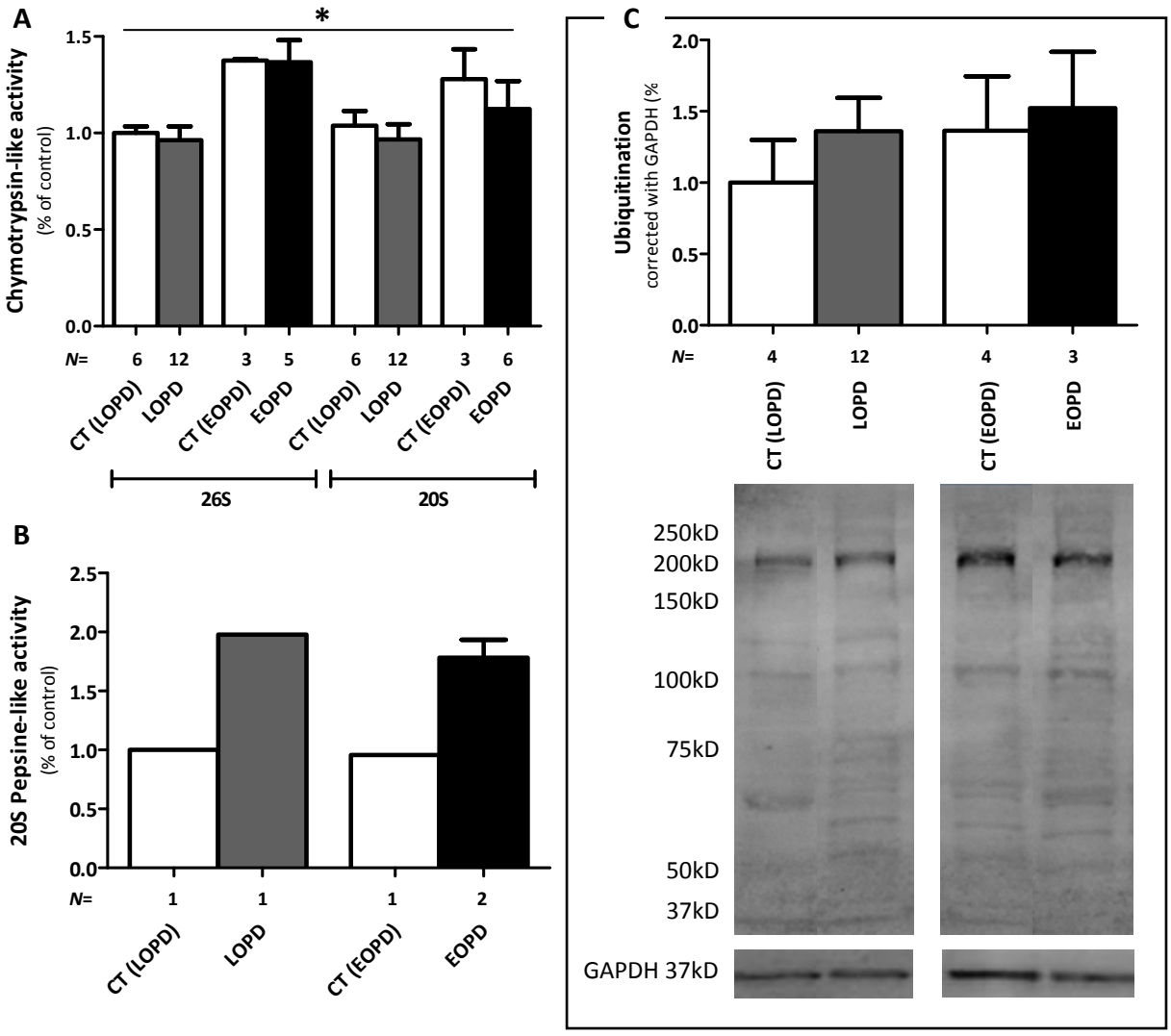

Figure 6. UPS function in PBMC of PD patients. (A) Proteasome 26S and 20S chymotrypsin-like activity. **P*<0,05 (B) Proteasome 26S and 20S PGPH-like activity. (C) Densitometry analysis of total ubiquitinated protein content and representative WB. (D) Ubiquitination influence on proteasome activity in LOPD patients: 26S chymotrypsin-like activity is positively correlated with ubiquitinated protein content. *N*=13, *Pearson r* = 0,4403, *P*=0,1321, r^2=0,1939; 20S chymotrypsin-like activity has a significant positive correlation with ubiquitinated protein content. *N*=13, *Pearson r* = 0,5584, **P*=0,0473, r^2=0,3118.

3.5. aSN aggregation in PD cellular models

Esteves and colleagues, showed an increased aSN oligomers formation in PD Cybrids compared to CT Cybrids (Esteves et al., 2010b). Indeed, aSN oligomerization is also increased in SH-SY5Y ndufa2 KD (P=0,0007, unpaired t test) when compared to respective control cell line. However, in PBMC of PD patients we can just observe a tendency to an increase oligomerization, because there is no significance due o high variability (Figure 7A and 7B). Treatment with lactacystin, as expected, seems to increase aSN oligomerization, markedly in SH-SH5Y ndufa2 KD cells (Figure 7A).

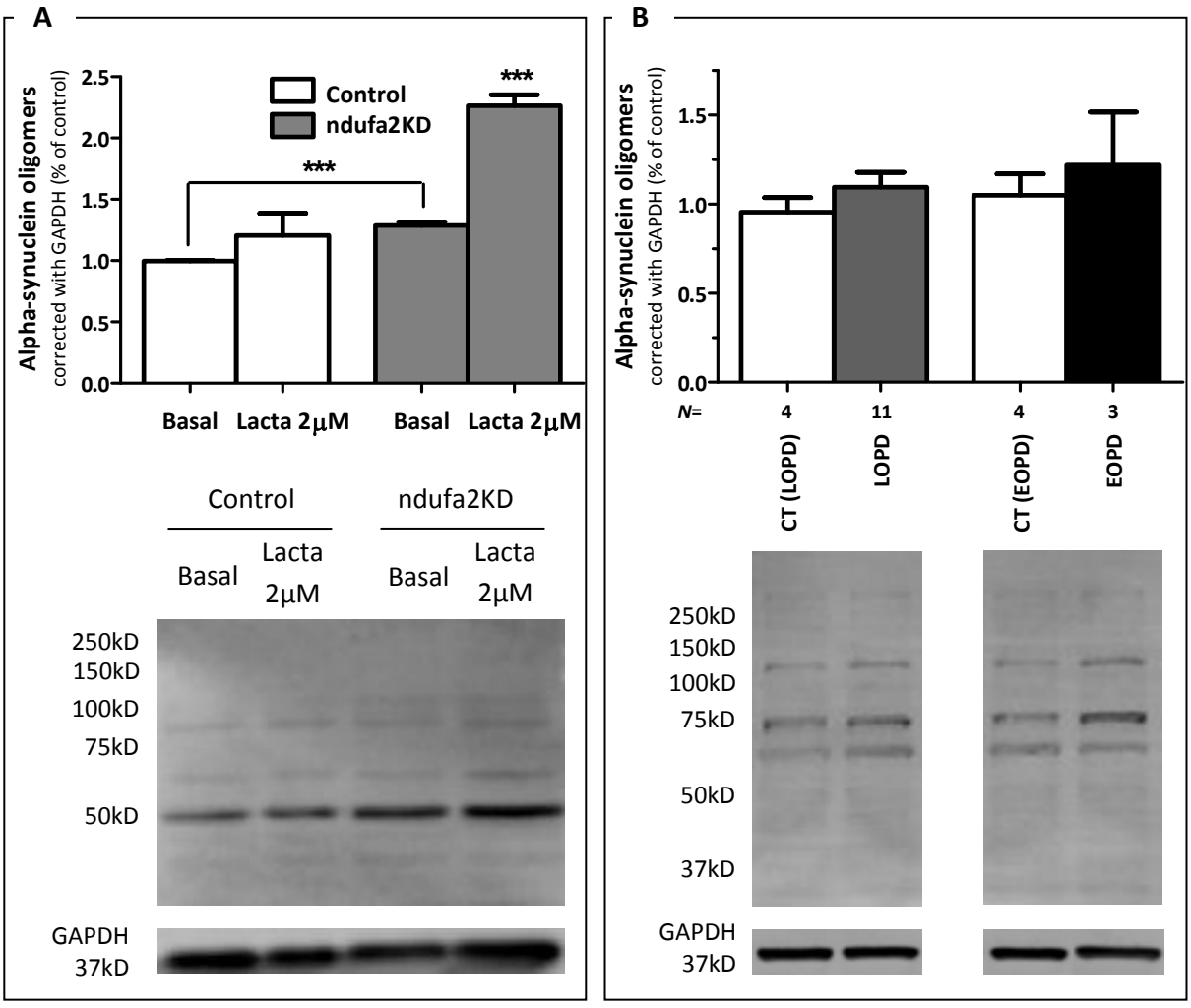

Figure 7. aSN aggregation in PD cellular models. (A) Densitometry analysis of triton-soluble aSN oligomers in SH-SY5Y ndufa2 KD cells and representative WB. N=3, ***P<0,001 (B) Densitometry analysis of triton-soluble aSN oligomers in PD patients PBMC and representative WB.

3.6. Ubiquitinated aSN in PD cell-line models

3.6.1. Ubiquitinated aSN in SH-SY5Y ndufa2 KD cells

There is a not statistically significant increase of ubiquitinated aSN in basal condition of SH-SY5Y ndufa2 KD cells comparing with parental cell-line. Lactacystin effect on control cell-line is favorable to accumulation of ubiquinated aSN. However, in SH-SY5Y ndufa2 KD cells, lactacystin inhibitor effect is paradoxically inverted in ubiquitinated aSN/aSN ratio.

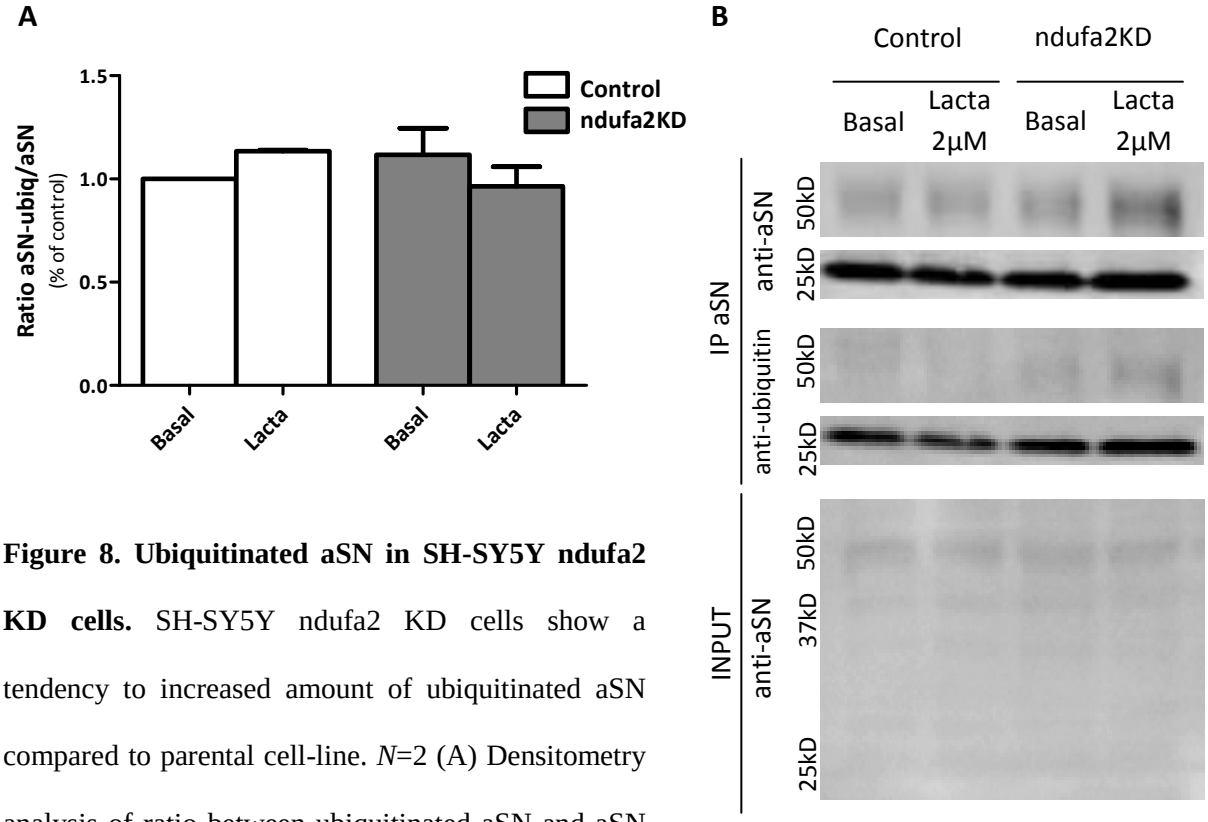

Figure 8. Ubiquitinated aSN in SH-SY5Y ndufa2 KD cells. SH-SY5Y ndufa2 KD cells show a tendency to increased amount of ubiquitinated aSN compared to parental cell-line. *N*=2 (A) Densitometry analysis of ratio between ubiquitinated aSN and aSN after aSN IP (B) Representative WB of aSN IP.

3.6.2. aSN ubiquitination in PD Cybrids

There are no evident differences in aSN ubiquitination between CT and PD Cybrids.

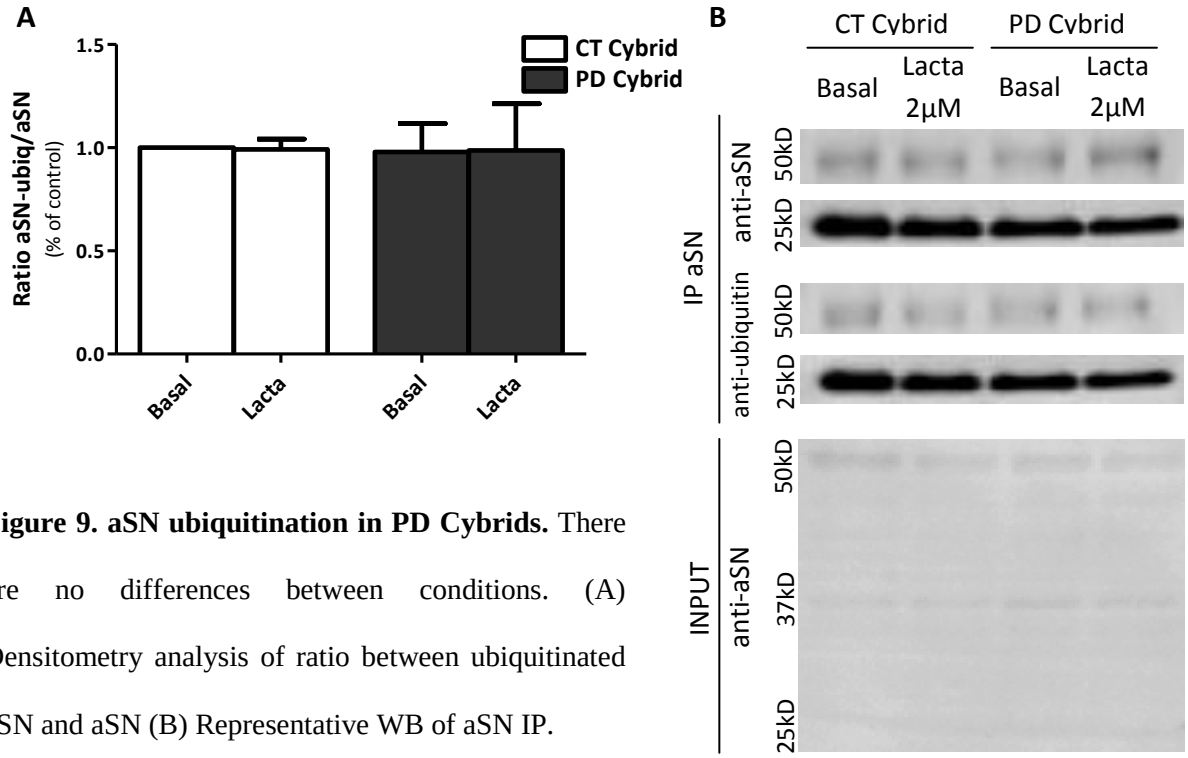

Figure 9. aSN ubiquitination in PD Cybrids. There are no differences between conditions. (A) Densitometry analysis of ratio between ubiquitinated aSN and aSN (B) Representative WB of aSN IP.

3.6.3. Correlation between aSN and total ubiquitination in PBMC

In PBMC of PD patients there is a statistically significant positive correlation between aSN and total ubiquitin content (*P*=0,0182).

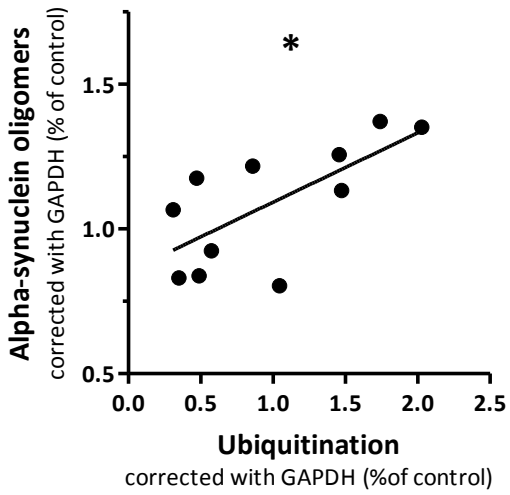

Figure 10. Correlation between aSN and total ubiquitination in PBMC of PD patients. aSN oligomers levels have a positive correlation with total ubiquitination. *N*=11, *Pearson r* =0,6924, **P*=0,0182, r^2=0,4795.

3.7. Correlation perspectives between parameters evaluated with PBMC model

3.7.1. Demographic characteristics of patients population

Due to high variability observed in values of parameters evaluated in the previous results, some correlation studies were performed, in order to better understand the influence of some demographic characteristics of patients population (Table I).

Table I. Demographic characteristics of control individuals and PD patients. Duration of disease and duration of L-DOPA treatment are presented in years.

Condition group	*N*	Gender ♂	Gender ♀	Age	Age of diagnostic	Duration of disease	Duration of L-DOPA treatment	UPDRS III	MMSE
CT (LOPD)	6	2	4	65,17± 3,31					
LOPD	14	9	5	74,295 ±7,39	64,64±10,2	9,64±7,75	7,27±6,10	45±9,06	26,08± 2,29
CT (EOPD)	4	2	2	54,75± 3,86					
EOPD	6	2	4	58,83± 3,19	47,17±1,47	11,67±2,42	10,5±4,32	44,5± 27,58	26,83± 0,41

3.7.2. Chymotrypsin-like proteasome activity

Corresponding to the major difference observed in the previous section related to proteasome activity analysis in PBMC of PD patients, where younger individuals seem to have higher chymotrypsin-like activity, there is a tendency to a negative correlation between age of individuals and chymotrypsin-like activities, both control and patients, except to 20S chymotrypsin-like activity in EOPD patients that has a positive correlation tendency (Figure 11). The other demographic features were accessed but there were neither significant correlations nor strong associations (data not shown).

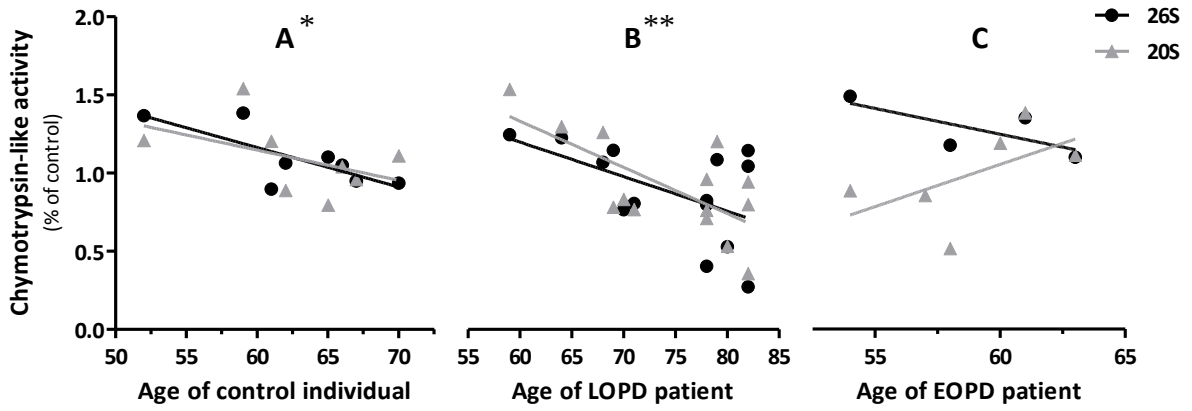

Figure 11. Correlation between Age and Chymotrypsin-like activity in PBMC. (A) Age in control individuals: 26S chymotrypsin-like activity has a significant negative correlation with age. *N*=8, *Pearson r* = -0,754, **P*=0,0307, r^2=0,5686; 20S chymotrypsin-like activity is negatively correlated with age. *N*=8, *Pearson r* = -0,468, *P*=0,2422, r^2=0,219 (B) Age in LOPD patients: 26S chymotrypsin-like activity is negatively correlated with age. *N*=14, *Pearson r* = -0,5275, *P*=0,0526, r^2=0,2783; 20S chymotrypsin-like activity has a significant negative correlation with age. *N*=14, *Pearson r* = -0,6864, ***P*=0,0067, r^2=0,4712 (C) Age in EOPD patients: 26S chymotrypsin-like activity is negatively correlated with age. *N*=4, *Pearson r* = -0,7365, *P*=0,2635, r^2=0,5424; 20S chymotrypsin-like activity is positively correlated with age. *N*=6, *Pearson r* = 0,5648, *P*=0,2429, r^2=0,3190.

3.7.3. Ubiquitination and aSN oligomers

Age at the time of participation in the study is again the independent variable from demographic characteristics that has stronger impact. Although there is no influence of this variable in the amount of ubiquitinated species and aSN oligomers on healthy individuals, in LOPD group of patients, there is a negative correlation between age and ubiquitination levels (Figure 12A). aSN/ubiquitin ratio is not correlated with duration of disease but is positively correlated with age, with an exponential nonlinear fit (Figure 12C and D). Interestingly, ubiquitination has a positive correlation with duration of disease (Figure 12B). Even though, aSN oligomers levels remain unchangeable in dependence of duration of disease, just with a very small positive slope in the linear regression, both depending on age or duration of disease. The other demographic features were accessed but there were neither significant correlations nor strong associations (data not shown).

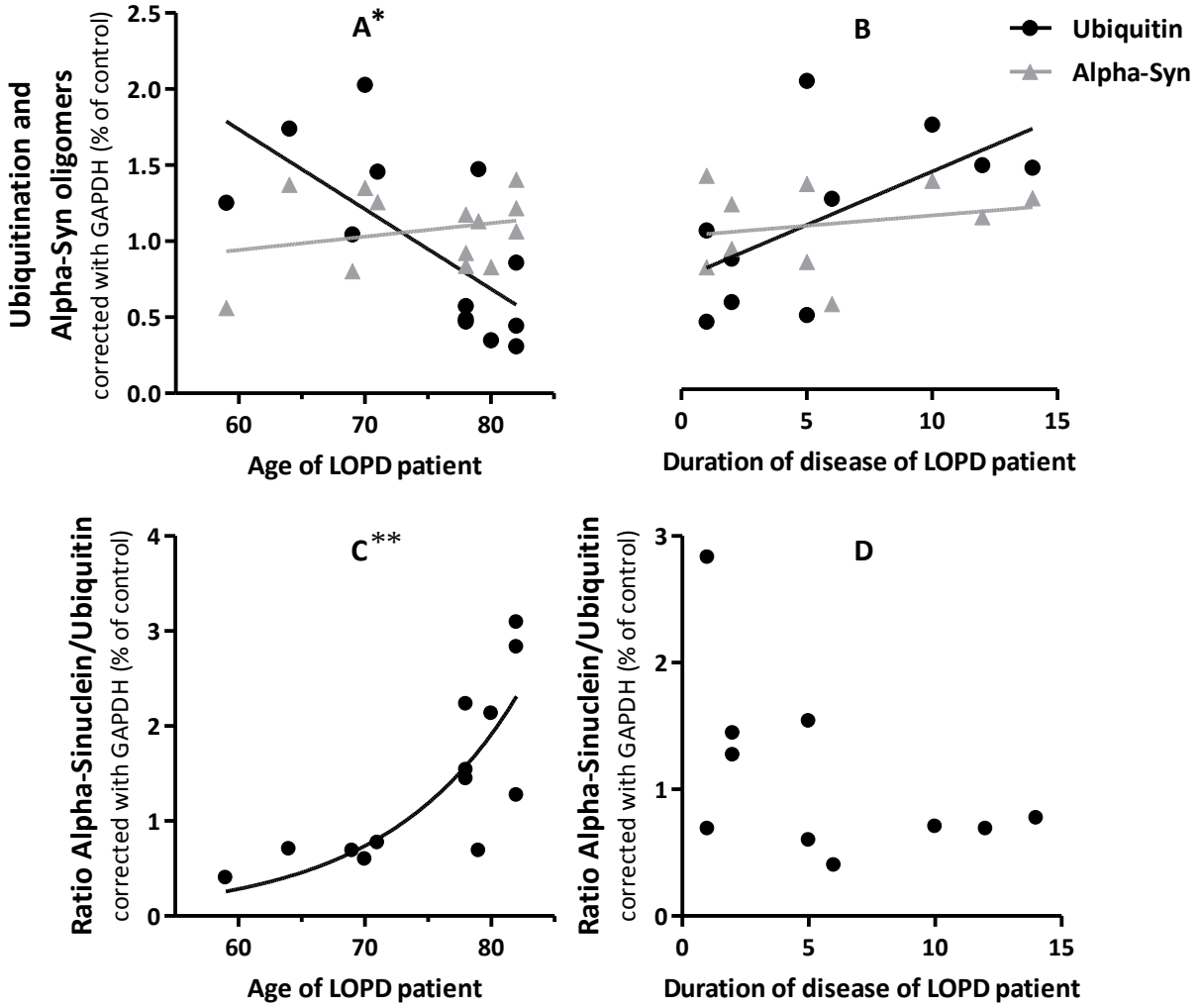

Figure 12. Correlations studies between demographic characteristics and Ubiquitination or aSN oligomers. Ubiquitin and aSN levels were corrected for GAPDH. (A) Age in LOPD patients: Ubiquitination has a significantly negative correlation with age. *N*=13, *Pearson r* = -0,6748, **P*=0,0114, r^2=0,4553; aSN has a very low and weak positive correlation with age. *N*=13, *Pearson r* = 0,2511, *P*=0,4079, r^2=0,06306 (B) Duration of disease in LOPD patients: Ubiquitination has a positive correlation with age. *N*=10, *Pearson r* = 0,6058, *P*=0,0634, r^2=0,3670; aSN has a very low and weak positive correlation with age. *N*=10, *Pearson r* = 0,2214, *P*=0,5387, r^2=0,04902 (C) Age in LOPD patients: aSN/ubiquitin ratio has a positive correlation with age. *N*=13, *Pearson r* = 0,736, ***P*=0,0041, r^2=0,5417 (D) Duration of disease in LOPD patients: aSN/ubiquitin ratio has a very weak negative correlation with duration of disease. *N* =10, *Pearson r* = 0,4997, *P*=0,1414, r^2=0,2497.

3.8. aSN secretion in plasma of PD patients

Quantification of aSN in plasma of PD patients was obtained by dot blot analysis. Although there is no significant difference between means, isolated comparison, using Unpaired t test, shows increased aSN secretion in the EOPD group comparing with the respective age-matched control group (*P*=0,0117) (Figure 13).

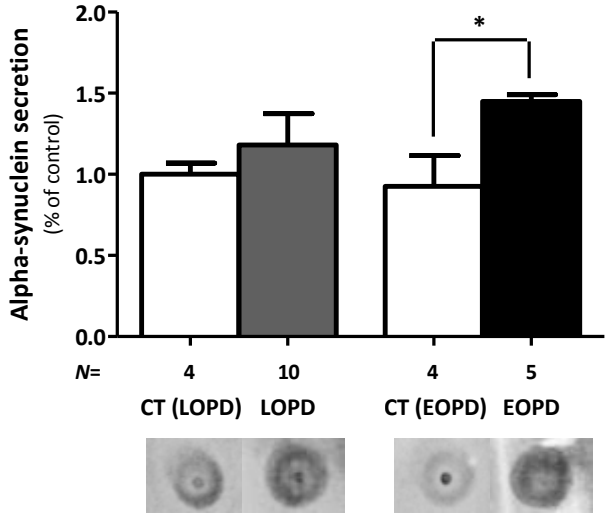

Figure 13. aSN quantification in plasma of PD patients. Densitometry analysis of aSN levels in plasma of PD patients and representative dot blot. There is no significant difference between means. **P*<0,05

3.9. Special cases – PD patients with identified mutation for familiar form of disease

3.9.1. Demographic characteristics of patients population

Due to the opportunity to observe the behavior in these specific cases of the enzymatic activities and protein post-translational modifications performed before, we decided to evaluate them in a different section. Demographic characteristics of these cases are shown in Table II.

Table II. Demographic characteristics of special cases and respective control individuals.

Condition group	*N*	Gender		Age
		♂	♀	
PARK2	**3**	**1**	**2**	**54,667±3,22**
PARK8	**2**	**0**	**2**	**55,5±9,19**
MD	**1**	**1**	**0**	**59**

3.9.2. UPS function in PBMC in patients with mutant forms of disease

PARK2 individuals show a decreased proteasome 26S chymotrypsin-like activity, considering that their activity values are out of the confidence interval (95%) of the group of subjects with EOPD (Figure 14A). Despite a slight decrease compared to EOPD group mean, concerning to 20S chymotrypsin-like activity and ubiquitinated protein content, there is no significant difference when compared to the values of that group (Figure 14B and C). PARK8 individuals have no difference in both proteasomal activities compared to EOPD. Though, 20S chymotrypsin-like proteasome activity seems to be higher than 26S and there is also a decreased amount of ubiquitin levels (Figure 14A, B and C). MD subject show higher activity in both proteasomal activities compared to EOPD group, although the activity value is out of the confidence interval to the correspondent group of PD patients (Figure 14A and B). Moreover, this patient has increased ubiquitinated protein content in PBMC (Figure 14C).

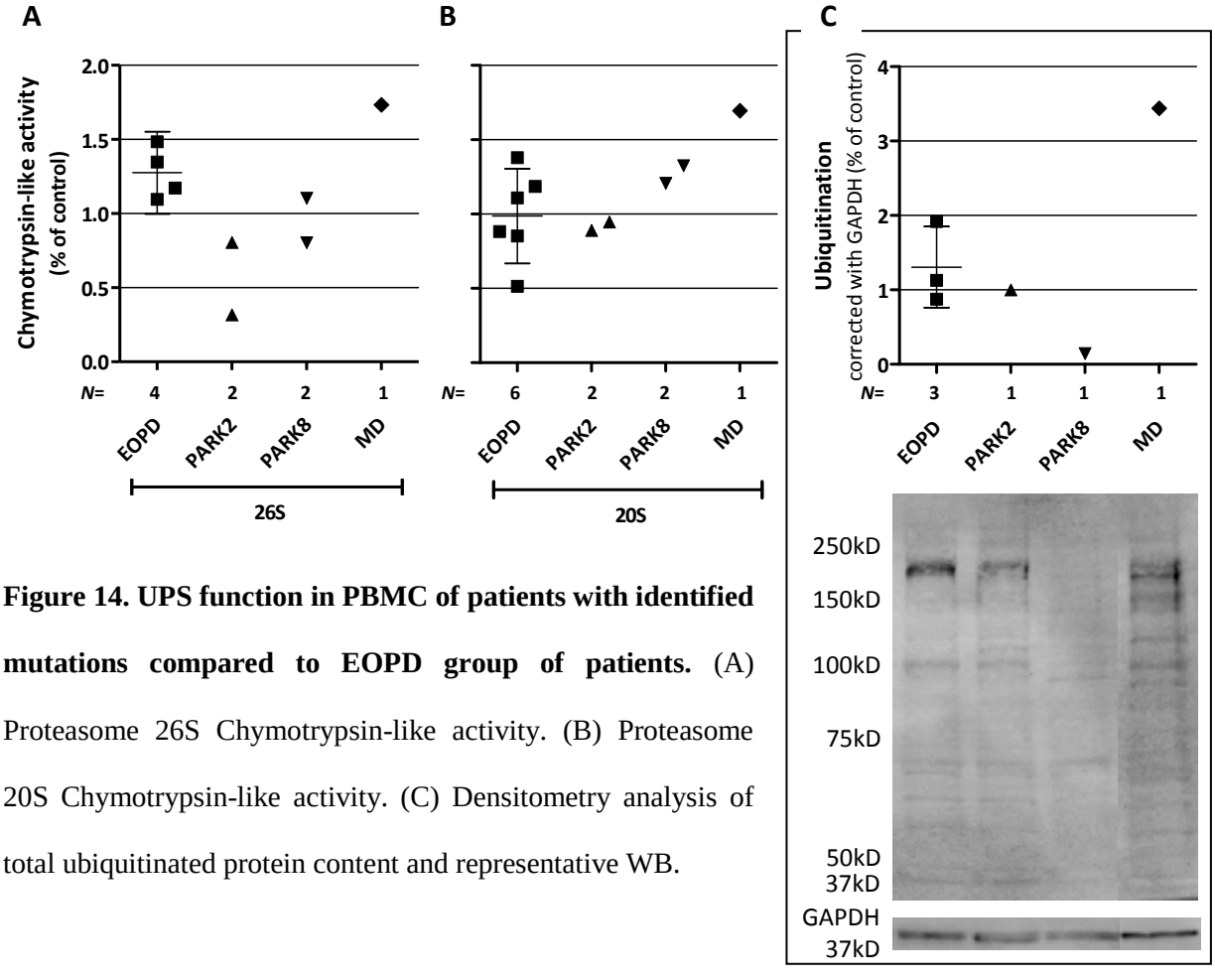

Figure 14. UPS function in PBMC of patients with identified mutations compared to EOPD group of patients. (A) Proteasome 26S Chymotrypsin-like activity. (B) Proteasome 20S Chymotrypsin-like activity. (C) Densitometry analysis of total ubiquitinated protein content and representative WB.

3.9.3. aSN oligomers and secretion in patients with mutant forms of disease

PARK2 individual shows an increased amount of aSN oligomers over the mean of EOPD group but still inside the confidence interval. Both PARK8 and MD have a decrease in the levels of aSN that are under the lower limit of confidence interval of EOPD group of patients (Figure 15A). Considering aSN levels in plasma, there are no values out of confidence interval of EOPD for any of the patients with mutant form of disease (Figure 15B).

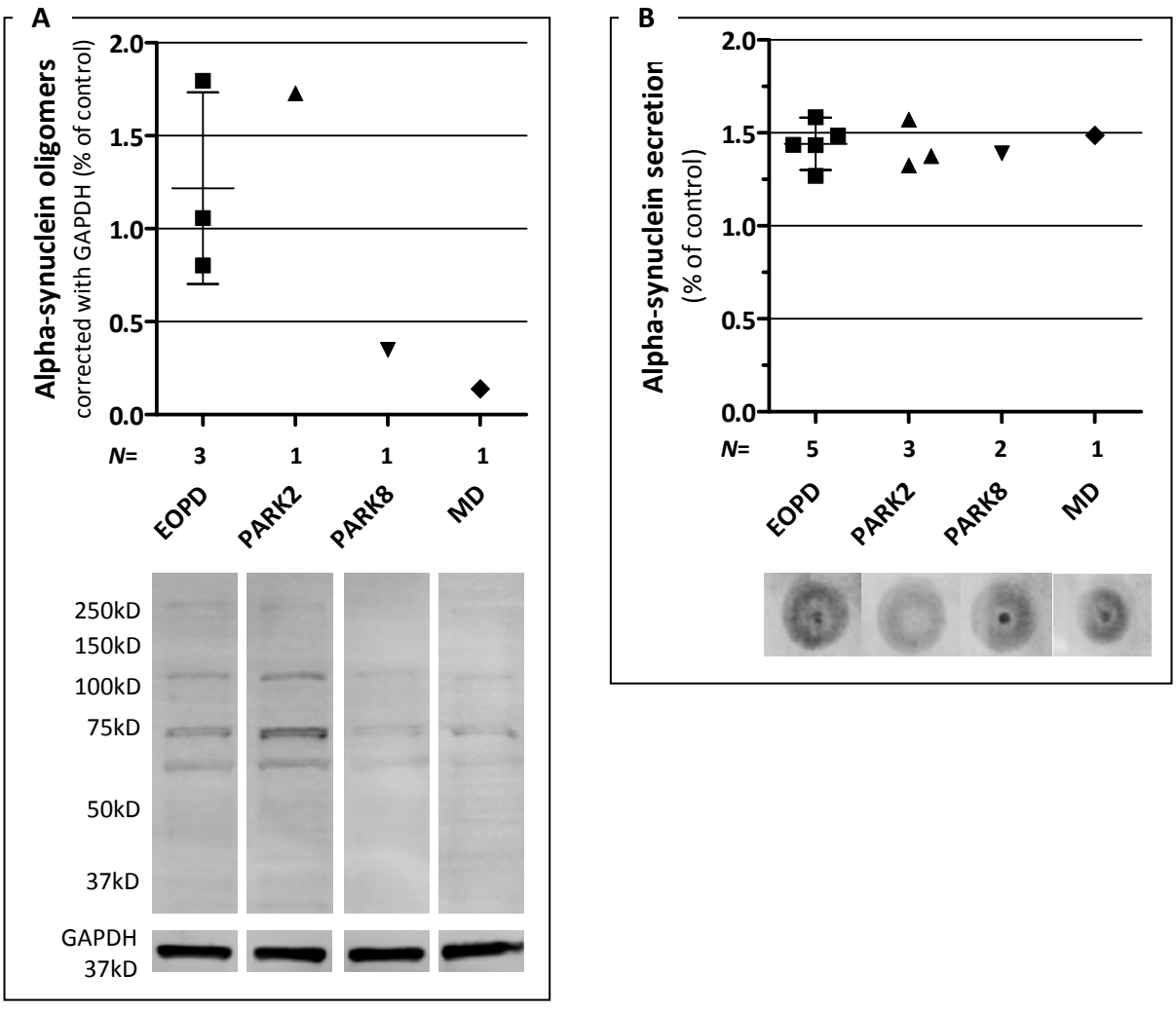

Figure 15. aSN aggregation and secretion in PBMC of patients with identified mutations compared to EOPD group of patients. (A) Densitometry analysis of aSN levels in PBMC of PD patients and representative WB. (B) Densitometry analysis of aSN secretion to plasma of PD patients and representative dot blot.

Chapter 4
Discussion

DAergic cell death in SNpc is well known to be the main cause of the disease, but the reason why these cells are progressively dying remains elusive. We believe that ETC CXI dysfunction is the major trigger of age related PD etiopathogenesis and that mitochondrial metabolic control is also involved in PD familial forms. It was previously observed by our group transversal alterations in different PD models, with alterations in the mitochondrial function, like SH-SY5Y ndufa2 KD cells, PD Cybrids, and platelets of PD patients.

There are several lines of evidence supporting this theory, such as genetic studies revealing the importance of mitochondria role in PD, mainly through pink1 and parkin genes, whose mutated forms have been linked to EOPD. CXI dysfunction was first discovered in SN of postmortem PD brain in 1989 (Schapira et al., 1989) and subsequently some additional studies confirmed similar results. Moreover, different studies reported a decrease of relevant subunits of CXI in PD human brain (Keeney et al., 2006; Mizuno et al., 1989). Interestingly, MPTP (1-methyl--phenyl-1,2,3,6-tetrahydropyrine), a neurotoxin capable to induce PD symptoms in humans (Langston et al., 1983), is a mitochondrial CXI inhibitor. In addition, CXI deficiency was also found in other PD patients tissues, as platelets and lymphoblasts (Barroso et al., 1993; Schapira, 1994). To address the potential causes of CXI defect, namely if it was due to an environmental toxin or to an alteration of mitochondrial or nuclear DNA, the cytoplasmic hybrid (cybrid) technique, first described in 1989 (King and Attardi, 1989), has been applied and the outcome indicates that the CXI defect in PD appears to be genetic and arising from mtDNA. Supporting this theory, is that mitochondria in vulnerable PD neurons are under greater stress condition that increases the probability of mtDNA mutation (Soong et al., 1992). Moreover, another incontestable piece of evidence of mitochondrial dysfunction in PD has come from conditional knockout mice, termed "MitoPark" mice, the first animal model showing the slow progressive degeneration of DA neurons seen in PD (Ekstrand et al., 2007).

All these results are consistent with the involvement of respiratory chain dysfunction in PD pathogenesis. Thus, we propose that SH-SY5Y ndufa2 KD cell line is PD cellular model since

it has CXI dysfunction (Figure 3). Accordingly, a progressive mitochondrial dysfunction process leads to a loss of ATP, decreased calcium buffering capacity and increased oxidative stress acting synergistically to promote DAergic cell death.

Oxidative stress can compromise the integrity of vulnerable neurons and thus contribute to neuronal degeneration. The source of the increased oxidative stress observed in PD is unclear but may derive from mitochondrial dysfunction, increased DA metabolism, increased reactive iron, impairment of antioxidant defenses pathways, and/or due to the highly oxidative intracellular environment within DAergic neurons (revised by Cardoso et al., 2009). A mitochondrial defect generates excessive ROS/RNS formation resulting in neuronal damage through protein aggregation (Nakamura and Lipton, 2009). This is a point of intersection between mitochondria and UPS function, since mitochondria, producing excessive ROS, may affect UPS activity. Thus, even mitochondrial dysfunction is a major factor, UPS regulation may also be either associated as a consequence of mitochondrial dysfunction, or as a causative factor when it is impaired.

Protein degradation by the UPS consists in a tightly regulated process, starting with target-protein tagging with a polyubiquitin chain by ubiquitin ligases E3 in an ATP-dependent manner and ending with degradation by the 26S proteasome, which also requires ATP to assemble 19S and 20S subunits (Goldberg, 2003). The proteasome is a large protease complex that eliminates intracellular misfolded, oxidized or aggregated proteins (Ciechanover and Brundin, 2003). Accordingly with our results in PBMC of both control individuals and LOPD patients (Figure 11A and 11B), other groups showed that there is proteasomal loss of function with aging, reflected on a decrease in proteasome subunits expression, activity and response to oxidative stress (Bulteau et al., 2000; Keller et al., 2000). Furthermore, we showed an age dependent decrease of total ubiquitin content (Figure 12A) as well as exponential increase of aSN/ubiquitin ratio, also consistent with UPS function deterioration with aging (Figure 12C).

In addition to the existence of EOPD forms caused by mutations in genes that codify proteins of UPS pathway, such as parkin and UCHL1, the co-localization of proteasome subunits in LBs (Ii et al., 1997) and also the presence of ubiquitinated proteins in LB may indicate UPS involvement in PD, since ubiquitin is the signal protein for degradation by UPS. Beside UPS impairment being evidently related with some EOPD forms, it was also reported a proteasomal dysfunction in the SN in LOPD (McNaught and Jenner, 2001).

However, the results in our models suggest that there is no proteasomal dysfunction and, interestingly, in SH-SY5Y ndufa2 KD cells there is a tendency to an up-regulation of proteasome activity in 20S chymotrypsin-like and in PGPH-like activities, both 26S and 20S (Figure 4A and 4B). Although this could mean a cell rescue attempt by increasing degradation rate of oxidized proteins, we must consider that, even if this is a proposed chronic model of the disease, it is not strictly representative of aging in human beings. Thus, it could be important to further research in this model how proteasome function is being upregulated in order to better understand how protein quality control can be improved as prevention or treatment of disease. Moreover, transversal to the three PD cellular models, there are elevated levels of total ubiquitination content (Figure 4C, 4E and 6C). This can be explained by UPS over request or UPS dysfunction, both correlated with ubiquitinated aSN accumulation observed in SH-SY5Y ndufa2 KD cells (Figure 8) and highly suggestive in PBMC of LOPD (Figure 10). Since there is no reduction of proteasome activity compared to control basal condition in any of the used models, we believe that proteasome enzymatic function is preserved as well as 20S ATP-independent degradation pathway that is responsible to degrade smaller misfolded proteins without ubiquitin tagging. Thus, aSN oligomers formation could be probably explained by insufficiency of normal clearance activity due to increased request or by any other alteration in UPS pathway previous to enzymatic degradation of the substrate, as protein tagging and/or ubiquitin recognition that are ATP-dependent processes.

This hypothesis is compatible with the ubiquitinated species accumulation and ubiquitin monomer increased levels observed. Preliminary results of aSN IP reveal that lactacystin treatment in SH-SY5Y ndufa2 KD cells does not induce increased amounts of ubiquitinated aSN as it is observable in the parental cell line, what can be correlated with a predominant ATP-independent degradation pathway in SH-SY5Y ndufa2 KD cells (Figure 8).

Accordingly to this proposal, increase of proteasome enzymatic activity in our models is followed by increased levels of total ubiquitin content, as we can see in SH-SY5Y ndufa2 KD cells (Figure 4C and 4E) and in younger groups of individuals, CT(EOPD) and EOPD (Figure 6C). Moreover, in LOPD patients, total ubiquitination content is positively correlated with 20S chymotrypsin-like activity (Figure 6D). This could mean an enzymatic response to a previous stimulus of higher rate of ubiquitination. There is also more availability of ubiquitin monomer in SH-SY5Y ndufa2 KD cells (Figure 4D) what could represent UPS impairment but also higher degradation rate or increased ubiquitin expression. It could be also interesting to further study this ubiquitin content accumulation to better understand if it is due to a dysfunction or up-regulation of ubiquitination machinery.

Even with an UPS up-regulation, this is not enough to avoid aSN oligomerization since we can observe aSN accumulation in the three cellular models (Figure 7). Moreover, it was reported that expression of mutant aSN significantly reduces chymotrypsin-like, trypsin-like and PGPH-like activities of the proteasome (Tanaka et al., 2001). Thus, it is expectable that proteasomal function decrease with aging due to aSN slow accumulation and that proteasome activity up-regulation is an early event in the lifetime since it is only observed in younger groups of individuals, despite a higher mean of duration of disease in EOPD group when compared to LOPD group (Table I). We were not able to find any correlation between aSN levels and proteasome activity in LOPD group (data not shown).

Considering aSN accumulation, once UPS is not efficiently clearing this high rate of protein misfolding, other alternatives must be requested. Toxin-induced parkinsonism is not associated with typical LBs formation, thus suggesting that aSN aggregates are not a cause of the disease but probably a chronic mechanism of cell protection against soluble oligomeric aSN toxicity. These oligomers can aggregate in bigger structures with other proteins to form LBs or being secreted through cell membrane to extracellular space. Our results show increased levels of aSN in plasma of patients, mainly in those suffering from EOPD (Figure 13). This probably represents a cellular mechanism to avoid soluble oligomeric aSN toxicity and it could be of great interest if we can understand that this is an early process in aging and disease progression, because it may be used as a method of diagnostic or staging of disease.

Moreover, autophagy, a process that can remove deficient mitochondrial and protein aggregates, is also involved in PD etiopathogenesis. Thus, it would be very interesting to describe autophagy regulation in our models. Lactacystin worked as negative control of proteasome activity and conduced to ubiquitinated proteins accumulation as well as monomer levels increase in SH-SY5Y cells. Moreover, treatment of SH-SY5Y cells with lactacystin induces accumulation of higher amounts of ubiquitinated proteins than those observed in basal condition of SH-SY5Y ndufa2 KD cells. This fact is in agreement with presence of dysfunctional UPS in PD or with a shift in cellular ubiquitin tagging in order to address misfolded proteins to other degradation pathways as autophagy, once UPS is inhibited. Accordingly, it was previously proposed that systemic administration of proteasome inhibitors in rat induced a progressive PD model. After a latency period of 1–2 weeks rats developed the characteristic symptoms of the disease and also showed, in the postmortem analysis, striatal DA depletion and DAergic cell death in SNpc (McNaught et al., 2004). Furthermore, proteasome inhibition with lactacystin in cells expressing mutant aSN increased mitochondria-dependent apoptotic cell death (Tanaka et al., 2001).

Considering our and others studies, there are several lines of evidence that suggest a cross-talk between mitochondria and UPS in PD (revised by Branco et al., 2010). Some authors claim that mitochondrial compromise is the primary event followed by proteasome impairment and consequent aSN aggregation. However, it was reported that after proteasome inhibition in dopaminergic neuronal cells, there is a prominent accumulation of polyubiquitinated proteins that are likely to be related with activation of mitochondrial apoptosis and consequent neuronal loss (Sun et al., 2009). An increase of polyubiquitinated proteins in mitochondria may be indicative of the potential role of mitochondria as an early key sensor of UPS impairment and accumulation of misfolfed ubiquitinated proteins. In this point of view, proteasomal dysfunction seems to appear as the causal disturb, followed by mitochondria participation in the molecular mechanism of the disease (Sun et al., 2009). It would be interesting to further investigate in our models how proteasomal inhibition can influence mitochondrial function.

Our group demonstrated an effective correlation between mitochondrial dysfunction and proteasomal impairment, suggesting that they act synergistically and not only exclusively by themselves. We reported that MPP^+ induced in NT2 human teratocarcinoma cells a marked increase in ubiquitinylated protein levels, free radicals generation and a decrease in ATP levels. These results indicate that mitochondrial deficits may adversely affect ATP-dependent proteasomal degradation. Accordingly, we also showed a reduction in proteasomal activity (chymotrypsin and PGPH-like activities) in NT2-MPP^+ treated cells after 24h and in NT2-ρ0 (mitochondrial DNA depleted) cells under basal conditions (Domingues et al., 2008). The evident discrepancy between NT2-MPP^+ and ndufa knockdown cells may reflect that MPP^+ treated cells are an acute toxic model while ndufa2 KD cells represent a chronic mitochondrial dysfunction model whereas an UPS up-regulation reflects a cell rescue phenomenon.

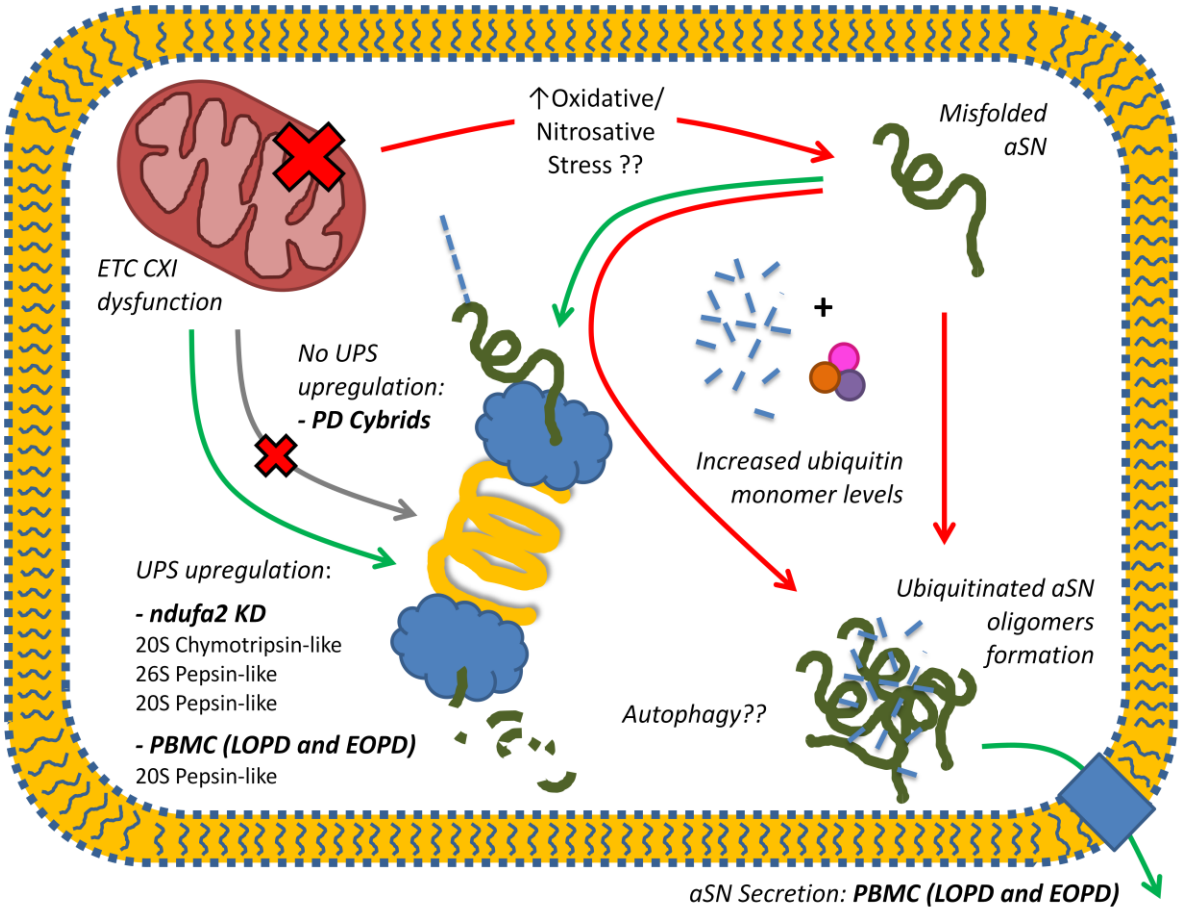

Figure 16. Schematic representation of UPS and mitochondrial interplay.

Considering our hypothesis that mitochondrial dysfunction is the main trigger of the etiopathogenesis of the disease, ETC CXI impairment previously observed by our group, is the leading event that cause SH-SY5Y ndufa2 KD cells, PD Cybrids and indirectly platelets of PD patients cellular alterations. As a consequence of this modification, oxidative/nitrosative stress conduces to conformational changes in proteins, such as aSN. These proteins are detected as dysfunctional and tagged by ubiquitin ligases to degradation. Ubiquitin tagging allows the recognition by intracellular protein degradation systems as proteasome or autophagosome and then physiologically degraded. However, protein quality control system seems to be over requested and misfolded aSN tends to accumulate through oligomers formation. Even so, there is an attempt of cell rescue by a tendency to up-regulation of proteasomal enzymatic activities. Moreover, in PBMC of PD patients there is a slight increase of aSN secretion.

As previously referred, genetic approaches in familial forms of PD provided some evidences. Analysis of these genes products lead us to conclude that mitochondria and proteasome are deeply involved in the molecular mechanisms of PD pathogenesis. Moreover, in some genetic forms both mitochondria and proteasome are involved simultaneously due to the relation of their protein product with both, which somehow mean a probable interplay between them.

Parkin (PARK2) is a 465-aminoacid (a.a.) polypeptide (~51 kDa) that plays an important role in the UPS as ubiquitin E3 ligase (Shimura et al., 2000). Mitochondrial dysfunction and increased apoptosis were shown in *Drosophila* parkin null mutant or in overexpressed parkin mutation models (Greene et al., 2003). Parkin is a protein that may have a role in maintenance of the outer mitochondrial membrane integrity (Darios et al., 2003). Accordingly, parkin function was related with mitochondrial dynamics (Riparbelli and Callaini, 2007). Moreover, it seems to be involved in mitochondria trafficking since it was shown that parkin may bind and stabilize microtubules (Yang et al., 2005). Parkin was also reported to be recruited to impaired mitochondria and induce their autophagy, what means that degradation of abnormal mitochondria in PARK2 is reduced, contributing to neuronal death (Narendra et al., 2008).

Additionally, parkin protein is an E3 ubiquitin-protein ligase, so mutations in the gene of parkin, originate lack of enzymatic activity of this enzyme and consequent misfolded proteins accumulation. We can see that from the three individuals reported in this study with this mutation, the two who have been measured the proteasome activity show a decreased ubiquitination-dependent proteasome enzymatic activity with increased amount of aSN oligomers, despite there is no significant ubiquitination levels reduction (Figure 14 and 15). Some studies revealed that over-expression of parkin, using viral vectors, may be effective against aSNopathy (Lo Bianco et al., 2004; Yamada et al., 2005; Yasuda et al., 2007). Thus, parkin over-expression may be a therapeutic target, due to increased UPS activity and altered proteins and dysfunctional mitochondria clearance, avoiding aggregates-induced cell death.

PARK8 was firstly described and mapped to chromosome 12p11.2-q13.1 in 2002 of a LOPD autosomal dominant transmission family (Funayama et al., 2002), being later identified as the causative gene for PARK8-linked familial PD (Paisan-Ruiz et al., 2004). The clinical manifestations in patients with LRRK2 mutation mainly resemble sporadic PD with good response to levodopa (Funayama et al., 2002). Despite approximately 20 putative pathogenic mutations have been described in LRRK2 gene (Lu and Tan, 2008), LRRK2 G2019S mutation, the most prevalent and the one identified in the two patients included in this study, is located within the kinase domain and exhibit increased activity (Gloeckner et al., 2006; West et al., 2005). Dysregulated kinase activity may explain the core damaging effect of LRRK2 in neurons (Cookson et al. 2007).

The LRRK2 protein is a 2527 a.a. polypeptide (280 kDa), consisting of leucine-rich repeats (LRR), Ras complex proteins followed by the C-terminal of Roc, mitogen-activated protein kinase kinase kinase (MAPKKK) and WD40 domains (Marin, 2006; Mata et al., 2006).

LRRK2 might play a role in cell division and development (Marin et al., 2008). In the rodent brain, LRRK2 is widely distributed including the SNc, caudate putamen, and olfactory bulb (Biskup et al., 2006; Higashi et al., 2007b; Melrose et al., 2007). In the human brain, recent in situ hybridization and immunohistochemical analyses revealed that LRRK2 also localizes within various brain regions associated with PD pathology (Higashi et al., 2007a).

Therefore, LRRK2 supposedly has important functions in broad areas of the brain as well as the nigro-striatal DAergic pathway. In cells, LRRK2 protein is mainly found in the cytoplasm. However, LRRK2 protein is also present in membranous organelles as interacting with cytoskeleton and trafficking proteins (revised by Hatano et al., 2009). The interaction between LRRK2 and parkin was also reported (Smith et al., 2005).

Results in these patients revealed a predominance of ATP-independent activity; even it is not different from the EOPD group, with reduced amount of ubiquitination and aSN oligomers.

The patient suffering from MD is a 59 years-old man, who has clinical symptoms of EOPD, such as dystonia of the left lower limb and bradykinetic-rigid presentation with rest tremor of left predominance. DaTSCAN study confirms reduction of DAergic neurons on right striatal region, compatible with the clinical syndrome and corroborating EOPD diagnostic. The patient presented a good response to levodopa treatment. Moreover, genetic studies to Hereditary Spastic Paraplegia type 4 (*SPG4*), PARK2 and PARK8 were negative. Thus, mitochondrial DNA screening was performed due to high degree of suspicion. In muscle biopsy cells there were identified multiple mtDNA deletions and in lymphocytes was found ETC complex IV activity reduction. Despite there are no evident family history, considering that the patient also suffers from myopathy, confirmed by electromyography, bilateral neurosensory hearing loss and Diabetes mellitus we strongly suspect of POLG1-related disorder (Orsucci et al., 2011). The patient was proposed to study of this mutation. Thus, we consider this case as another example of the influence of mitochondrial dysfunction in UPS regulation. We can observe highly increased proteasome activity and ubiquitination levels when compared to the EOPD group, which we correlate with SH-SY5Y ndufa2 KD cells results. In this case there is also a reduction of aSN oligomers, what could mean that proteasome up-regulation is being effective in misfolded aSN degradation despite neuronal loss due to mitochondrial dysfunction. It could be also interesting evaluate autophagy function as well.

To sum up, some final remarks must be considered. We believe that some points of this work could have been improved, especially concerning the PBMC model. We found a great variability inside each group of controls and patients and a higher number of participants would reduce these differences giving stronger statistical significance to the results and tendencies observed. In addition, control individuals could have been even more age matched

since there are still some age differences between control groups and respective PD groups, mainly between CT(LOPD) and LOPD, where we can find a gap of almost 10 years.

Moreover, some additional cellular alterations could be evaluated in our models, such as ATP and ROS levels that could provide a better understanding of the connection between mitochondrial dysfunction and UPS modulation. However, our findings (Table III) support that cross-talk between mitochondria and proteasome is likely to be a two ways dead-road inside the cell. There are major evidences that UPS is involved in the age dependent mechanism of disease progression, so recognition of mitochondrial and UPS interplay may open a new window to PD therapeutics.

Table III. Summary of results.↑/↓ increased/decreased value, = not difference, - not studied, **P*<0,05, ***P*<0,01, ****P*<0,001, compared to respective control condition from control cellular model.

PD Models		CX I activity	UPS activity					aSN olimers	Ubiq-aSN	aSN secretion
			Chymotrypsin		PGPH		Ubiquitination			
			26S	20S	26S	20S				
SH-SY5Y ndufa2KD		↓**	=	↑**	↑**	↑**	↑	↑***	↑	-
PD Cybrids		↓**	=	=	=	=	↑***	↑**	=	-
PBMC	LOPD	↓* platelets	=	=	-	↑	↑	↑	-	↑
	EOPD	-	=	=	-	↑	↑	↑	-	↑*
	PARK2	-	↓*	=	-	-	=	↑	-	↑
	PARK8	-	=	=	-	-	↓	↓	-	↑
	MD	↓CXIV	↑	↑	-	-	↑	↓	-	↑

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Appendix

Creation of cybrids cell-line and cell culture

NT2ρ0 cells were briefly agitated in polyethylene glycol with platelets from the human subjects (Cardoso et al., 2004). Seven days after plating the resulting mixture in T75 flasks and ρ0 growth medium, the medium was changed to cybrid selection medium. NT2ρ0 cells lack intact mtDNA, do not possess a functional ETC, and are auxotrophic for pyruvate and uridine (Cardoso et al., 2004; Swerdlow et al., 1997). Maintaining cells in selection medium removes ρ0 cells that have not repopulated their mtDNA with platelet mtDNA. After selection was complete, the resultant cybrid cells were switched to cybrid expansion medium. Cells were plated at $0{,}25\times10^6$cells/ml for measurement of proteasome activity and WB analysis.

NADH-ubiquinone oxidoreductase assay

ETC CXI activity was determined by a modified version of Ragan et al. (1987), which follows the decrease in NADH absorbance at 340 nm that occurs when ubiquinone (CoQ1) is reduced to form ubiquinol. The reaction was initiated by adding CoQ1 (50 lM) to the 30 _C reaction mixture. After 5 min, rotenone (10 lM) was added and the reaction was followed for another 5 min. CXI activity was expressed both as nanomoles per minute per milligram of protein, as well as the ratio of CXI activityper citrate synthase activity.

Fluorimetric proteasomal activity analysis

Upon treatment cells were washed twice in ice-cold PBS, and 100µl of lysis buffer (1mM EDTA; 10mM Tris-HCl, pH 7,5; 20% glycerol; 4mM DTT) was added to each well, and placed at 4°C. Cells were then scraped and frozen three times, with subsequent centrifugation at 13000g, for 10min, at 4°C. Supernatants were collected and protein concentrations were assayed using the Bradford protein assay (Bio-Rad, Hercules, CA, USA) (Stocchi et al., 1985). In a 96-multiwell plate, 50µg of protein was incubated with proteasome activity buffer (0,5mM EDTA and 50mM Tris-HCl, pH 8) and 50µM N-Succinyl-Leu-Leu-Val-Tyr-AMC (Suc-LLVY-AMC) or 400 µM Z-Leu-Leu-Glu-βNa, which were used as substrate to measure

the chymotrypsin-like or peptidyl-glutamyl peptide hydrolytic-like (PGPH) proteolytic activities, respectively. This enzyme activity was assayed by continuous recording of the fluorescence activity released from fluorogenic substrates using a Spectramax GEMINI EM fluorocytometer (Molecular Devices), and with excitation and emission wavelengths corresponding to 380 and 460nm, respectively, for 1h at 37°C.

Immunoblotting

Cells were washed in ice-cold PBS and lysed in 1% Triton X-100 containing hypotonic lysis buffer (25mM HEPES, pH 7,5, 2mM MgCl2, 1mM EDTA and 1mM EGTA supplemented with 2mM DTT, 0,1mM PMSF and a 1:1000 dilution of a protease inhibitor cocktail). Cell suspensions were frozen three times in liquid nitrogen and centrifuged at 20000g for 10min. The resulting supernatants were removed and stored at -80°C. Protein concentrations were determined by Bradford protein assay. For the analysis of ubiquitination levels and aSN aggregates, equal amounts of protein in supernatants (Triton soluble fractions) were collected and were loaded onto 7% and 10% SDS-PAGE, respectively, after 5min at 100ºC. For the analysis of NDUFA2 protein amount the same procedure was performed. After transfer to PVDF membranes (Millipore, Billerica, MA, USA) previously activated, the membranes were incubated for 1h in Tris-buffered saline (TBS) solution containing 0,1% Tween 20 and 5% nonfat milk for ubiquitination analysis and 5% bovine serum albumin (BSA) for aSN oligomers quantification, followed by an overnight incubation with the respective primary antibodies at 4°C with gentle agitation (1:100 anti-aSN LB509 from Zymed Laboratories Inc; 1:200 anti-ubiquitin SC-9133 from Santa Cruz Biotechnology, Inc; 1:1000 NDUFA2 antibody generously donated by Dr. Leo G. J. Nijtmans from Nijmegen Center for Mitochondrial Disorders, Laboratoy of Paediatrics and Neurology, Radboud University Nijmegen Medical Center, The Netherlands; 1:2500 monoclonal anti-GAPDH antibody from Chemicon International or 1:10000 monoclonal anti-alpha-tubulin antibody from Sigma were

used for loading control. Membranes were washed with TBS containing 0,1% Tween 20 three times (each time for 10min), and then incubated with the appropriate secondary antibody (1:20000 anti-mouse or anti-rabbit IgG alkaline phosphatase linked, from GE Healthcare, UK) for 1h30min at room temperature with gentle agitation. After three washes specific bands of interest were detected by developing with an alkaline phosphatase enhanced chemical fluorescence reagent (ECF from GE Healthcare, Buckinghamshire, England). Fluorescence signals were detected using a Biorad Versa-Doc Imager, and band densities were determined using Quantity One Software.

Dot Blot assay

Dot Blot assay was done as previously described with modifications (Domingues et al., 2008) Protein concentration of plasma samples of PD patients and control individuals was determined by the method of Bradford using BSA as protein standard (Bradford, 1976).

After PVDF membrane (Millipore, Billerica, MA, USA) pretreatment, it was placed on the top of a dry sheet of filter paper. One hundred micrograms of protein, in a final volume of 5 μl, were laid in dots in specific zones of the membrane. Once the dots were dried, nonspecific binding was blocked for 1h at 4°C using 5% nonfat milk for ubiquitination analysis or 5% BSA for aSN oligomers with 0,1% Tween 20 in TBS. Membranes were incubated overnight at 4ºC with gentle agitation with primary antibody (1:100 anti-aSN LB509 from Zymed Laboratories Inc; 1:200 anti-ubiquitin sc-9133 from Santa Cruz Biotechnology, Inc). After 3 washes in TBS containing 0,1% Tween 20, the membrane was incubated with a 1:20000 dilution of secondary antibody for 1h30min at room temperature followed by 3 washes in TBS containing 0,1% Tween 20. After three washes dots were detected by developing with an alkaline phosphatase enhanced chemical fluorescence reagent (ECF from GE Healthcare, Buckinghamshire, England). Protein dots were visualized using a VersaDoc imaging system (Bio-Rad) and quantified using Quantity-One software (Bio-Rad).

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