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Alterações transcricionais e redox em células estaminais humanas da doença de Huntington

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular realizada sob a orientação da Prof.ª Doutora Sandra Maria Tavares da Costa Rebelo, Professora Auxiliar convidada do Departamento de Ciências Médicas da Universidade de Aveiro, e co-orientação científica da Prof.ª Doutora Ana Cristina Carvalho Rego, Professora Auxiliar com Agregação da Faculdade de Medicina da Universidade de Coimbra.

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o júri

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palavras-chave Doença de Huntington; iPSC; NSC; mitocôndria; transcrição; stresse oxidativo

resumo

A doença de Huntington (HD, “Huntington’s disease”) é uma doença neurodegenerativa autossómica dominante, causada pela repetição de sequências CAG no gene *HTT.* As manifestações clínicas da doença incluem alterações motoras, cognitivas e psiquiátricas, e atualmente não existe cura para a HD. A utilização de células estaminais pluripotentes induzidas (iPSC, ”induced-pluripotent stem cells”) e células estaminais neurais (NSC, “neural stem cells”) fornecem um modelo adequado para o estudo dos eventos iniciais conducentes à neurodegenerescência na HD. Assim, este trabalho teve como objetivo analisar as alterações de transcrição que envolvem a biogénese mitocondrial e a resposta ao stresse oxidativo em células HD-iPSC e HD-NSC, comparativamente a células controlo.Foi avaliada a expressão de PGC-1α (“peroxisome proliferator-activated receptor gamma coactivator 1-alpha”), do fator de transcrição mitocondrial A (TFAM) e subunidades dos complexos da cadeia respiratória mitocondrial e de enzimas que regulam a atividade da piruvato desidrogenase (PDH) em HD-iPSC e HD-NSC. Os resultados mostraram uma diminuição dos níveis de mRNA de PGC-1α, TFAM e subunidades do complexo III (CYC1, MT-CYB e UQCR10), e um aumento de mRNA da subunidade ND1 do complexo I e da cinase1 da PDH (PDK1) em HD-iPSCNas HD-NSC apenas se observou uma diminuição nos níveis de mRNA de PGC-1α. Em células HD-iPSC e HD–NSC detetaram-se também níveis aumentados de peróxido de hidrogénio, uma espécie reativa de oxigénio (ROS, “reactive oxygen species”), sem contudo ocorrerem alterações na acetilação no resíduo de lisina 68 da superóxido dismutase 2 (SOD2) nas HD-iPSC; curiosamente, observou-se uma diminuição significativa dos níveis de mRNA de UCP2 (”uncoupling protein 2”) em HD-iPSC e HD-NSC. De forma a avaliar a resposta antioxidante, analisámos ainda os níveis proteicos e de mRNA do Nrf2 (”nuclear factor erythroid 2–related factor 2”) e da subunidade catalítica da enzima gama-glutamilcisteína ligase (GCLc) e heme oxigenase 1 (HO-1). Não foram encontradas alterações nos níveis de mRNA ou proteicos de Nrf2, apesar de se ter verificado uma tendência para um aumento da sua fosforilação no resíduo de serina 40 (P(Ser40)-Nrf2) em células HD-iPSC, sugerindo uma resposta ao aumento de ROS. Verificou-se ainda um aumento da expressão de GCLc nas HD-iPSC. Os resultados demonstram que a alteração transcricional de proteínas envolvidas na biogénese mitocondrial e de subunidades de complexos mitocondriais, assim como elevados níveis de ROS, a que se associa um aumento de GCLc e uma redução de UCP2, constituem potenciais eventos iniciais envolvidos na patogénese da HD.

keywords Huntington's disease, iPSC, NSC, transcription, mitochondria, oxidative stress

abstract

Huntington´s disease (HD) is an autosomal dominant neurodegenerative disorder caused by an abnormal expansion of CAG repeats in the *HTT* gene. Clinical manifestations of the disease include motor, cognitive and psychiatric changes, and currently there is no cure for HD. The use of induced-pluripotent stem cell (iPSC) and neural stem cell (NSC) provide reliable models to study early events involved in HD neurodegeneration.Thus, in this study we aimed to determine changes in transcripts related with mitochondrial biogenesis and the response to oxidative events in HD-iPSC and HD-NSC, when compared with the respective control cells. We investigated the expression of the transcription factor peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and its downstream targets, namely TFAM (mitochondrial transcriptional factor A) and subunits of mitochondrial complexes, and enzymes that regulate the activity of pyruvate dehydrogenase (PDH) in both HD-iPSC and HD-NSC. Our analysis revealed reduced mRNA levels of PGC-1α, TFAM and mitochondrial and nuclear-encoded complex III subunits (CYC1, MT-CYB and UQCR10) and enhanced mRNA levels of ND1 subunit of complex I and PDH kinase 1 (PDK1) in HD-iPSC. Interestingly, apart from PGC-1α, unchanged mRNA levels of other targets were observed in HD-NSC. In both HD-iPSC and HD-NSC we observed increased levels of hydrogen peroxide, a reactive oxygen species (ROS), but unchanged acetylation at Lys68 of superoxide dismutase 2 (SOD2 or Mn-SOD); nevertheless, we observed a significant reduction in the expression of uncoupling protein 2 (UCP2) in HD-iPSC and HD-NSC. To evaluate the antioxidant response, we further measured protein and mRNA levels of nuclear factor erythroid 2–related factor 2 (Nrf2) and two of its downstream targets, namely γ-glutamylcysteine ligase catalytic subunit (GCLc) and heme oxygenase 1 (HO-1). We did not find changes in mRNA expression nor protein levels of Nrf2, but we observed a tendency for increased Nrf2 phosphorylation at Ser40 (p-Nrf2) in HD-iPSC, suggesting a slight response to ROS. Concomitantly, we found an increase in GCLc expression in HD-iPSC. Our results evidence reduced transcriptional changes associated with decreased mitochondrial biogenesis and complexes subunits, as well as enhanced ROS levels linked to increased GCLc and reduced UCP2 as potential early events in HD pathogenesis.

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LIST OF ABBREVIATIONS

ΔΨm	Mitochondrial membrane potential
3-NP	3-Nitropropionic acid
AP-2	Assembly protein complex-2
AMPK	AMP-activated kinase
AMP	Adenosine monophosphate
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ARE	Antioxidant response elements
BDNF	Brain-derived neurotrophic factor
CAT	Catalase
CREB	cAMP-responsive element binding
Cul3	Cullin-3
$O_2^{\bullet -}$	Superoxide anion
Drp1	Dynamin related protein 1
ER	Endoplasmic reticulum
ESC	Embryonic stem cells
ETC	Electron transport chain
HO-1	Heme oxygenase 1
hESC	Human embryonic stem cells
GCLc	γ-Glutamylcysteine catalytic heavy chain
GCLm	γ-Glutamylcysteine regulatory light chain
GPx	Gutathione peroxidase
GST	Glutathione S-transferase
GSSG	Oxidized glutathione

GSH	Reduced glutathione
GRed	Glutathione reductase
H_2O_2	Hydrogen peroxide
HTT	Huntingtin protein
HTT	Human huntingtin gene
Htt	Mouse huntingtin gene
HD	Huntington's disease
iPSC	Induced pluripotent stem cells
Keap1	Kelch like ECH associated protein 1
mHTT	Mutant huntingtin protein
mtDNA	Mitochondrial DNA
mRNA	Messenger RNA
MSN	Medium spiny neurons
MAPK	Mitogen activated protein kinase
NPC	Neural precursor cells
Nrf2	Nuclear factor erythroid 2–related factor 2
NRF1/2	Nuclear respiratory factor ½
NAD+	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NSC	Neural stem cells
NMDA	*N*-methyl-D-aspartate
nDNA	Nuclear DNA
OCR	Oxygen consumption rate
O_2	Molecular oxygen
OXPHOS	Oxidative phosphorylation

PDK1/2/3/4	Piruvate dehydrogenase kinase 1/2/3/4
PDC	Pyruvate dehydrogenase complex
PDP1/2	Pyruvate dehydrogenase phosphatase 1/2
PDH	Pyruvate dehydrogenase
PGC -1α	Peroxisome proliferator-activated receptor gamma coactivator-1α
$PKC_{l/\delta}$	Protein kinase C (L/δ)
PolyQ	Polyglutamine
Prx1/2/6	Peroxiredoxin 1/2/6
$ONOO^-$	Peroxynitrite
ROS	Reactive oxygen species
ROOH	Organic peroxide
Sirt1	Sirtuin 1
SOD1/2/3	Superoxide dismutase 1/2/3
Taldo1	Transaldolase 1
TFAM	Mitochondrial transcription factor A
t-BHQ	tert-Butylhydroquinone
TGF-β	Transforming growth factor β
Trx	Thioredoxin
TrxR	Thioredoxin reductase
UPS	Ubiquitin-proteasome system
UCP1/2/3/4/5	Uncoupling protein 1/2/3/4/5
Wnt	Wingless
WT	Wild-type

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1 - INTRODUCTION

1.1- Huntington´s disease

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder first described by George Huntington as a "Hereditary Chorea" in 1872. HD affects approximately 1 in 10,000 individuals in most of European population (Krawczak et al. 1991). It is characterized by motor disturbance, cognitive impairment and psychiatric manifestations (Martin and Gusella 1986). Although not all the patients develop the same symptoms, one of the HD hallmark are distinctive choreoathetoid movements (hyperkinesia). In the majority of HD patients, symptoms begin between the age of 40-50 and tend to deteriorate with the progression of the disease (Myers 2004). Until 1993, the origin of HD was unknown, but in that year *The Huntington´s Disease Collaborative Research Group* (1993) was able to describe, for the first time, the definite genetic mutation causative of HD. The *HTT* gene in chromosome 4 encodes for the protein huntingtin (HTT) protein, which normally presents up to 35 CAG repeats in the exon 1. In HD, an abnormal expansion of CAG repeats leads to the production of aberrant form of HTT protein (stretch of polyglutamines (polyQ)) (Figure 1.1), which interferes with the regular function of the protein in multiple cell sites. Healthy individuals contain between 11 to 34 CAG repeats whereas individuals at risk of HD present a length of 35 to 39 CAG repeats and tend to have HD clinical manifestation late in life (late onset). CAG repeats higher than 39 correspond to a fully-penetrant mutation with symptoms occurring at middle age. In rare cases of juvenile forms, the polyQ stretch is larger than 60 glutamines, accelerating the disease progression and symptoms appearance. For such high number of glutamines, the size of polyQ and the age of onset (juvenile *versus* late-onset) was shown to be inversely correlated (Gusella and MacDonald 2006). In patients with more than 50 CAG repeats, the correlation with age onset is higher although the triplet repeat only explains less than 50% of age of onset variations (Stine et al. 1993). This correlation is not so strong for individuals with polyQ stretch between 35 and 50, for whom the age of onset is explained in only 10% of the cases by the repeat size (The Huntington's Disease Collaborative Research Group et al., 1993, Stine et al. 1993, Duyao et al. 1993). The weaker correlation of late onset and the size of polyQ stretch might be due to environmental factors. Therefore, patients with small extended polyQ stretch may be more susceptible to these factors, triggering the disease and influencing the age of symptoms onset (Wexler et al. 2004).

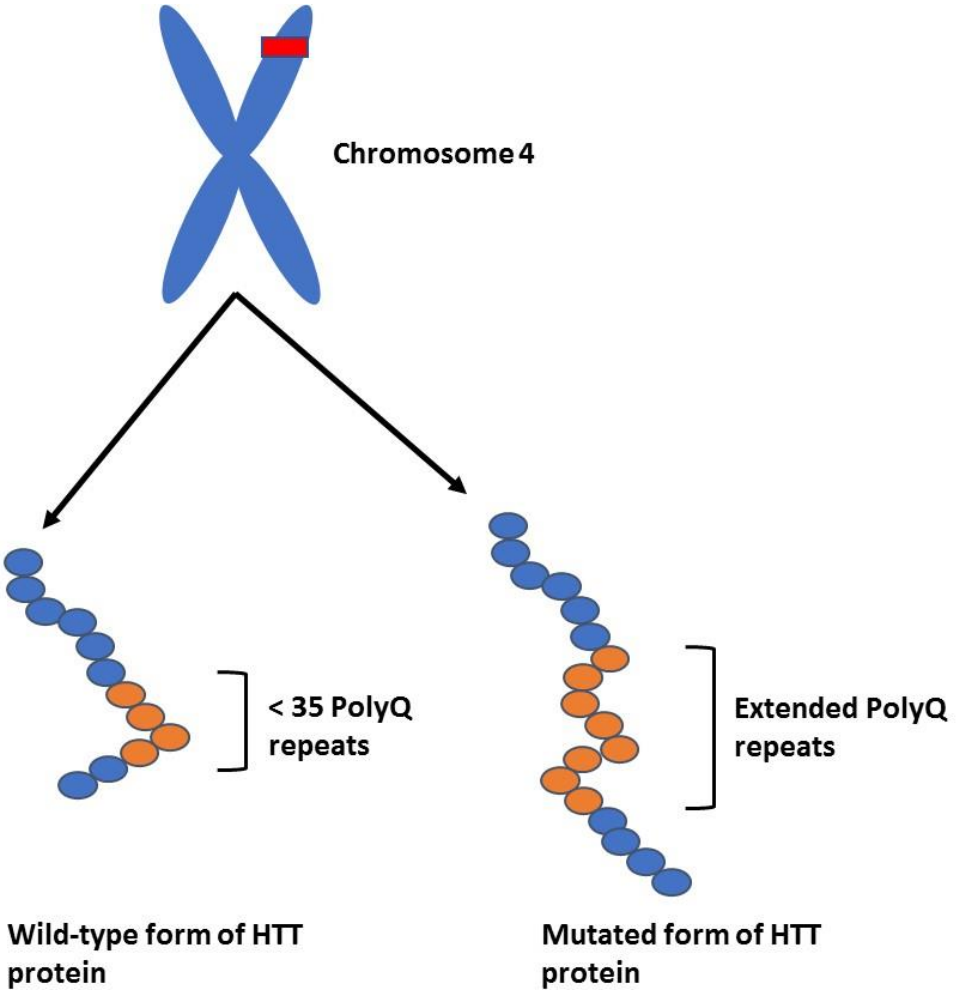

Figure 1.1 – Representation of aberrant form of huntingtin protein with a stretch of polyglutamine associated with Huntington's Disease.

In early stages of the disease there is a progressive degeneration of the basal ganglia specifically the striatum nucleus (putamen and caudate) and the cortical regions. The loss of medium spiny projection neurons interferes with the indirect pathway of movement, which ultimately leads to thalamic inhibition and motor symptoms such as hyperkinesia (Rosas et al. 2002). The impairment of motor control and the involvement of HTT in HD is consistent with reports that both cortex and striatum express higher levels of HTT when compared to other regions such as hypothalamus and brainstem (Li et al. 1993, Sharp et al. 1995). The exact mechanism that leads to the preferential neurodegeneration of neurons in the striatum and cortex over neurons in other brain areas is unknown, although some hypotheses have emerged to explain this phenomenon. According to the "neurotrophin disorder hypothesis" (Zuccato and Cattaneo 2009), the specific neurodegeneration occurs due an impairment of the neurotrophin BDNF (brain-derived neurotrophic factor), which is produced in cortical

afferent neurons and transmitted to striatal neurons, and thus required for striatal neuronal survival (Zuccato et al. 2001). BNDF is an important modulator of neurogenesis, especially in adult olfactory bulb, striatum and thalamus (Zigova et al. 1998, Benraiss et al. 2001, Pencea et al. 2001). In addition, BNDF is thought to participate in proliferation (Katoh-Semba et al. 2002), although it may also be relevant in promoting the survival of newly generated neurons (Lee, Duan, and Mattson 2002). The reduction of wild-type (WT) HTT, which is involved in axonal transport, decreases the transport of BDNF, leading to striatal and cortical neurodegeneration in HD (Gauthier et al. 2004). Indeed, BNDF has also a cytoprotective role in the brain as previously described by our group, by preventing apoptosis induced by caspase-3 activation, and decreasing cytochrome c release and nuclear fragmentation in cortical neurons treated with 3-nitropropionic acid (3-NP), an irreversible inhibitor of succinate dehydrogenase, that mimics HD pathology (Almeida et al. 2009). Additionally, the cytoprotective effect of BDNF was demonstrated in HD knock in mouse striatal cells (ST*Hdh*$^{Q111/Q111}$) (Silva et al. 2015).

Another hypothesis, the “excitotoxicity hypothesis” is based on the overactivation of extrasynaptic N-methyl-D-aspartate (NMDA) glutamatergic receptors in specific neuronal subpopulations (Hardingham and Bading 2010, Zeron et al. 2002) and on impairment in glutamate handling (Behrens et al. 2002). This hypothesis suggests that medium spiny neurons, but not interneurons, which are spare in HD, are sensible to an increase of glutamate receptor stimulation leading to an hyperactivation of NMDA receptors and consequent neuronal death (Calabresi et al. 1998).

Lastly, the “mitochondrial dysfunction and energetic deficits” hypothesis postulates that impairment of respiratory chain and in enzymes related to energy metabolism are relevant intiating factors in HD. This hypothesis is supported by a reduction in mitochondrial complexes activities in post mortem samples of caudate/putamen derived from HD patients (Browne et al. 1997, Gu et al. 1996) and a reduction in aconitase activity in both caudate and putamen (Sorolla et al. 2008, Tabrizi et al. 1999).

1.1.1 – The huntingtin protein – wild-type and mutant forms

HTT is an ubiquitously expressed protein, enriched in the brain (Trottier et al. 1995, Fusco et al. 1999, Block-Galarza et al. 1997). The intracellular location of this protein in neurons includes dendrites, cell bodies and axons (Sharp et al. 1995). The biological functions of HTT are still under debate, but has been associated to axonal

intracellular trafficking, endocytosis, membrane recycling and synaptic communication (Engqvist-Goldstein et al. 2001, Wu 2004, McMahon and Mills 2004). Early attempts to inactivate the *Htt* gene, using a mouse homolog, *Hdh*, demonstrated that the deletion of the gene is lethal during early stages of development (Dragatsis, Efstratiadis, and Zeitlin 1998). HTT protein participates in the transport of nutrients across extraembryonic membranes contributing to the development of the embryo and upon inactivation induces embryonic lethality (Duyao et al. 1995, Nasir et al. 1995, Zeitlin et al. 1995). Therefore, inactivation of Htt expression seems to affect not only migration, proliferation and survival in several brain regions, but also mitotic spindle orientation (Godin et al. 2010). Thus, some brain areas seem to rely more on Htt than others to complete differentiation, turning them more susceptible to neurodegeneration in HD (Reiner et al. 2001).

The HTT protein has also a neuroprotective role by preventing apoptotic neuronal death. This protein protects from pro-apoptotic signals induced by Bik and Bak proteins, as well as apoptosis induced by caspase-9, acting upstream of caspase-3, suggesting an interference on caspase-9 activation (Rigamonti et al. 2000, Zhang et al. 2006, Luo and Rubinsztein 2009). Increased cell death associated with caspase-3 activation in HD was reported in striatal cells expressing mHTT (Rosenstock et al. 2011).Additionality, the loss of WT HTT may also contribute to HD pathology, by reducing its neuroprotective role (Ho et al. 2001, Leavitt et al. 2001, Leavitt et al. 2006, Zhang et al. 2003, Rigamonti et al. 2000). HTT is a substrate of multiple proteases, includingcaspases, calpain and endopeptidases (Kim et al. 2001, Gafni and Ellerby 2002, Goldberg et al. 1996). Previous reports indicate that WT HTT decreases the cleavage of full-length mutant HTT (mHTT) and thereby reduces the apoptotic process induced by the toxic fragment (Ho et al. 2001). Thus, overexpression of WT HTT ameliorates the clinical manifestation in transgenic YAC128 mouse model; however, overexpression alone is not capable of reversing HD progression (Van Raamsdonk et al. 2006). Similar results were obtained by Leavitt and co-authors in the same transgenic mice model, where an increase in WT HTT reduced the formation of mHTT fragments and subsequent apoptotic cascade (Leavitt et al. 2001). The loss of HTT levels has been also shown to affect striatal size and mice life-span, when compared with mice with normal WT HTT protein levels (Van Raamsdonk et al. 2005, Leavitt et al. 2001). Therefore, a reduction in the expression of WT HTT can have a role in disease progression.

mHTT is known to interact with different proteins and organelles in an irreversible manner and acquires toxic properties (Gauthier et al. 2004, Landles and Bates 2004, Wanker et al. 1997), as illustrated in Figure 1.2. Among the caspases that target mHTT protein, caspase-6 is responsible for originating cytotoxic N-terminal fragments with 586

amino acids (Graham et al. 2006, Wong et al. 2015, Aharony et al. 2015)that tend to aggregate forming intranuclear and cytoplasmic inclusions (Davies et al. 1998). The formation of intracellular aggregates can be a cellular defence mechanism against misfolded protein. These proteins impair the proteasome forming aggregates, inducing cell sequestration to probably avoid toxicity. Accordingly, it seems that the activation of the ubiquitin-proteasome system (UPS) is unable to fully respond to protein aggregation (DiFiglia et al. 1997, Wang et al. 2008). A study conducted by DiFiglia (1997) observed a relationship between the location of mHTT aggregates and polyQ length. mHTT with longer polyQ presented predominantly nuclear inclusions, whereas the most common form, with lower polyQ repeats, showed more prevalent aggregates in the cytoplasm (DiFiglia et al. 1997). Overexpression of mHTT has been shown to reduce the intracellular trafficking, which compromises neuronal viability (Yanai et al. 2006). The increased number of protein aggregates composed by mHTT toxic fragments, monomers and oligomers can recruit WT HTT from the cytosol into the nucleus and to protein inclusions, which might contribute to the loss of axonal transport in HD (Cattaneo et al. 2001, Wheeler et al. 2000). Moreover, these aggregates can induce endoplasmic reticulum (ER) stress by inhibiting the ubiquitin-proteasome system, activating ER-stress dependent apoptosis and leading to neuronal degeneration (Bence, Sampat, and Kopito 2001, Nishitoh et al. 2002, Kouroku et al. 2002). Apoptosis activation may occur in early stages or even in a pre-symptomatic stage of the disease, triggered by the cleavage of mHTT by caspase-6 and the resultant cytotoxic fragment with 586 amino acids. Classically, the mutation associated to HTT is assumed to be implicated in several processes that culminate in neurodegeneration and in the clinical features of HD (Figure 1.2). Possibly, the loss of function of WT HTT plus the gain of toxic function of mHTT play key roles in HD pathogenesis and contribute to selective neuronal degeneration in HD.

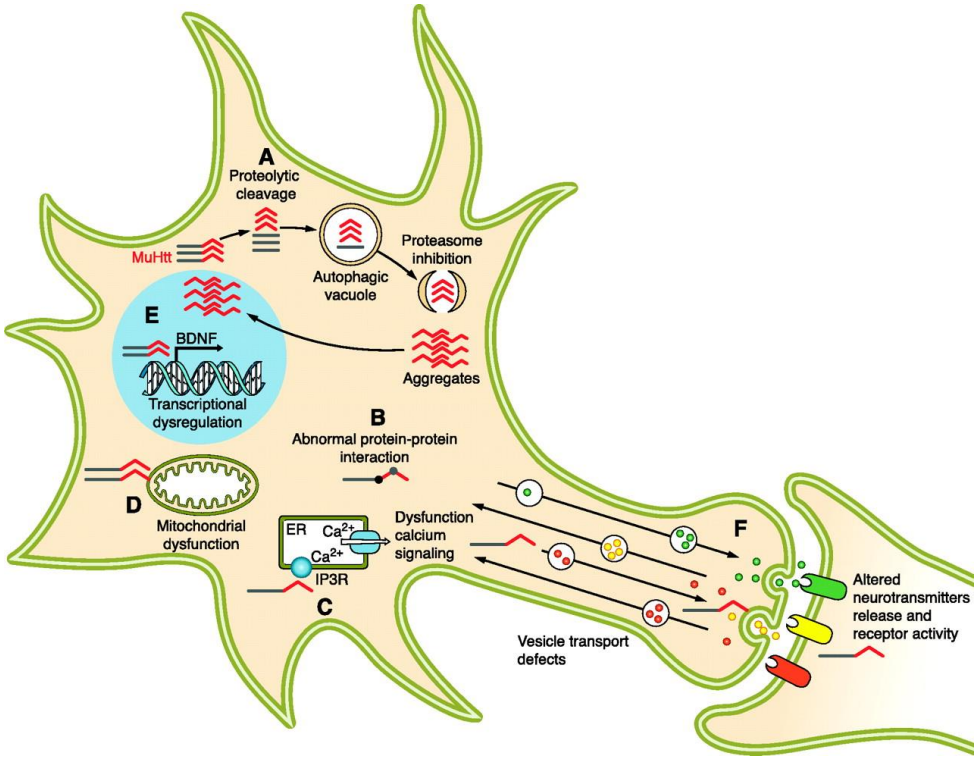

Figure 1.2 – Molecular Mechanisms implicated in Huntington´s disease. A – Abnormal HTT formation causes protein aggregation due to impaired protein misfolded clearance. B – Alterations in mHTT protein conformation alters protein interactions and acquire new functions. C – mHTT fragments containing the poly Q stretch can cause impairment of calcium handling due abnormal interaction with inositol 1,4,5-triphosphate receptor 1 in the endoplasmatic reticulum which promotes calcium release this organelle. D – mHTT causes direct mitochondrial dysfunction through direct binding or indirectly by affecting mitochondrial biogenesis. E – The cytotoxic fragments are cleaved in the cytosol in an attempt to restrain the full-lenght mHTT-induced cytotoxicity can translocate into the nucleus and interfere with transcriptional activity of different genes. F – mHTT also interferes with vesicle transport and with neurotransmitters release (Zuccato, Valenza, and Cattaneo 2010)

1.2 – An overview of induced-pluripotent stem cells as HD cell models

Induced pluripotent stem cells (iPSC) are pluripotent stem cells first generated by Takahashi, by reprogramming human differentiated cells into a pluripotent state through the use of four transcription factors, namely OCT4, SOX2, c-Myc and Klf4 (Takahashi et al. 2007). iPSC were demonstrated to have similar properties as human embryonic stem cells (hESC) regarding morphology, gene expression and proliferation rate (Takahashi et al. 2007). Since the first generation of iPSC, several attempts were made to reproduce age-associated neurological diseases. The first successful generation of iPSC from HD patients was achieved by Park (Park et al. 2008), by reprogramming patient's fibroblasts

with 72 CAG repeats into iPSC. In this work, Park and colleagues differentiated HD-iPSC into neural precursor cells (NPC). Later in 2010, Zhang and co-workers characterized this HD-iPSC model and stablished the first protocol to convert HD-iPSC into medium spiny neurons (Zhang et al. 2010). In this report, HD iPSC-derived NSC showed an increase on caspase 3/7 activity and altered ERK activation. Thereafter, a study conducted in several HD-iPSC lines with different CAG repeats demonstrated that the CAG length is not affected during reprogramming and neither the growth rate or neuronal differentiation, however an increase in lysosomal activity was observed (Camnasio et al. 2012). An increase in lysosomal activity was also demonstrated by another group in neurons derived from HD-iPSC (Nekrasov et al. 2016).

In 2012 the HD consortium generated several iPSC lines derived from HD patients and further characterised these cells. This study indicated alterations in cell-to-cell adhesion, energetic defects (e.g. decreased adenosine triphosphate and adenosine diphosphate ratio (ATP/ADP) levels), reduced actin filaments and altered electrophysiological properties correlated with CAG repeats (Consortium 2012). Alterations in spindle orientation have also been described in NSC derived from HD-hESC and altered distribution within the spindle pole of HTT protein interactors, namely dynein, $p150^{Glued}$ and NuMA (Lopes et al. 2016).

Another study addressed the importance of BDNF on medium spiny neurons and showed that HD iPSC-derived NPC are sensible to BDNF withdrawal, as shown by an increase on excitotoxicity induced by glutamate (Mattis et al. 2015). This report was consistence with previous observations of an increased susceptibility to cell stressors of HD-derived cell lines (Consortium 2012).

Moreover, several pathways were shown to be dysregulated in HD-iPSC, including mitogen activated protein kinases (MAPK), wingless (Wnt) and transforming growth factor β (TGF-β) signaling, as well as altered expression of p53 (An et al. 2012, Chae et al. 2012, Ring et al. 2015, Consortium 2012, Szlachcic et al. 2015). These results corroborate previous reports of altered signaling in early stages of the disease and further support the use of HD-iPSC model for the study of HD pathogenesis.

In a recent paper, the HD iPSC Consortium indicated that several genes involved in striatal neural maturation were altered in HD-iPSC lines, which could compromise neural differentiation (Consortium 2017).

Regarding oxidative-stress and mitochondrial dysfunction, Chae and co-workers (2012) observed in a proteomic analysis of HD-iPSC at undifferentiated stages, an upregulation of 26 proteins associated to oxidative stress. Among these proteins are peroxiredoxin 1, 2 and 6 (Prx1, Prx2 and Prx6, respectively), proteins involved in

antioxidant cellular response. Other proteins, such as superoxide dismutase 1 (SOD1 or Cu/Zn-SOD), glutathione S-transferase (GST) and glutathione peroxidase (GPx) were downregulated in HD-iPSC (Chae et al. 2012), being strong indicators of increased levels of reactive oxygen species (ROS). Later, another study reported an upregulation of SOD1, but unchanged Prx1 messenger RNA (mRNA) levels in HD-iPSC derived from a juvenile HD patient with 109 CAG repeats (Szlachcic et al. 2015). Defects in calcium handling were also identified following glutamate stimuli in low repeats (37 and 51 CAGs) ESC-derived forebrain neuronal cultures (Niclis et al. 2013). Notably, Guo and colleagues identified alterations in mitochondrial dynamics, a decrease in mitochondrial membrane potential (ΔΨm), energetic defects and an increase in ROS production in HD iPSC-derived neurons (Guo et al. 2013). The authors observed an increased mitochondrial fragmentation associated with hyperactivation of dynamin-related protein (Drp1), a protein related with mitochondrial fission, which may impair mitochondrial functionality. In the same study, an increase in mitochondrial ROS production was also observed in several HD cell lines, including HD iPSC-derived medium spiny neurons (Guo et al. 2013). Thus, metabolic defects such as decreased ATP levels, along with alterations on ΔΨm may result from the metabolic shift that iPSC face during reprogramming (Varum et al. 2011). iPSC seem to rely more on glycolysis than oxidative phosphorylation for metabolic outcome, favouring a decrease in ΔΨm that is essential to prevent full functionality and promote the glycolytic pathway. Although these alterations in ΔΨm and energy production have been observed in WT iPSC, in HD-iPSC these alterations are more pronounced and may indicate mitochondrial defects at early stages of the disease.

Some research groups focused on the use of iPSC for cell replacement therapy, while others attempted to genetically correct HD-iPSC and reverse the phenotype prior to autologous cellular therapy. An and co-workers generated the first HD-iPSC corrected model resorting to homologous recombination (An et al. 2012). Upon correction, the HD-iPSC model reverted the alterations found in signaling pathways, proliferation and changes in cadherin and TGF-β family genes mRNA when compared with uncorrected HD-iPSC. Neural stem cells (NSC) derived from corrected HD-iPSC also decreased caspase 3/7 activity and improved mitochondria bioenergetics shown by an increase in oxygen consumption rates (OCR) when compared to NSC derived from non-corrected HD-iPSC (An et al. 2012). Transplantation of corrected HD iPSC-derived NSC into the striatum of 6 weeks old R6/2 mice demonstrated the capacity to repopulate the striatum with medium spiny neurons (MSN) (An et al. 2012). These results seemed promising when considering the therapeutic use of iPSC. Nonetheless, some problems occurred,

namely as the formation of mHTT aggregates in differentiated neurons derived from iPSC after 12 weeks transplantation, suggesting that this approach alone is not enough (Jeon et al. 2012, Jeon et al. 2014). The exact mechanism that leads to the formation of aggregates in the neurons derived from transplanted NPC was not identified, although non-cell autonomous communication may play a role in the transmission between host-to-graft (Jeon et al. 2016).

HD also affects glial cells, as shown by increased cytoplasmic vacuolation in astrocytes in the absence of stressors (Juopperi et al. 2012). The increase of vacuolation was also observed in lymphoblasts from peripheral blood of HD patients, indicating that although medium spiny neurons are mainly affected in HD, other cells outside the central nervous system also face alterations induced by mHTT(e.g. Nagata et al. 2004). Some of the discoveries made in patient's derived HD-iPSC indicate altered cell growth and cell adhesion, oxidative stress, impaired protein clearance and changes in signaling pathways. Overall, these observations reveal changes that occur in early pre-symptomatic stages of the disease. Summing up, increasing evidences are emerging that transplantation of iPSC alone is not capable of curing the disease, and thus possible future treatments must combine gene therapy with iPSC-derived NPC transplantation.

1.3 – Mitochondria: From physiological function to HD impairment

1.3.1 – Mitochondria as the powerhouse of cells

Mitochondria are subcellular organelles in eukaryotic cells involved in different functions aiming to maintain cellular homeostasis. These organelles have been implicated in several cellular processes such as apoptosis, autophagy, immunity, ROS and ATP production, thermogenesis, calcium homeostasis (for review (McBride, Neuspiel, and Wasiak 2006, Prasai 2017, Dejean et al. 2006)). Fatty acid oxidation, calcium storage and energy production are among the vast identified roles of mitochondria. In mammals, the main function of mitochondria is energy production in aerobic conditions, by converting ADP to ATP through oxidative phosphorylation (OXPHOS). This process is based on the transfer of electrons among mitochondrial respiratory chain complexes I to IV, from a donor to a final acceptor, molecular oxygen (O_2). The transfer of electrons between complexes is an exergonic process that provides energy for proton pumping from the matrix into the inter-membrane space across the mitochondrial inner membrane. The re-entrance of protons in the mitochondrial matrix through ATP synthase (complex V) generates a proton motive force allowing the

production of ATP. The ΔΨm generated by the electron flow coupled to the proton flux across the mitochondria inner membrane allows for a more efficient production of ATP than other metabolic pathways, such as glycolysis.

1.3.2 – Intracellular sources of reactive oxygen species

In physiological levels, ROS have a beneficial effect on cells specially in antimicrobial defence, cell signaling, and immunity (for review (Sena and Chandel 2012)). By contrast, increased levels of ROS induce damage in enzymes, membrane lipids and nucleic acids due to its high reactivity. Mitochondria is the main source of ROS, through complex I and III (Duchen 2004, Cui et al. 2006), however other intracellular sources exist like xanthine oxidase in the cytosol (Ardan, Kovaceva, and Cejková 2004, Kelley et al. 2010). During electron transport chain (ETC), ROS are formed by the reaction of oxygen with a 'leaking' electron, originating the superoxide anion ($O_2^{\bullet-}$), the main source of ROS in mitochondria. Alterations in the reduction state of the electron's donors, proton gradient and ΔΨm trigger ROS production. Particularly, in tissues that rely specially on mitochondria as the main supplier of energy, such as neurons, the increase in ROS production can cause severe damage, ultimately inducing apoptosis and degeneration. In fact, unaltered ROS levels and decreased ΔΨm was previously reported in our laboratory in peripheral blood cells from HD patients (Almeida et al. 2008), however, in human HD-iPSC, an increase in ΔΨm linked to ATP synthase reversal was observed (*Lopes C., PhD thesis, 2015*), which may be indicative that mitochondrial modifications and altered ROS production are part of the mechanisms involved in HD pathological features.

Mitochondrial dysregulation has been consistently reported in HD pathogenesis. Alterations in mitochondrial complex II activity were initially reported in the 90's in post-mortem analysis of the striatum (caudate nucleus) in HD patients (Mann et al. 1990, Browne et al. 1997). The use of mitochondrial complex II irreversible inhibitor, 3-NP, reproduced neuropathological features of HD by the selective degeneration of MSN (Beal et al. 1993). The loss of complex II activity was also described both in *in vitro* and *in vivo* HD models (Stahl and Swanson 1974, Gu et al. 1996, Turner, Cooper, and Schapira 2007, Benchoua et al. 2006). Defects in mitochondrial complexes activity also extend to complexes I, III and IV (Gu et al. 1996, Browne et al. 1997). In R6/2 HD mouse model a reduction in complex IV activity was observed (Tabrizi et al. 2000). Similar results were obtained in R6/2 mice after 3-NP injections (Bogdanov et al. 1998). In the same study, an increase of ROS production was observed (Bogdanov et al. 1998).

Impairment of complex I and IV and a decrease in ATP synthase activity was found by Napoli and collaborators (2013) in homozygous and heterozyous HD knock in mouse striatal cells (ST*Hdh*$^{Q111/Q111}$ and ST*Hdh*$^{Q111/Q7}$).

1.3.3 – Relevance of transcriptional activity in cellular redox homeostasis and glucose metabolism

Mitochondrial biogenesis involves extensive coordination between mitochondrial and nuclear genome, the latter required for replication of mitochondrial DNA (mtDNA). Peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) is a key transcriptional regulator that plays a role not only in mitochondrial biogenesis, but also in thermogenesis, fatty acid oxidation and glucose metabolism (Puigserver and Spiegelman 2003, McGill and Beal 2006). The transcription of mitochondrial genes is tightly regulated by the interaction of PGC-1α with two nuclear transcriptional factors, nuclear respiratory factor 1 and 2 (NRF1 and NRF2, respectively) (Uittenbogaard and Chiaramello 2014). These downstream effectors enhance the expression of mitochondrial complexes by the interaction with nuclear genes that encode mitochondrial complexes subunits. NRF1 and NRF2 also regulate other nuclear-encoded mitochondrial proteins, essential for mitochondrial biogenesis, thermogenesis and antioxidant response (Uittenbogaard and Chiaramello 2014). Upstream, several pathways have been implicated in the regulation of PGC-1α, by controlling its translocation to the nucleus, through phosphorylation or acetylation. Some studies report that certain stimuli such as exercise or starvation activates the adenosine monophosphate (AMP)-activated kinase (AMPK), which is activated by enhanced intracellular AMP/ATP levels, and directly phosphorylates PGC-1α, engaging its translocation to the nucleus and promoting mitochondrial biogenesis (Birkenfeld et al. 2011, Jäger et al. 2007, Cantó and Auwerx 2009). AMPK is also capable of indirect activation of PGC-1α via Sirtuin1 (Sirt1). AMPK upregulates the intracellular levels of NAD+, a regulator of Sirt1 activity. When active, Sirt1 promotes deacetylation of PGC-1α and activates the downstream pathway (for review (Li, Hou, and Hao 2017)). Another important transcription factor is the mitochondrial transcription factor A (TFAM). This transcription factor acts as a downstream effector of PGC-1α involved in mitochondrial biogenesis through regulation of mtDNA copy number and ,consequently, the regulation of mitochondrial-encoded complex subunits (Ekstrand et al. 2004, Ribeiro et al. 2014). TFAM is an abundant protein in mitochondria required for maintenance of normal levels of mtDNA and may play a role in organizing and compacting mtDNA (Larsson et al.

1998). This close relationship between nuclear DNA (nDNA) and mtDNA is important to regulate and maintain mitochondrial function and emphasizes the importance of transcription in the context of cellular homeostasis.

A link between PGC-1α and HD has been stablished. Some groups identified single nucleotide polymorphisms in the PGC-1α gene associated with HD age of onset (Taherzadeh-Fard et al. 2009, Che et al. 2011, Weydt et al. 2009). Similar conclusions were obtained for the downstream effectors of PGC-1α, NRF-1 and TFAM (Taherzadeh-Fard et al. 2011). These reports point a strong relation between HD pathology and mitochondrial transcriptional dysregulation, reinforcing the mitochondrial involvement in HD neuropathology.

Interaction of mHTT with transcription factors seems to interfere with normal transcriptional activity. Aberrant form of the HTT protein represses CRE-mediated transcriptional activity of PGC-1α in HD mouse striatal cells ST*Hdh*$^{Q111/Q111}$ (Cui et al. 2006). The downregulation of PGC-1α was also observed in brown adipose tissue, altering thermogenic response, and in the striatum of transgenic HD mouse model (N171-82Q) (Weydt et al. 2006, Chaturvedi et al. 2010). PGC-1α knockout mice presented movement disorders, mitochondrial dysfunction and striatal degeneration that mimicked HD pathology (Lin et al. 2004). Moreover, overexpression of PGC-1α rescued neurodegeneration induced by mHTT (Chiang et al. 2010). Knockdown of mHtt protein also affected the transcriptional activity by enhancing PGC-1α and its upstream regulators in striatal cells ST*Hdh*$^{Q111/Q111}$ (Chaturvedi et al. 2012). Administration of resveratrol, a Sirt1 promoter, was shown to have a neuroprotective role in ST*Hdh*$^{Q111/Q111}$ by enhancing PGC-1α activity and reducing neuronal death (Parker et al. 2005). Moreover, *in vitro* experiments using isolated striatal MSN derived from HD knock-in mice with 140 CAGs showed a decrease in PGC-1α mRNA levels, whereas in striatal interneurons it was upregulated (Cui et al. 2006). These lines of evidence support the selective neurodegeneration of MSN and the transcriptional implications in HD pathogenesis.

At the transcriptional level, downregulation of PGC-1α pathway can also have a significant impact on OXPHOS by regulating the mitochondrial complexes biogenesis, and promoting the bioenergetic metabolic shift towards fatty acid oxidation (Gleyzer, Vercauteren, and Scarpulla 2005, Feige et al. 2008, Hallows, Lee, and Denu 2006). Pyruvate dehydrogenase (PDH) or pyruvate dehydrogenase complex (PDC) is composed by four components: PDH itself (E1 subunit); the dihydrolipoyl transacetylase (E2 subunit); the dihydrolipoamide dehydrogenase (E3 subunit) and the E3-binding protein (Jha and Suk 2013). This complex catalyzes the irreversible conversion of

pyruvate to acetyl coenzyme A, stablishing an interface between glycolysis, the TCA cycle and OXPHOS (Harris et al. 2002). The complex is tightly regulated by phosphorylation/inactivation at the PDH (E1) subunit at three serine residues (Ser232, Ser293, and Ser300); phosphorylation at any of these sites causes decreased PDH activity (For review (Zimmer et al. 2016)). In humans, the pyruvate dehydrogenase kinase (PDK), responsible for PDH phosphorylation, is composed by 4 isoforms, PDK1-4 that are widely distributed, and each isoform respond to different stimuli (Bowker-Kinley et al. 1998). PDK1 is the most abundant isoform and is responsible for reversal phosphorylation of PDH_E1 subunit at the three Ser sites (Kolobova et al. 2001). Dephosphorylation of PDH_E1 subunit at the serine residues described above is catalyzed by pyruvate dehydrogenase phosphatases 1-2 (PDP1-2), which promote enzyme activation and favour OXPHOS (Bowker-Kinley et al. 1998). The modulation of glucose metabolism by these enzymes in starvation or fasting and at a transcriptional level is very important for the cell to guarantee its survival and avoid energy depletion (Gudiksen et al. 2016). Under stress signal, PGC-1α response also induce alterations in energy production by promoting the expression of PDK4 (Gudiksen and Pilegaard 2017). Downregulation of transcriptional factors such as PGC-1α, as described above, can contribute to HD pathology at different levels and might explained some of the mitochondrial dysfunctions reported in HD, reaffirming its role in the course of the disease.

1.3.4 – Uncoupling proteins

During the process of OXPHOS, responsible for ATP generation, energy is partially dissipated as heat. Part of this heat is generated through the re-entry of protons into the mitochondrial matrix. This process is partially regulated through uncoupling proteins 1-5 (UCP1-5) by creating a proton leak across the inner mitochondrial membrane that dissipates the proton motive force and reduces ATP formation by OXPHOS, and therefore reducing ROS formation (Fleury et al. 1997, Echtay et al. 2002). UCP family is composed by several members with high homology, but with different location and cellular function. UCP1 is mainly present in brown adipose tissue and is associated with the regulation of thermogenesis upon specific stimuli such as cold exposure (Golozoubova et al. 2001). UCP3 is mainly present in mammalian skeletal muscle and participates in the control of proton flux and ATP generation during rest and exercise, although it may also participate in the dowregulation of ROS generation in this tissue (Erlanson-Albertsson 2003). The poorest described members of this family are UCP4 and UCP5, present in the brain with an unclear function. Nevertheless, one of the most

studied uncoupling proteins is UCP2, which is widely distributed and also present in the brain (Horvath et al. 1999). Previously, it has been suggested that UCP2 has a role in insulin secretion and its activation decreases ROS production (For review (Erlanson-Albertsson 2003)). Additionally, UCP2 can act as a modulator of iPSC metabolism, through the control of metabolites influx in mitochondria and by controlling mitochondrial ROS production (Figueroa et al. 2010). Disruption of ΔΨm may illustrate a potential role for UCP2 in regulating the metabolic shift during differentiation process, as suggested by Zhang and colleagues (Zhang et al. 2011), which further reinforces the role of mitochondria in iPSC reprogramming as reported by our group (Lopes and Rego 2017). Depending on the differentiation state of the cell, different levels of UCP2 can be observed, conferring specific metabolic and mitochondrial properties. Previous reports indicated that UCP2 has anti-apoptotic properties by reducing caspase-3 activity under hypoxia conditions (Mehta and Li 2009, Mattiasson et al. 2003). iPSC seem to regulate the major ATP source through inner mitochondrial depolarization mediated by UCP2. Zhang and co-authors (2011) suggested that UCP2 may have a double function in stem cells, by depolarizing the mitochondria, thus hampering ATP production through OXPHOS, thus promoting glycolysis and limiting the entry of pyruvate into mitochondria. Overall, the metabolic shift in cell differentiation involves alterations in mitochondrial structure and mitochondrial inner membrane depolarization promoted by UCP2 and ATP synthase reversal, as suggested in previous studies (Zhang et al. 2011, Esteves et al. 2014, Pecqueur et al. 2008). These reports are consistent with observations that indicate the preference for ATP production of glycolysis over OXPHOS in stem cells (Varum et al. 2011). During differentiation, UCP2 levels decrease, reducing mitochondrial uncoupling and depolarization, therefore facilitating the transition from glycolysis to OXPHOS and allowing the cells to increase the ATP levels (Zhang, Marsboom, et al. 2014). Like in cancer cells, which present high rates of proliferation and biosynthesis, stem cells also share these properties (Orkin and Hochedlinger 2011, Kang, Shakya, and Tantin 2009). To maintain the pluripotent state, cells produce most of its ATP through glycolysis, which is also responsible for providing anabolic precursors that fuel key metabolic pathways such as the pentose phosphate pathway in order to maintain high proliferative rates and stemness (Prigione et al. 2010, DeBerardinis et al. 2008, Lunt and Vander Heiden 2011). The precursors provided by glycolysis are then used for lipid and nucleotide biosynthesis (Locasale and Cantley 2011).

Several studies describe the upregulation of UCP2 levels when endogenous ROS production is increased, indicating a neuroprotective effect of UCP2 and a role as an oxidative stress modulator, in accordance with the “uncoupling to survive” hypothesis

(Andrews, Diano, and Horvath 2005, Andrews et al. 2005); (Mattiasson et al. 2003). The close relationship between UCP2 and ROS was previously shown in several articles (Pi et al. 2009, Lee, Robson-Doucette, and Wheeler 2009). Knockdown of UCP2 was increased basal ROS levels in macrophages (Lee, Robson-Doucette, and Wheeler 2009). In cancer cells, overexpression UCP2 enhanced the efficient of therapeutics in drug-resistant cells by controlling ROS levels (Derdak et al. 2008). Additionally, it has been shown that deglutathionylation induced by ROS is responsible for the activation of proton leak (Mailloux et al. 2011). Even during physiological conditions part of the energy consumed in mitochondria is due UCP2 activation. This process is thought to promote a partial depolarization of mitochondria and allows the cell to maintain low ROS levels and prevent greater damage. The mechanisms that regulate UCP2 are still under debate, but it seems that different pathways may be involved. Chang and colleagues demonstrated that UCP2 gene expression was downregulated in symptomatic HD patient's leukocytes, which was not detected in pre-symptomatic patients (Chang et al. 2012). It seems that UCP2 gene expression is downregulated in early stages of the disease compromising ROS management. The PGC-1α transcriptional factor could be a potential mechanism to upregulate UCP2 levels under oxidative stress, since PGC-1α activation upregulates UCP2 levels as part of the antioxidant mechanisms (Chan et al. 2009). Thus, other mechanisms have also been implicated in UCP2 activation. The oxidation of GSH by ROS levels is a non-enzymatic reaction that protects from ROS, although GSH can directly react with ROS enhancing the glutathionylation of proteins (Pizarro and Ogut 2009) has also been observed as a process enhanced by increased hydrogen peroxide (H_2O_2), as mentioned by Mailloux and Harper (Mailloux and Harper 2011). The same group also demonstrated that glutathionylation/deglutathionylation modulates proton leak through UCP2 and UCP3, but not UCP1 (Mailloux et al. 2011).

Overall, UCP2 upregulation is thought to participate in the antioxidant response under stress signal, although the mechanisms implicated in this process are not fully understood, ROS levels seem to modulate UCP2 in a negative feedback loop.

1.4 – Antioxidant defences

ROS are by-products of normal metabolic reaction being the mitochondrial ETC the main source. As a response to increased ROS levels, cells activate several mechanisms to decrease cellular oxidative state and limit cell damage. One of the cellular responses to increased levels of ROS is an upregulation of antioxidant enzymes located in the cytosol or in specific cellular compartments, such as mitochondria. Although $O_2^{\bullet -}$ is not a strong oxidant, it is the precursor of most ROS, therefore has a

significant role in oxidative stress. The antioxidant mechanisms incorporate oxidative sensors that are responsible for detecting ROS and mediate intracellular signaling response.

SOD is an enzyme responsible for dismutating $O_2^{\bullet -}$ into H_2O_2. SOD presents different isoforms (SOD1, SOD2 and SOD3), being some organelle-specific, such as SOD2 (Mn-SOD) present in mitochondria, while others, like SOD1 (Cu/Zn-SOD) are mainly present in the cytosol but can also be found in the nucleus and outer mitochondrial membrane (Yonashiro et al. 2009, Marin et al. 2012). SOD3 is the only SOD isoform that can be found in the attatched to the plasma membrane (Fukai and Ushio-Fukai 2011). Prx and Trx are both sensors of H_2O_2 and responsible for the conversion of cytosolic H_2O_2 into water (for review (Rhee et al. 2012)).The H_2O_2 resulting from superoxide dismutation by SOD is converted into water by two different antioxidant enzymes: catalase (CAT) and GPx. The H_2O_2 is detoxified by CAT in the cytosol and peroxisomes (Kirkman and Gaetani 2007), and by GPx in the cytosol and mitochondria (Matés, Pérez-Gómez, and Núñez de Castro 1999) (Figure 1.3). The regulation of GPx is a complex process involving several enzymes. The levels of GSH and oxidized glutathione (GSSG) display a critical role in the regulation of GPx (Forman, Zhang, and Rinna 2009). The conversion of GSSG into GSH is a NADPH-depend process catalysed by glutathione reductase (GRed) (for review,(Deponte 2013)). The GSH/GSSG ratio is a good indicator of the cellular redox homeostasis and antioxidant response to oxidative stress (Benzi et al. 1992). Thus, disruption at the transcriptional level of SOD, CAT, GRed and GPx can jeopardize an effective antioxidant response. Several groups reported a link between PGC-1α pathways and antioxidant response system, possibly through direct regulation of antioxidant enzymes at the transcriptional level (Valle et al. 2005, Marmolino et al. 2010, Olmos et al. 2013). Among the PGC-1α-dependent antioxidant enzymes are SOD2, GPx and CAT (Finkel 2006). Impaired PGC-1α pathways is responsible for the downregulation of CAT, SOD1, GPx and SOD2 gene expression (St-Pierre et al. 2006), which under oxidative stress conditions might compromise the cellular redox homeostasis and contribute to the selective neuronal degeneration of MSN in HD.

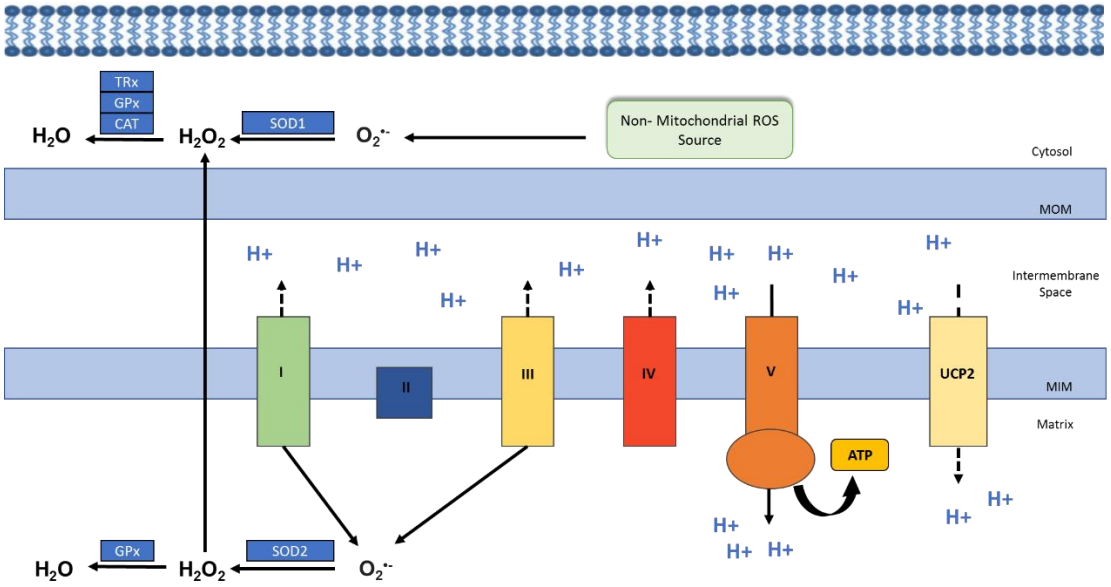

Figure 1.3 – Enzymatic cellular response to ROS production. Cytosolic and mitochondrial enzymatic ROS scavenger. Mitochondrial complex I and complex III are the two major ROS cellular sources, which can be kept inside the mitochondria or diffuse into the cytosol. In different cellular locations, specific enzymes are responsible for ROS removal and restore the redox homeostasis.

Similar to other neurodenerative disease such as Alzheimer's disease (Mota et al. 2015) or Parkinson's disease (Perfeito, Cunha-Oliveira, and Rego 2013), in HD increased ROS production and impaired antioxidant response have been extensively described in different models (Solans et al. 2006, Sorolla et al. 2008, Stack, Matson, and Ferrante 2008). In a study conducted by Sorolla and colleagues, post-mortem HD patient's striatum and cortex tissue exhibited an increase in GPx1 and Prx1-2 and Prx6 protein levels (Sorolla et al. 2008). Likewise, in R6/2 mice, an increased activity of SOD2 and SOD1 was observed at 19 weeks of age; however, at later stages, a decrease followed (Santamaría et al. 2001). Increased transcriptional levels of SOD2 and γ-glutamylcysteine ligase catalytic heavy chain (GCLc) was also observed in R6/2 mice with 8 weeks of age (Fox et al. 2004). Counterwise, in another study HD mouse striatal cells (ST*Hdh*$^{Q111/Q111}$) presented a decrease in GCLc protein levels and activity, but an increase in SOD1 and 2 protein levels (Ribeiro et al. 2014). The same contradictory information regarding antioxidant response was observed in HD-iPSC. Some groups reported an increase in antioxidant enzymes (SOD1, Prx1 and GPx1) in YAC128 derived-iPSC (Szlachcic et al. 2015). By contrast, a decrease in SOD1 expression was reported in human iPSC derived from a HD patient with 72 CAG repeats (Chae et al. 2012).

Taken together, similar to other neurodegenerative diseases, increased ROS levels have been observed in patient's samples and different cellular and animal HD

models. On the other hand, the characterization of enzymatic antioxidant response has been rather inconclusive, with groups reporting impairment only in later stages of HD, while other reporting no changes or downregulation in gene expression, protein levels or enzymatic activity. In HD, the antioxidant response under redox stress still needs to be clarified in order to understand if in early stages of HD the cell is capable to restore the redox balance or if the loss of redox homeostasis is an early event in pathology.

Another antioxidant response is mediated by Nuclear factor erythroid 2–related factor 2 (Nrf2), first described in 1994 by Moi (Moi et al. 1994). The binding of Nrf2 to antioxidant-response elements (ARE) induces the expression of phase II detoxifying enzymes (Rushmore and Pickett 1990, Itoh et al. 1997). Nrf2 is able to regulate the expression of proteins that directly or indirectly protect cells from ROS and electrophiles (Pietsch et al. 2003, Ishii et al. 2000, Kwak et al. 2003, Alam et al. 1999, Banning et al. 2005) and enhances the synthesis of GSH (Mulcahy and Gipp 1995, Lee et al. 2005, Harvey et al. 2009). The activation of Nrf2 under endogenous or exogenous stimuli is dependent on molecules associated to oxidative stress, namely H_2O_2, peroxynitrite (ONOO-) or organic peroxide (ROOH) (for review (Dinkova-Kostova et al. 2002, Talalay, De Long, and Prochaska 1988). ROS have the ability to modify cysteine residues in kelch like ECH associated protein 1 (Keap1, a cysteine-rich protein), inducing conformational changes and triggering the activation of Nrf2. Although Nrf2 activation under oxidative stress has a protective effect, in cancer cells it was demonstrated that Nrf2 compromises apoptosis activation, decreases autophagy and increases proteasomal degradation allowing cancer cells to proliferate (for review (Brigelius-Flohé and Flohé 2011)). Under physiological conditions, Nrf2 is sequestered by Keap1, which contains an adapter region for the Cullin-3 (Cul3)-based E3 ligase system in the cytosol. Keap1 targets Nrf2 for ubiquitination and proteasomal degradation (Zhang et al. 2004). Keap1 suffers conformational alterations upon exposure to electrophiles and oxidants, dissociating from Nrf2 and allowing its translocation to the nucleus (Boutten et al. 2010, Eggler, Gay, and Mesecar 2008, Surh, Kundu, and Na 2008). The non-canonical activation of Nrf2 is enhanced by the phosphorylation of p62, a stress-inducible protein, at serine 349, which increases its affinity to Keap1 and release the sequestered Nrf2 (Komatsu et al. 2010). This pathway is activated in response to specific stimuli, such as mitochondrial depolarization or bacterial infection (Ichimura et al. 2013), and recruits the Keap1 to the ubiquitinated cargo targeted for degradation (Fan et al. 2010). Furthermore, in the canonical pathway, Nrf2 can be phosphorylated at serine 40 by protein kinase C_δ (PKC_δ) and protein kinase C_I (PKC_I), causing Keap1 to release Nrf2 for translocation into the nucleus (Huang, Nguyen, and Pickett 2002, Li et al. 2004, Niture, Jain, and Jaiswal 2009,

Numazawa et al. 2003). Once in the nucleus, Nrf2 dimerizes with Maf proteins (MafF, MafG anf MafK) and recruits other proteins (Zhu and Fahl 2001) to form a complex that binds genes with an ARE in the promoter region. These genes encode phase II detoxification enzymes, such as CAT, heme-oxygenase-1 (HO-1), GPx, NAD(P)H quinone oxidoreductase 1 (NQO1), GST, sulfiredoxin (Srx), thioredoxin reductase (TrxR), Prx, γ-glutamylcysteine ligase regulatory light chain (GCLm) and GCLc (Zhang, Ding, et al. 2012, Tripathi and Jena 2010). HO-1 is one of the first activated enzymes under stress conditions (Calabrese et al. 2008) and is upregulated in response to oxidative/nitrosative stress (Willis et al. 1996) as an adaptive survival response (Vile et al. 1994, Bishop et al. 1999).

γ-Glutamylcysteine ligase (GCL) is a rate-limiting enzyme in a multiple step process for GSH biosynthesis. The enzyme contains two subunits, GCLc and GCLm (Griffith and Mulcahy 1999). GSH has a significant antioxidant relevance by converting H_2O_2 into water through the activity of GPx (for review (Marí et al. 2009). Therefore, the regulation of GCLc is extremely important to maintain redox homeostasis. Nrf2 regulates GSH by modulating the expression of GCLc and GCLm subunits (Moellering et al. 1998) and cysteine influx (Sasaki et al. 2002). Possibly, the imbalance in GSH/GSSG can also trigger Nrf2 activation, therefore upregulating GCLc expression and boosting GSH levels (Chia et al. 2010). Under ROS stress signal, the Nrf2 pathway activation is responsible for encoding key components of both disulfide systems (thioredoxin, Trx, and GSH systems) and thereby act as an important regulator of antioxidant response (Schmidt 2015).

In addition to the of Nrf2 as a regulator of redox homeostasis, recent developments evidence other important functions in stem cells. Several studies point Nrf2 as a regulator of proliferation and homeostasis in stem cells (Hochmuth et al. 2011, Tsai et al. 2013, Jang et al. 2014, Jang et al. 2016). Nrf2 activation delays human ESC differentiation and upregulates proteasomal activity through modulation of 19 proteasome Nrf2-dependent subunits out of 36 proteosome subunits (Jang et al. 2014). A high proteasomal activity is required to maintain pluripotency, and Nrf2 pathway might be essential to maintain the capacity of self-renewal and proliferation, characteristic of stem cells (Vilchez, Morantte, et al. 2012). Importantly, regulation of redox homeostasis and protein degradation can be a mechanism by which Nrf2 contributes to maintain stem cells identity.

Alterations in the cellular redox homeostasis in HD has been widely reported, suggesting the involvement of Nrf2-responsive genes in the antioxidant response. Nrf2 activation in HD has been controversial. Van Roon-mom and colleagues described for

the first time in PC12 HD cell model an increase in Nrf2-dependent antioxidant enzyme transcripts, except for HO-1 and transaldolase 1 (Taldo1) (van Roon-Mom et al. 2008). Concordantly, another study where 3-NP was administred to mice showed an increase in Nrf2 translocation to the nucleus and subsequent increase in HO-1 expression after 3-NP administration (Gao et al. 2015). However, in HD mouse striatal cells (ST*Hdh*$^{Q111/Q111}$), despite an increase in Nrf2 phosphorylation, no changes were found in the expression of Nrf2- target genes (Ribeiro et al. 2014). Moreover, in the same HD cell line, an impaired Nrf2 activation under stress conditions was reported due to a decrease of Nrf2 cytosolic repressors, Keap1 and p62, but no changes in Nrf2 levels were observed (Jin et al. 2013). Unaltered Nrf2 protein levels were also reported in the striatum of R6/1 mice at different weeks of age (8, 12 and 30 week-old), whereas in the cortex increased Nrf2 levels were observed (Rué et al. 2013). Overall, these data suggest that the role of Nrf2 pathway in HD pathogenesis is not yet completely understood. Thus, further research will be required to reveal the importance of Nrf2 signaling pathway and its implications in HD neuropathology.

1.5 – Objectives

In the past few decades significant advances in the comprehension of HD pathogenesis and disease underlying mechanisms have been made. Despite the previous findings, the understanding of the molecular mechanisms and pathophysiological processes that lead to HD is far from being complete. HD patients present different ages of onset also differing regardingthe clinical progression, which hinders the complete comprehension of the disease. This implicates that there is still a major need in extensive research to reveal the biological processes that play part in disease progression. The discovery of altered mechanisms at early stages of disease is important for clinical and therapeutic purposes, allowing to identify potential biomarkers for early diagnosis and the use of more effective therapies to attenuate the course of the disease. Taking this into account, the use of stem cell models, in particular iPSC, can contribute for the understanding of impaired mechanisms in early stages of the disease.

It has been recognized that mHTT has a significant impact on cellular homeostasis by interfering with the regular activity of organelles, proteins and DNA (Bossy-Wetzel, Petrilli, and Knott 2008). In HD, elevated ROS production is considered a hallmark of disease in symptomatic stages and has been observed in several models of HD (Browne and Beal 2006, Sorolla et al. 2008, Lim et al. 2008). Moreover, elevated ROS activates transcriptional factors responsible for antioxidant and metabolic

response. The importance of PGC-1α as a transcriptional factor in the regulation of mitochondrial complexes biogenesis, thermoregulation, glucose metabolism and antioxidant enzymes response mediated by the downstream effectors TFAM and NRF1/2 was previous established (Scarpulla, Vega, and Kelly 2012). Thus, mHTT was previously reported to bind to PGC-1α (Cui et al. 2006). Additionally, the expression of detoxifying and antioxidant enzymes is regulated by the Nrf2 signalling pathway (Zhu et al. 2005), which is activated following ROS stress signal and further translocation of Nrf2 to the nucleus (Hsieh et al. 2009). Therefore, in the present study, we aimed to characterize transcriptional activity, as well as alterations occurring in mitochondrial biogenesis, metabolic shift and cellular redox state in human HD-iPSC and derived HD-NSC *versus* respective control cells. For this purpose, we defined the following specific objectives:

I. Determine transcriptional activity related with mitochondrial biogenesis

We evaluated the mRNA expression of PGC-1α and TFAM. Considering that previous results from our lab indicated a reduction in complex III activity and in PDH activity analysed through increased phosphorylation at Ser residues in HD iPSC (Lopes et al., *unpublished data*), we also analysed the mRNA expression of selective nuclear- and mitochondrial-encoded subunits of complexes I, III and IV and regulatory enzymes of PDH, namely PDK1 and PDP1.

II. Evaluate cellular ROS levels and ROS-related response proteins

This part of the study was focused on cellular redox homeostasis. We measured cellular ROS levels before and after mitochondrial complex III inhibition with antimycin A. As part of the redox homeostasis maintenance, we assessed the protein levels of antioxidant enzymes (e.g. SOD2 and Lys acetylated SOD2) and UCP2 mRNA levels. Moreover, we assessed Ser phosphorylated and total Nrf2 in cellular and nuclear extracts, as well as the mRNA levels of its downstream effectors (e.g. GCLc and HO-1).

2 – METHODS

2.1 – Materials

Murine embryonic fibroblasts (MEFs) were acquired at AMSBIO® (Abingdon, U.K.). Matrigel was obtained from Corning® (Amsterdam, The Netherlands). DMEM/F12, Lipofectamine 3000, Geltrex and Knockout serum replacement, Amplex® Red hydrogen Peroxide/Peroxidade Assay Kit were purchased from Invitrogen (Carlsbad CA, USA). Nonessential amino acids, L-glutamine, 2-mercaptoethanol, XAV939, Y-27632, protease inhibitors (chymostatin, pepstatin, A, leupeptin, antipain), Antimycin A, hydrogen peroxide (H_2O_2), PMSF (phenylmethanesulfonyl fluoride), butylhydroquinone (t-BHQ) were purchased at Sigma-Aldrich Corporation (St. Louis, MO, USA).

Neurobasal medium, Hoechst 33342, Accutase, N-2 Supplement (100X) and B27® Supplement (50X) were obtained from Life Technologies (Eugene, OR, USA). Human basic fibroblast growth factor (FGF2), human epidermal growth factor (EGF), mitomycin C and Dorsomorphin were obtained from Tebu-bio (Yvelines, France). SB431542 was from Tocris Bioscience (Bristol, UK). Dulbecco's Modified Eagle's Medium (DMEM) culture medium, Fetal Bovine Serum (FBS), penicillin/streptomycin and Opti-MEM were purchased from GIBCO (Paisley, UK). was provided by Promega Corporation (Madison, WI, USA).

PureZOL® RNA Isolation Reagent, iQ™ SYBR® Green Supermix, Bio-Rad Reagent were supplied by Bio-Rad Laboratories (Hercules, CA). NZY First-Strand cDNA Synthesis Kit from NZY tech (Lisbon, Portugal). Primers were obtained from SabVida (Portugal).

Enhanced ChemiFluorescence reagent (ECF) was from GE Healthcare (Little Chalfort, UK). Primary antibodies for P(Ser40)-Nrf2, Nrf2, Acetyl(Lys68)-SOD2, SOD2 were obtained from Abcam (Cambridge, UK). HDAC1 was obtained from Millipore (MA, USA). α-tubulin, actin B were purchased from Sigma Aldrich (St. Louis, NO, USA). Secondary antibodies (anti-rabbit and anti-mouse) and Pierce Firefly Luc One-Step Glow Assay Kit were purchased from ThermoFisher Scientific (Waltham, MA, USA).

2.2 – Cell culture of human HD and control iPSC and NSC

To conduct this study, we used heterozygous human induced pluripotent stem cell line designated HD4-iPSC (XY, passages 4-30) with a normal allele (19 CAG repeats) and an extended allele (72 CAG repeats). The HD4-iPS was generated by Park and colleagues (Park et al. 2008) and gently provided by Prof. George Daley (Harvard Medical School, Boston, MA, USA). The wild-type cell line, AMS4-iPSC (XY, passages 7-30), was generated by de Almeida and collaborators (CNC, Faculty of Medicine, University of Coimbra).

Cells were maintained on a layer of mitotically inactivated murine embryonic fibroblasts (MEFs) for several passages or allowed to grow under feeder-free conditions on Matrigel® or Geltrex® on 6-well plates. MEFs were obtained from AMSBIO®, expanded for 3 passages and inactivated with mitomycin C. iPSC grow in medium containing DMEM/F12 supplemented with 20% Knockout serum replacement (KSR), 2 mM glutamine, 1 mM nonessential amino acids, 1% penicillin/streptomycin, 100 μM 2-mercaptoethanol and 10 ng/mL recombinant human FGF2. iPSC cultures were checked daily under the microscope and abnormal/differentiated colonies were manually removed. The medium was replaced daily, and when cultured without feeders the medium had to be conditioned in MEFs for 24 hours and filtered with a 0.22 μm filter prior to change. Cultures were passaged every 7 to 14 days by manual dissection upon attaining 90% confluence. iPSCs were differentiated into neural stem cells (NSCs) based on dual SMAD inhibition SB431542 (Lefty/Activin/transforming growth factor beta - TGFSB431542 (Lefty/Activin/transforming growth factor beta – TGFβ inhibitor), dorsomorphin (bone morphogenetic protein – BMP inhibitor) and XAV – 939 (β-catenin-transcription inhibitor and axin stabilizing agent) (Chambers et al. 2009), (Delli Carri et al. 2013), (Nicoleau et al. 2013). Neural induction occurred between day 0 and day 10-12. N2 medium consists in a mixture of two basal media (DMEM/F12 and Neurobasal, 1% N2 (100x), 2 mM L-glutamine, 100 μM nonessential amino acids, 100 μM 2-mercaptoethanol, 1% penicillin/streptomycin and 2% B27 (50x)). From day 0 to day 5, cells were maintained in KSR medium without FGF2 and incubated with 5 μM dorsomorphin, 10 μM SB431542 and 1 μM XAV-939. Medium was replaced every day. From day 5 to day 10-12, the medium was gradually replaced by 75% KSR + 25% N2 medium, 50% KSR + 50% N2 medium, until reaching 100% N2 medium with 5 μM dorsomorphin 10 μM SB431542 and 1 μM XAV-939 (Nicoleau et al. 2013); (Delli Carri et al. 2013). Prior to day 10-12, morphological changes are visible under microscope with the formation of characteristic macroscopic rosettes. Cultures were then replated in basement membrane matrix coated 6-well plates. To detach the cells, 500 μL of 1X

Accutase® diluted in pre-warm DMEM/F12 was added to medium and incubated at 37ºC in 5% CO_2, for 15-20 minutes. Then, cells were collected and centrifuge at 1000 rpm for 3 minutes at room temperature, resuspended in 200 µL of media and addedto the plate, after which cells were allowed to adhere for 30 minutes. This step is repeated with 500 µL of media. After 2 hours, N2 medium supplemented with 10 ng/mL FGF2 and 10 ng/mL EGF was added for a total volume of 2 mL per well. Cultures were maintained at 37ºC in 5% CO_2 overnight and medium was replaced daily and passaged every 2-3 days for no more than 10 passages.

2.3 – Immunocytochemistry

Neural stem cells were washed with pre-warmed PBS 1x (137 mM Nacl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4) followed by permeabilization with PHEM (5mM HEPES, 60 mM PIPES, 10 mM EGTA, 2 mM $MgCl_2$, pH 7.0 with KOH) 0.1% Triton X-100 for 30 seconds, and then fixed with 4% PAF/PHEM (20 minutes at room temperature). Cells were rehydrated with PBS/0.1% Triton X-100, three times, blocked in 3% BSA/PBS for 30 minutes and then incubated with primary antibody overnight at 4ºC. The following primary antibodies used were: anti-SOX2 (1:200) and anti-Nestin (1:200). Secondary antibodies and DAPI counterstain were applied for 1 hour at room temperature. Confocal analysis was performed on a Leica TCS SP2 microscope (Leic Micrisystems).

2.4 – Total RNA extraction, RT-PCR and quantitative RT-PCR

RNA was extracted according to PureZol® RNA Isolation protocol. Briefly, media from iPSC and NSC was removed, cells were washed with PBS once and scrapped in PureZol®. Chloroform was added to induce phase separation and RNA was colleted. Next, RNA was precipitated with isopropyl alcohol and the pellet washed with 75% ethanol . The final pellet was air-dried for 30 minutes and ressuspended in RNAse-free water (DPEC-treated water). After, RNA was quantified using NanoDropR spectrophometer and accepted as pure if the samples had a A260/A280 ratio between 1.8 and 2.0.

cDNA was obtained using NZY First-Strand cDNA Synthesis Kit. 1 µg of RNA was used for reverse transcriptase reaction. cDNA synthesis was performed according to manufacturer's protocol: 5 minutes at 25ºC, 30 minutes at 50ºC and 5 minutes at 85ºC. Finally NZY RNAse H (*E.coli*) was added and incubated at 37ºC for 20 minutes.

Prior to the qRT-PCR, all primers' efficiency was validated. Quantative Real-Time PCR was performed using iScript SybrGreen on a CFX96 Touch™ Real-Time PCR Detection System. The total reaction mixture (10 µL) contained 0.3 µM of forward and reverse primer, 5 µL of iQ™ SYBR® Green Supermix and 2.5 µL of cDNA for a final concentration of 50 nM. qRT-PCR was performed according to manufacturer's protocol: enzyme activation at 95ºC for 3 minutes, followed by 40 cycles of denaturation at 95ºC for 15 seconds, annealing/extension for 45 seconds at the specif primer´s annealing temperature, and the melt curve from 55 to 95ºC for 5 seconds/step with a 0.5ºC of increment.

All PCR samples were run in triplicates and the average of C(t) values were considered for calculation. Expression values were calculated using ΔΔC(t) method. For each gene specific primers sequence were used, as described in Table 1, and quantified relative to tubulin and 18S.

Table 1 – Primers Sequence and characterization

GENE	**Forward Primer (5'-3')**	**Reverse Primer (5'-3')**	**Annealing Temperature (ºC)**
UCP2	AGC CCA CGG ATG TGG TAA AG	CTC TCG GGC AAT GGT CTT GT	58
TFAM	AGA GCA GTC TGG GAG TAG GG	TGC TGC ATT TGT CCC GAG AT	57
Cox3	CAC CAC CTG TCC AAA AAG GC	CTA GGG GAT TTA GCG GGG TG	59
ND1	AAA CCC GCC ACA TCT ACC AT	GCC TAG GTT GAG GTT GAG CA	57
MT-CYB	ACC CCC TAG GAA TCA CCT CC	GCC TAG GAG GTC TGG TGA GA	58
UQCR10	TGG GCG TCA TGT TCT TCG AG	CTC TAC TCC ACT GTC ACC CC	58
CYC	CAC TGC GGG AGG GTC TCT AC	CAT CAT CAA CAT CTT GAG CCC C	58
NRF2	CAG TCA GCG ACG GAA AGA GT	AAG TGA CTG AAA CGT AGC CGA	57
HO-1	ACC CAT GAC ACC AAG GAC CAG A	GTG TAA GGA CCC ATC GGA GAA GC	59
GCLc	GTG GAT GTG GAC ACC AGA TG	GCG ATA AAC TCC CTC ATC CA	54
PGC-1α	GTC ACC ACC CAA ATC CTT AT	ATC TAC TGC CTG GAG ACC TT	61
18S	GAG GAT GAG GTG GAA CGT GT	TCT TCA GTC GCT CCA CGT CT	58
Tubulin	CCA GGG CTG TGT TTG TAG ACC	CAA TAG TGT AGT GTC CAC GGG C	57
PDP1	CCT CGT CGG GAA GAA TCG TT	CCT CAC AAC TTT GGA CGG GT	60
PDK1	GGA CTT CGG GTC AGT GAA TGC	CGC AGA AAC ATA AAC GAG GTC T	60

2.5 – Western blotting

Cells were washed in PBS and scrapped in 200µL of lysis buffer (50 mM Tris-HCl pH 7.5, 0.1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 50 mM NaF, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 0.1% (v/v) β-mercaptoethanol, 250 µM PMSF, and 10 mg/mL aprotinin and leupeptin) supplemented with 1 µg/mL of protease inhibitors and okadaic acid. For total extracts, the cells were homogenized and incubated in ice prior centrifugation at 14 000 rpm for 15 minutes. The supernatant was collected and protein quantified using Bio-Rad Protein Assay.

For the isolation of nuclear and cytoplasmic fractions a Nuclear/Cytosol Fractionation Kit was used according to manufacturer's instructions.

Samples were denaturated at 95ºC for 5 minutes with SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulphate, 5% glycerol, 0.01% bromophenol blue and 100 mM DTT). After, 30 or 75 µg of protein were loaded into 12% or 10% gels, subjected to SDS/PAGE and transferred onto polyvinylidene difluoride (PVDF) Hybond-P membranes. Then, membranes were blocked, at room temperature, in 5% BSA/TBS-T buffer (20 mM Tris-HCl, 0.15M M NaCl, 0.1% Tween 20) for 90 minutes and incubated with primary antibodies overnight at 4ºC. Membranes were then washed in TBS-T and incubated with secondary antibody (anti-rabbit or anti-mouse, 1:5000 or 1:10000), at room temperature, for 90 minutes, and developed using ECF fluorescent reagent. Immunoreactive bands were visualized with ChemiDoc™ Touch Imaging System (BioRad®, Hercules, USA). Data was presented as the ratio of phosphorylated protein/total protein, acetylated protein/total protein or total protein/actin or tubulin. Western Blotting data was quantified using ImageLab (BioRad Technologies, USA).

Table 2 - List of primary Antibodies used in the study

Antibody	Molecular Weight	Concentration	Secundary Antibody
Phospho-Nrf2 (S40)	100 kDa	1:5000	Anti-Rabbit
Nrf2 (Total)	100 kDa	1:1000	Anti-Rabbit
AcetylSOD2 (K68)	25 kDa	1:100	Anti-Rabbit
SOD2 (Total)	24 kDa	1:5000	Anti-Rabbit
α-Tubulin	57 kDa	1:5000	Anti-Mouse
Actin B	42 kDa	1:5000	Anti-Mouse
HDAC1	65 kDa	1:1000	Anti-Rabbit

2.6 – Cellular H_2O_2 production

In order to evaluate total H_2O_2 ROS production, the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit was used according to manufacturer's instructions. The Amplex® Red reagent in contact with H_2O_2 produces resorufin, a red-fluorescent oxidation product with a excitation wavelengh of 571 nm and an emission of 585 nm. Briefly, iPSC were detached by trypsin, collected and allowed to rest for 30 min in KSR. Subsequently, samples were centrifuged 3 min, 1000 rpm at RT and washed with Krebs medium. Media was then replaced by Krebs solution with 10 µM Amplex®Red reagent and 0.5 U/mL Horseradish (HRP) peroxidase. Fluorescence was measure for a total time of 40 minutes.

NSC were cultured for 24 hours in 96-well assay plates coated with Geltrex in neural maintainance medium. Prior to the assay, cells were washed in HBSS and the measurement is performed in the presence of 10 µM Amplex®Red reagent and 0.5 U/mL Horseradish (HRP) peroxidase.

As a positive control, iPSC and NSC were challenged for 2 hours with 100 µM H_2O_2 prior to fluorescence acquisition. Resultant fluorescence was analyzed on a Microplate Spectrofluorometer Gemini EM with excitation at 570 nm and collection at 585 nm. The results were calculated as RFU per mg of protein for NSC or per 500.000 cells for iPSC.

2.7 – Data and statistical analysis

All statistical analyses were performed on GraphPad 5.0 software (San Diego, CA, USA). Data are presented as the mean ± SEM for the indicated number of independent experiments. Statistical significance was analysed using Student's t-test, Mann-Whitney U test or one-way ANOVA.

3 – RESULTS

3.1 – Characterization of human HD and control iPSC and NSC

The characterization of AMS4-iPSC and HD-iPSC was already stablished in a previous work (*Lopes C., PhD thesis, 2015*). In that same study both cell lines exhibited pluripotency markers (OCT4 and SOX2) and HD-iPSC expressed mHTT with 72 CAG repeats. We further differentiated HD4 and AMS4 iPSC lines into NSC through a neural induction protocol, previously described by Delli Carry (Delli Carri et al. 2013). NSC are multipotent cells, characterized by the self-renewing capacity promoted by specific transcriptional factor, like SOX2. NSC also present specif markers such as nestin, an intermediate filament protein expressed during early development but absent in differentiated counterparts . Immunocytochemistry analysis with the pluripotent marker, SOX2, which is also expressed in early stages of neural development, and ectoderm marker, Nestin, demonstrated the successful conversion into NSC of both HD4-NSC and AMS4-NSC (**Figure 3.1**).

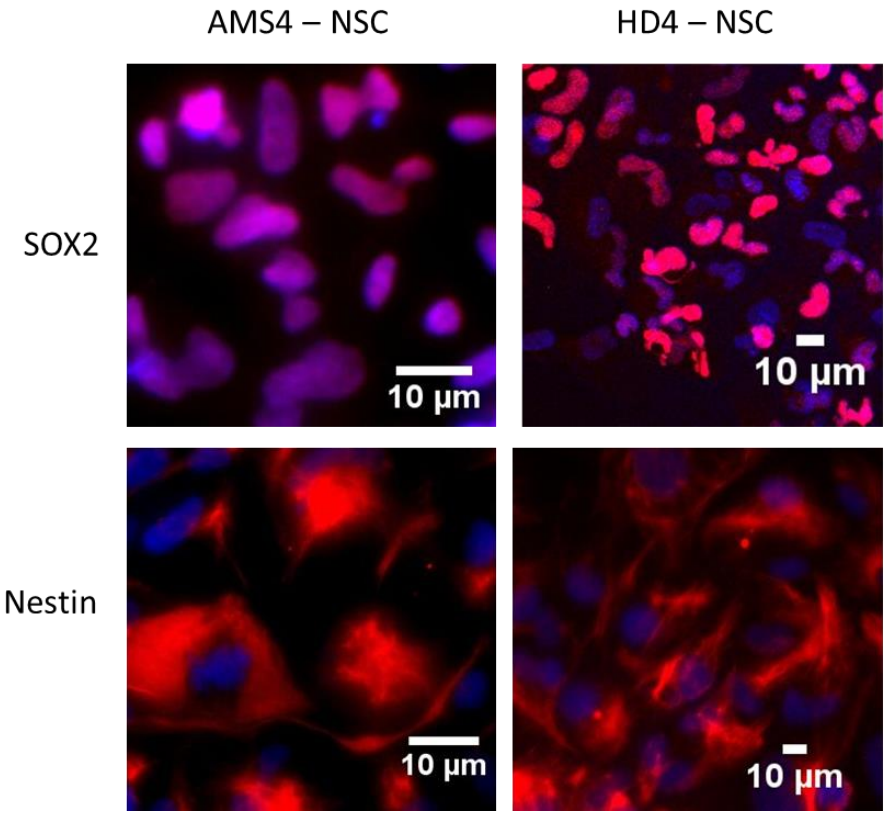

Figure 3.1 – Characterization of NSC lines. AMS4-NSC and HD-NSC were immunostained for stem cell marker, SOX2, and for ectoderm marker, Nestin. Scale bar of 10 µm.

3.2 – Evaluation of PGC-1α mRNA levels and its downstream targets

Several lines of evidence point to a general transcriptional dysregulation in HD (Kumar, Vaish, and Ratan 2014). As described before, the dysregulation of PGC-1α pathway and its downstream effectors have been reported in several HD models (Weydt et al. 2006, Chaturvedi et al. 2010, Cui et al. 2006). PGC-1α is a master regulator of mitochondrial biogenesis and oxidative stress response; therefore PGC-1α downregulation can compromise mitochondrial biogenesis, leading to mitochondrial defects in HD. Thus, we assessed the mRNA levels of PGC-1α and its downstream target, TFAM (a mitochondrial transcription factor responsible for mtDNA replication) in human HD-iPSC and derived HD-NSC. We observed a significant decrease in PGC-1α levels in both HD-NSC and HD-iPSC, when compared to the respective control cells (**Figure 3.2.**). Similarly, mRNA levels of TFAM were also downregulated in HD-IPSC; however, no changes were found in HD4-NSC, when compared with AMS4-NSC (**Figure 3.2**).

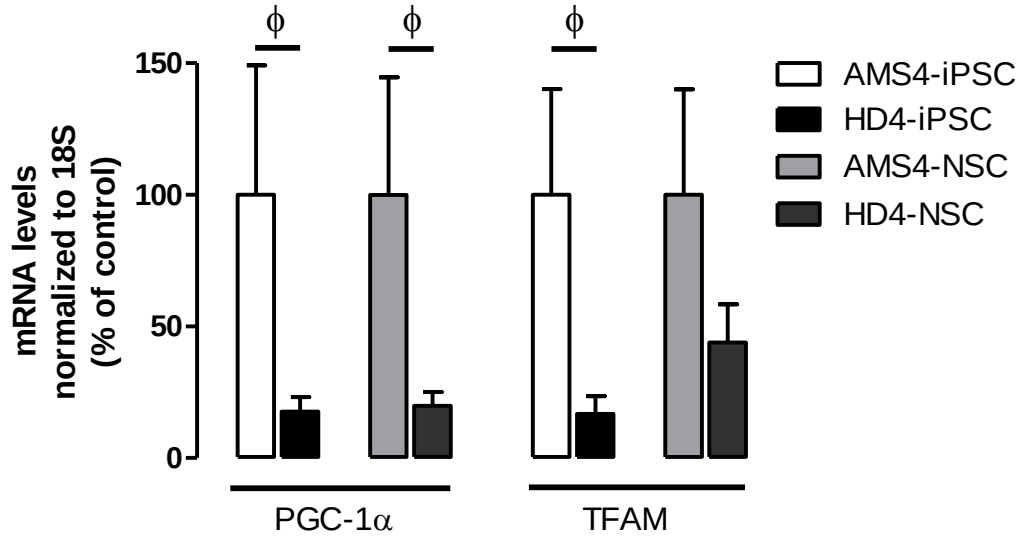

Figure 3.2– Expression of mitochondrial-linked transcription factors, PGC-1α and TFAM, in human HD iPSC and NSC lines. The mRNA levels of PGC-1α and TFAM were analysed in HD (HD4) and control (AMS4) iPSC and NSC lines. Results were normalized to 18S and presented as the percentage of the respective controls (AMS4-iPSC and AMS4–NSC). Values are expressed as mean ± SEM of 3 to 7 independent experiments, run in triplicates. Statistical analysis: ϕp<0.05 using Mann's Whitney U test.

Previous results from our lab showed reduced complex III activity in HD-iPSC and HD-NSC (C. Lopes and co-authors, *unpublished data*); therefore, we analysed the mRNA levels of nuclear encoded CYC1 and UQCR10, and mitochondrial-encoded MT-CYB complex III subunits (**Figure 3.3**). mRNA expression of these subunits was significantly reduced in HD-iPSC *versus* control cells. Interestingly, CYC1, UQCR10 and MT-CYB were significantly upregulated in HD-NSC, when compared with HD-iPSC. Concordantly with these differences between iPSC and NSC, CYC1 and UQCR10 mRNA levels in AMS-NSC were also significantly increased, when compared with AMS-iPSC. These data suggest that the expression of some complex III subunits is compromised in early stages of HD and may be possibly responsible for the impairment in complex III activity.

Additionally, we evaluated the mRNA levels of other mitochondrial-encoded complexes I and IV subunits, respectively, ND1 and COXIII that are also potentially regulated by TFAM (**Figure 3.3B**). Our analysis revealed a significant increase in ND1 in HD-iPSC *versus* control, but unchanged mRNA content in NSC lines. Moreover, the mRNA expression analysis of complex IV subunit, COXIII, revealed no significant changes in both cell lines (iPSC and NSC) when compared with the respective control. When compared both HD cell lines, HD-iPSC and HD-NSC, we observed a significant downregulation of COXIII in HD-NSC (**Figure 3.3B**).

Here, we were able to demonstrate that although the expression of PGC-1α and TFAM is compromised in HD- iPSC and NSC, not all mitochondrial-encoded subunits are affected by this downregulation. Expression of Complex III subunits was severely compromised and correlated with downregulation of PGC-1α and TFAM. Conversely, complex I and complex IV subunits were apparently less affected by the downregulation of these transcriptional factors.

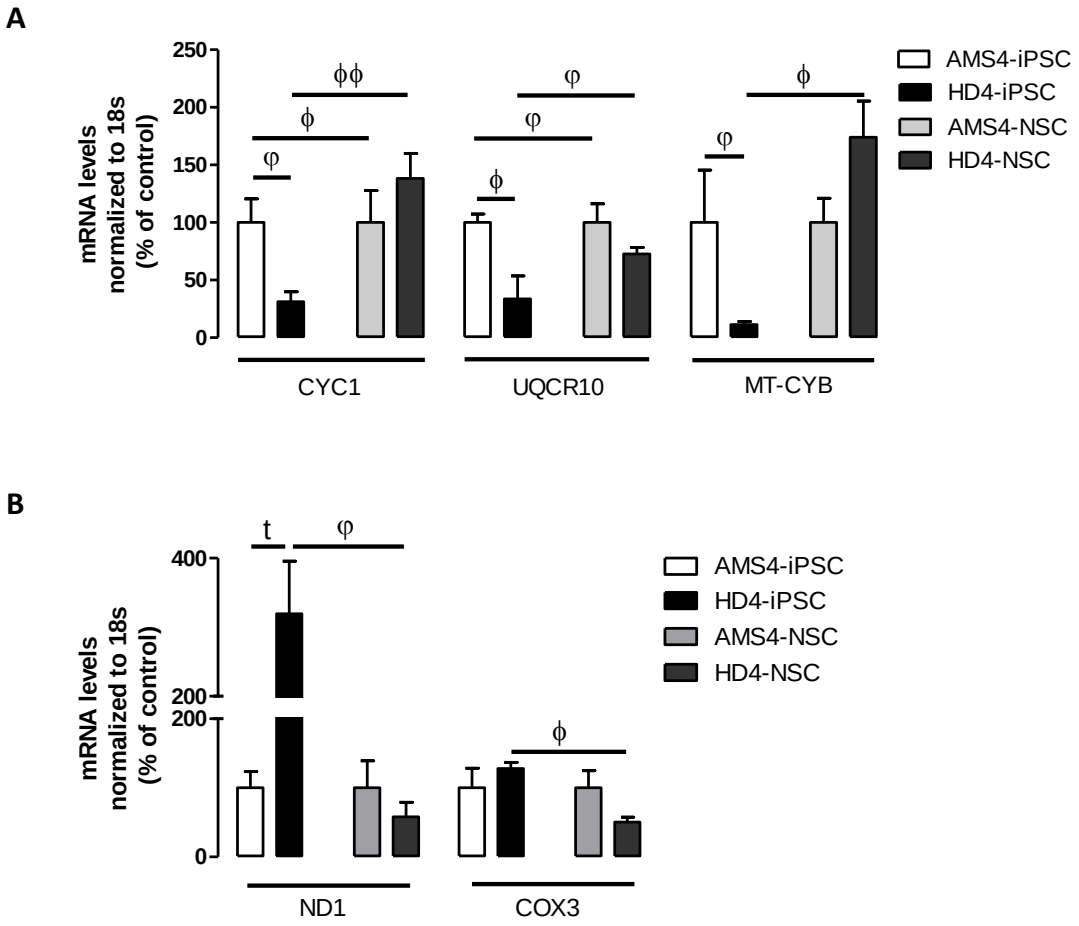

Figure 3.3 – Selective mitochondrial complexes I, III and IV subunits expression in human HD iPSC and NSC lines. A – Levels of mitochondrial- and nuclear-encoded complex III subunits, UQCR10; CYC1 and MT-CYB in HD iPSC and NSC *versus* control (AMS4) cell lines. **B** – Levels of mitochondrial-encoded complex subunit I (ND1) and complex IV subunit (COXIII) in HD and control iPSC and NSC. Results were normalized to 18S and presented as the percentage of WT. Values are expressed as mean ± SEM of 4 to 5 experiments, run in triplicates. Statistical analysis: $^{t}p<0.05$ *versus* control cells (Student's t-test); $^{\phi}p<0.05$ $^{\phi\phi}p<0.01$ (Mann's Whitney U test); $^{\varphi}p<0.05$ (one-way ANOVA test).

3.2.1 – Evaluation of nuclear encoded PDK1 and PDP1 in iPSC and NSC lines

During metabolic reprogramming of hiPSC, where a shift from OXPHOS to glucose metabolism occurs, several mechanisms have been suggested to facilitate the metabolic transition, by reducing ATP production and limiting respiration. One of the mechanisms that regulates the glucose metabolism is the inactivation of PDH complex, which reduces the conversion of pyruvate into acetyl-Co A and thus pyruvate entry in mitochondria. The regulation of PDH complex is mediated by nuclear encoded PDKs and PDPs. Previously

in our lab it was shown that HD-iPSC and HD-NSC displayed increased phosphorylation of PDH at Ser232, 293 and 300 (C. Lopes and co-authors, *unpublished data*). The increased phosphorylated levels of PDH in these cell lines led us to evaluate the mRNA levels of PDK1 and PDP1, which are responsible for the phosphorylation and dephosphorylation at these serine residues (**Figure 3.4**).

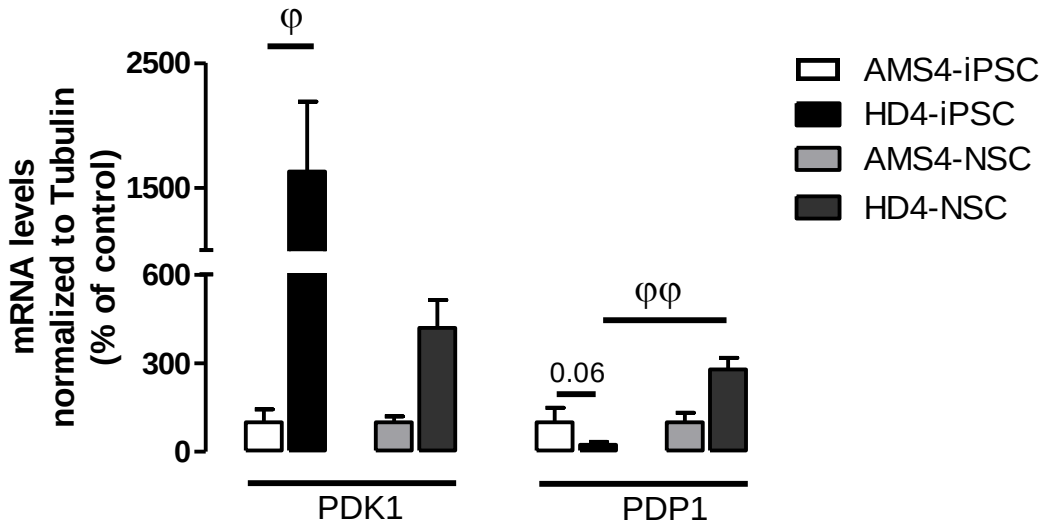

Figure 3.4 – Evaluation of mRNA levels of nuclear encoded PDK1 and PDP1 in human HD-iPSC and NSC lines. Results were normalized to tubulin and presented as the percentage of control. Values are expressed as the mean ± SEM of 3 to 5 experiments, run in triplicates. Statistical analysis: p=0.0635 *versus* control cells (Mann's Whitney U test); $^{\varphi}p<0.05$; $^{\varphi\varphi}p<0.01$ (one-way ANOVA).

Our mRNA analysis showed a significant increase in PDK1 in HD-iPSC, as compared to AMS-iPSC. Despite no significant differences in NSC lines, there was a tendency for an increase in HD-NSC (**Figure 3.4**). The mRNA of PDP1 has also changed in HD-iPSC. Our observations indicate a tendency for a decrease in HD4-iPSC, as compared to control cells. In NSC, we observed no statistical differences between both NSC lines, although there was a significant increase in PDP1 mRNA levels in HD-NSC when compared with HD4-iPSC (**Figure 3.4**). The increased PDH phosphorylation verified earlier in HD-iPSC could be explained by the increased levels of PDK1 and the downregulation of PDP1. These evidences point to PDH inactivation by phosphorylation, favouring glycolysis over OXPHOS for ATP production.

3.2.2 – Analysis of UCP2 mRNA levels in iPSC and NSC lines

Elevated levels of UCP2 appear to be required for maintaining the metabolic profile of stem cells; upon differentiation, a decrease in UCP2 has been related with enhanced mitochondrial activity (Zhang, Nuebel, et al. 2012). Activation of UCP2 is one of the mechanisms adopted by cells as part of the antioxidant response under conditions of higher production of mitochondrial ROS. The reduction in ΔΨm induced by UCP2 attenuates mitochondrial ROS production, facilitating the restoration of redox homeostasis. Taking this into consideration, we measured UCP2 mRNA levels in iPSC and NSC lines. In HD-iPSC, UCP2 mRNA levels showed a tendency for a decrease, when compared with control cells (**Figure 3.5**). Regarding NSC, UCP2 mRNA levels were significantly lower in HD–NSC *versus* control cells, as shown in **Figure 3.5**. The reduction in UCP2 expression levels might indicate a partial dysregulation of the antioxidant response under increased ROS production.

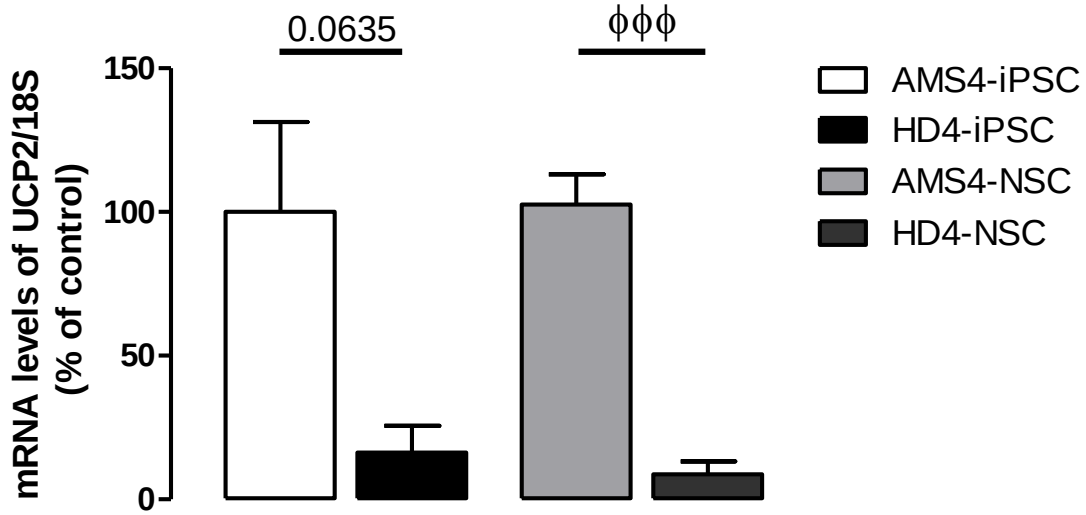

Figure 3.5– Expression of UCP2 in human HD-iPSC and NSC lines. The mRNA levels of UCP2 were evaluated in HD iPSC and NSC *versus* control (AMS4) cell lines. Results were normalized to 18S and presented as percentage of control. Values are expressed as mean ± SEM of 4 to 5 independent experiments, run in triplicates. Statistical analysis: ϕϕϕ p<0.001 *versus* control conditions (Mann's Whitney U test).

3.3 – Evaluation of intracellular H_2O_2 production in iPSC and NSC lines

Increased ROS levels is a common hallmark of several neurodegenerative disorders (Lin and Beal 2006). In HD, several studies reported increased ROS levels in samples from HD patients and in animal models of the disease (Sorolla et al. 2008, Browne and Beal 2006, Stack, Matson, and Ferrante 2008, Lim et al. 2008). These

results suggested alterations in the redox status of cells and, over-time, increased ROS production might be a possible mechanism leading to neurodegeneration in HD. Taking this into consideration, we evaluated cellular H_2O_2 levels using a fluorimetric probe, Amplex Red, to detect H_2O_2 in HD and control iPSC and NSC (**Figure 3.6A** and **3.6B**). The analysis of H_2O_2 production overtime revealed an increase in H_2O_2 levels in HD-iPSC or HD-NSC, as compared with AMS-iPSC (**Figure 3.6Ai, Bi)**). We also found enhanced H_2O_2 production rate in HD-iPSC, as compared to AMS4-iPSC (**Figure 3.6Aii)** and **Bii)**). However, a significant decrease in H_2O_2 production rate was found in HD-NSC *versus* AMS-NSC (**Figure 3.6Bii)**).

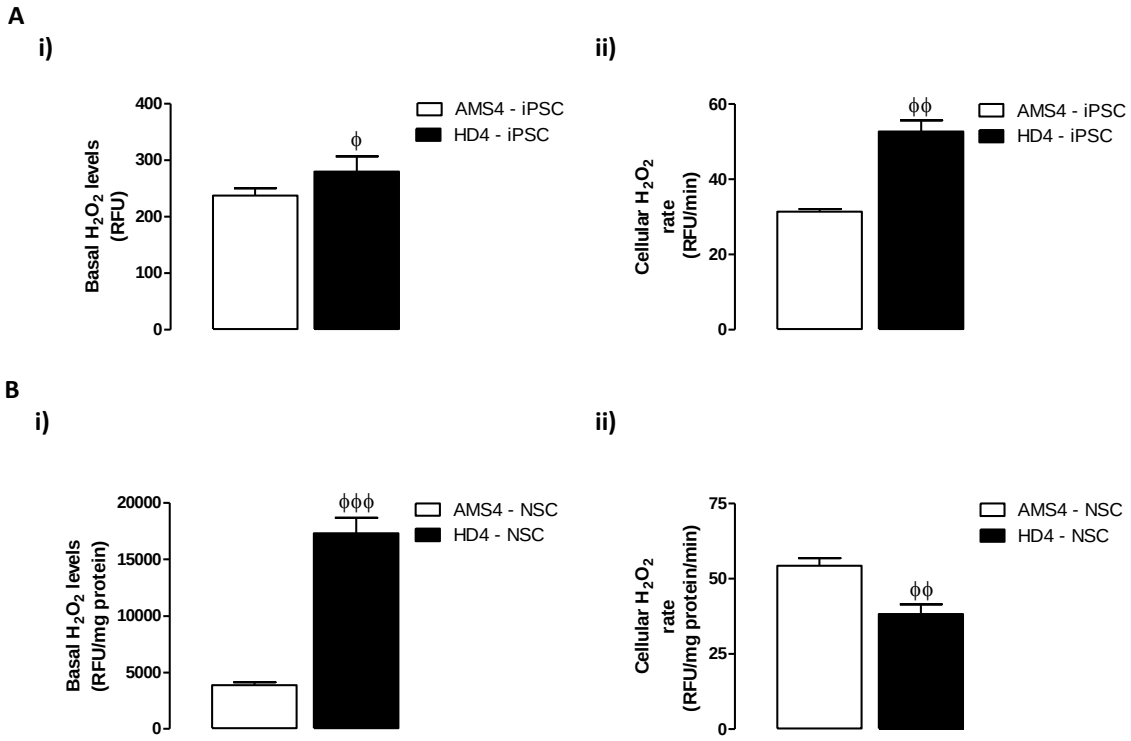

Figure 3.6 – Cellular H_2O_2 levels in human HD iPSC and NSC lines. H_2O_2 levels were measured by monitoring the fluorescence of resofurin using a Microplate Spectrofluorometer Gemini EM (excitation 570 nm; emission 585 nm). Fluorescence was measured for a total of 40 minutes. **Ai)** and **Bi)** - Basal H_2O_2 levels in iPSC and NSC respectively. H_2O_2 production over time was evaluated by the slope of each condition (**Aii; Bii**). In **Bi**, the basal levels are the mean of the 4 first readings normalized for the mg of protein. In **Bii**, the results were plotted as the difference between the last value achieved and the first basal value normalized to protein. Data are expressed as mean ± SEM of eindependent experiments, run in triplicates. Statistical analysis: $^{\phi}p<0.05$ Mann-Whitney U-test). [RFU=relative fluorescence units]

3.4 – Study of SOD2 protein and acetylation levels in HD-iPSC

Following increased ROS production, we determined the protein levels of SOD2, the organelle specific detoxifying enzyme responsible for converting $O_2^{\bullet-}$ into H_2O_2. The protein analysis revealed no differences between HD-iPSC and control cells (**Figure 3.7A**). Since SOD2 acetylation at Lys68 was shown to be linked to enzyme inactivation (Liu et al. 2015), we also evaluated the relative acetylated SOD2 protein levels. Again, unaltered relative acetylated protein levels were found between HD4–iPSC and AMS4-iPSC **(Figure 3.7B**)

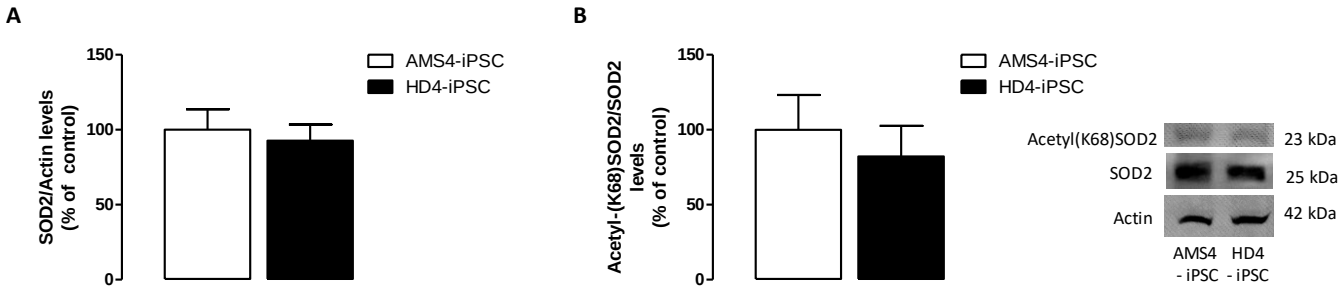

Figure 3.7 – Total and acetylated SOD2 protein levels in total extracts from HD and AMS iPSC lines. A – Protein levels of Acetyl(K68)SOD2/SOD2. **B** – Protein levels of SOD2 normalized to actin. Protein levels were assessed by Western Blotting in total extracts from HD *versus* control (AMS) iPSC. Results are expressed as percentage of control cells. Data are the mean ± SEM of 4 to 5 experiments.

3.5 – Nrf2 response under oxidative stress in HD-iPSC

The Nr2 pathway is an important cellular defence mechanism against oxidative stress. Under oxidative stress signals, Nrf2, initially sequestered in the cytosol by Keap1 (Wakabayashi et al. 2003), is phosphorylated at serine 40 (p-Nrf2) and translocated to the nucleus. In this organelle, it induces the expression of genes that participate directly or indirectly in the antioxidant response (Huang, Nguyen, and Pickett 2002).

Firstly, we assessed the mRNA levels of Nrf2 in iPSC and NSC (**Figure 3.8**). Our results indicate unchanged Nrf2 mRNA levels in both HD cell lines, when compared with the respective controls.

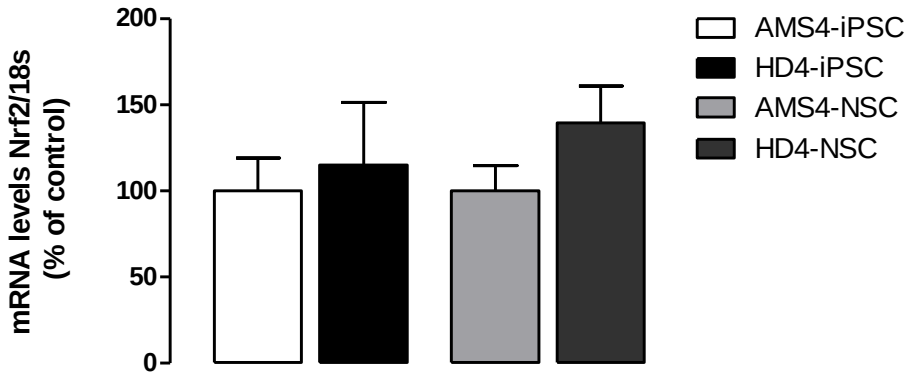

Figure 3.8 – Nrf2 gene expression in human HD-iPSC and NSC lines. The mRNA levels of Nrf2 was evaluated in HD iPSC and NSC lines *versus* control cells. Results were normalized to 18S and presented as the percentage of control (AMS4-iPSC and AMS4–NSC). Values are expressed as mean ± SEM of 4 to 5 experiments, run in triplicates.

Then, we used nuclear and cytosolic fractions to determine alterations in Nrf2 and P(Ser40)-Nrf2 protein levels, in order to understand if the Nrf2 pathway responds to elevated ROS production. In the nuclear enriched fraction, we observed no significant differences in the levels of p-Nrf2/Nrf2 in HD-iPSC (**Figure 3.9Ai**); however, in the cytosolic fraction, the ratio p-Nrf2/Nrf2 underwent a slight increase (although not statistically significant) in HD-iPSC, when compared to control cells (**Figure 3.9Bi**). Nevertheless, no changes in total Nrf2 protein levels were determined in the nuclear or cytosolic fraction of HD-iPSC, when compared to the control (**Figure 3.9 Aii)** and **Biii**). Although we did not detect significant changes in Nrf2 in the nucleus, a slight tendency for increased cytosolic p-Nrf2 suggested increased translocation and activity of Nrf2 in the nucleus, potentially controlling the expression of ARE-regulated genes.

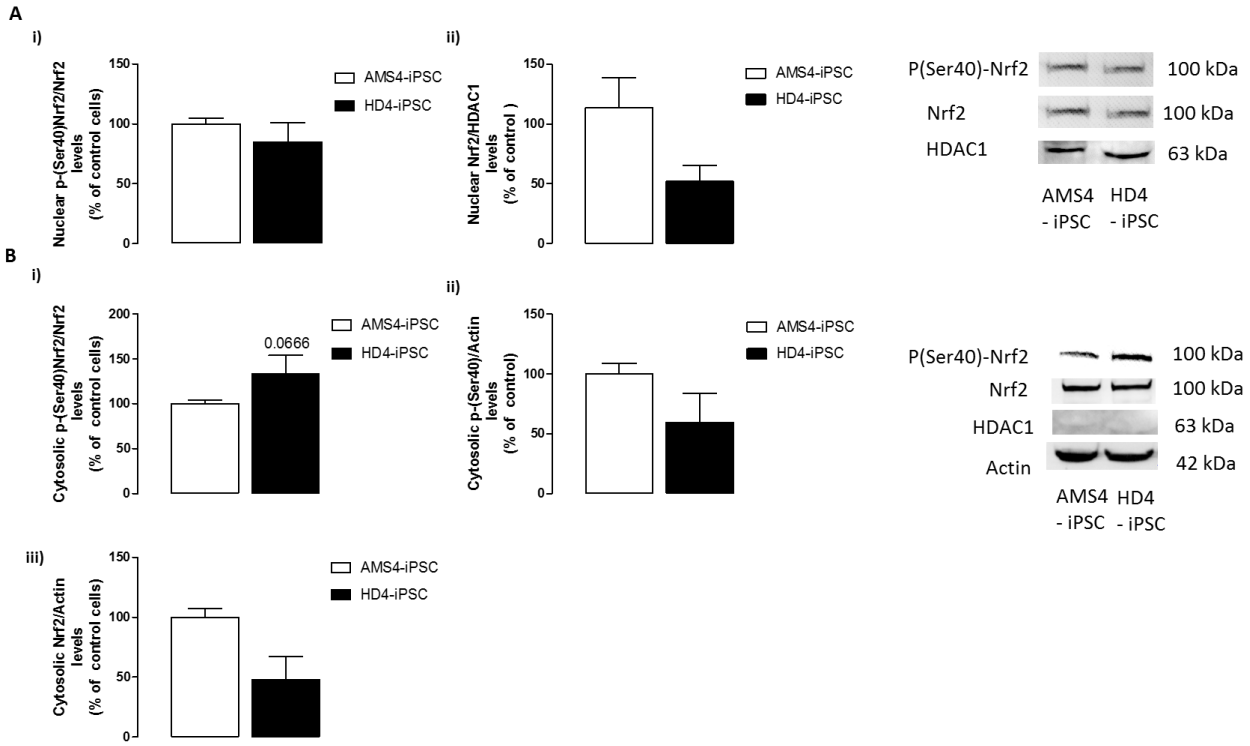

Figure 3.9 – Total and phosphorylated Nrf2 protein levels in nuclear and cytosolic fractions from HD and AMS iPSC lines. Ai) - Ratio of p-Nrf2 to Nrf2 protein levels in nuclear enriched fraction of AMS4 *versus* HD4 iPS cells. **Aii)** – Total Nrf2 level in nuclear enriched fraction normalized to HDAC1 of AMS4 *versus* HD4 iPSC cells. **Bi)** - Ratio of p-Nrf2 to Nrf2 protein levels in cytosolic enriched fraction of AMS4 *versus* HD4-iPSC cells. **Bii)** – p-Nrf2 level in cytosolic enriched fraction normalized to Actin of AMS4 *versus* HD4-iPSC cells. **Biii)** Total Nrf2 level in cytosolic enriched fraction normalized to Actin of AMS4 *versus* HD4 iPSC cells. Protein levels were assessed by Western blotting in nuclear and cytosolic fractions from iPSC. Results are expressed as the percentage of control cells and presented as the mean ± SEM of 4 to 6 experiments. Statistical analysis by the Mann's Whitney U test (**Bi**).

3.5.1 – Gene expression of Nrf2-dependent phase II antioxidant enzymes in iPSC and NSC lines

The Nrf2 response under oxidative stress encodes phase II antioxidant enzymes responsible for detoxifying the intracellular environment, participating in intracellular redox homeostasis (Chen and Kunsch 2004). Since HO-1 is one of the first activated enzymes under stress condition, it might indicate the cellular redox state. Because the glutathione antioxidant defence system is a good indicator of cellular redox status in the cell and GSH biosynthesis through GCL is also Nrf2-dependent, we also assessed the mRNA levels of GCLc levels. Our results indicate no significant alterations in the mRNA expression of HO-1 in both HD iPSC and NSC. Of interest, we found a significant

upregulation of GCLc mRNA levels in HD-iPSC, when compared with AMS-iPSC (**Figure 3.10B**). In contrast with the observation in iPSC, we observed no changes in the expression of GCLc between HD-NSC and AMS-NSC (**Figure 3.10B**). These results indicate that antioxidant defence mechanism mediated by Nrf2 pathway may be activated particularly in HD-iPSC.

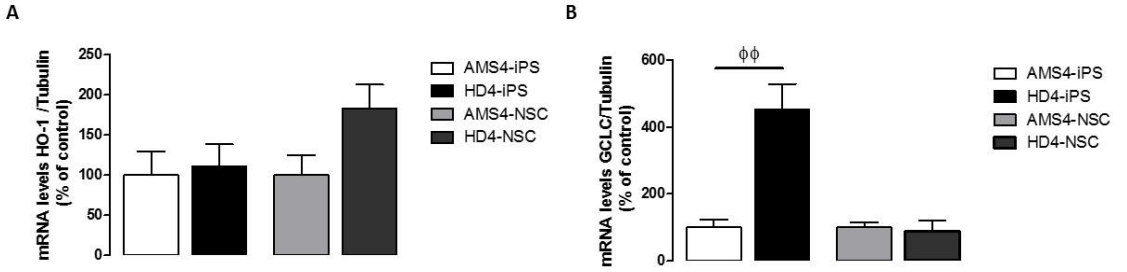

Figure 3.10– mRNA levels of Nrf2-dependent genes, HO-1 and GCLc in human HD-iPSC and NSC lines. A – mRNA levels of HO-1 in HD iPSC and NSC lines. **B** – mRNA levels of GCLc in HD iPSC and NSC lines. Results were normalized to tubulin and presented as the percentage of control. Values are expressed as the mean ± SEM of 3 to 6 experiments, run in triplicates. Statistical analysis: $^{\phi\phi}$ $p<0.01$ *versus* control (Mann's Whitney U test).

4 – DISCUSSION

In the present study we observed a decrease in both nuclear and mitochondrial encoded complex III subunits expression linked to the dowregulation of transcriptional factors, namely PGC-1α and TFAM in HD-iPSC. Secondly, we identified in HD-iPSC altered expression of PDH regulatory enzymes, PDK1 and PDP1, favouring PDH inactivation, which may promote glycolysis over OXPHOS. At last, the observations in cellular redox homeostasis indicate increased ROS levels in both HD-iPSC and HD-NSC. On the other hand, the antioxidant defences mechanisms evaluated (Nrf2 pathway; SOD2; UCP2) did not respond to oxidative stress, or were downregulated, with the exception of GCLc, in HD cell lines pointing to the loss of redox homeostasis as an early event in HD.

Stem cells, like cancer cells, present high proliferation rate, capacity of self-renewal and rely mostly on glycolysis for energy production (Varum et al. 2011, Boggs et al. 1982). The metabolic shift between OXPHOS and aerobic glycolysis is important to maintain the high proliferation capacity (Varum et al. 2011) The alteration in energy metabolism is extremely import to maintain stemness and is facilitated by reversal of ATP synthase which reduces ΔΨm, protecting the cells from ROS production, decreases ATP production and reduces mitochondrial activity (Prigione et al. 2010). Thus, mitochondrial characterization in the stem cell models revealed preferential perinuclear location, altered shape and morphology and poorly developed cristae, indicators of less active state, in opposition to adult somatic cells (Chung et al. 2010, Prigione and Adjaye 2010, St John et al. 2005, Facucho-Oliveira and St John 2009, Suhr et al. 2010). Furthermore, characterization of HD-iPSC revealed reduced ATP levels, lower OXPHOS activity and reduced oxygen consumption in HD-iPSC and HD-NSC (*Lopes C., PhD thesis*).

Mitochondrial dynamics, antioxidant defence and mitochondrial biogenesis have a common regulator,PGC-1α and its downstream effectors. Specifically, mitochondrial biogenesis is a complex process that requires the regulation of nDNA and mtDNA (Chakrabarti et al. 2011). In response to elevated ROS levels, PGC-1α induces the transcription of TFAM in the nucleus, which in term upregulates mitochondrial copy number. Most of mitochondrial complex subunits are nuclear encoded, however, some of the subunits are encoded in the mitochondria. The transcriptional activity of PGC-1α is blocked by mHTT (Cui et al. 2006). This interaction affects PGC-1α activity and subsequent mitochondrial biogenesis. PGC-1α is sensible to different cellular stimulus, such as starvation, calcium levels and, most important, oxidative stress. This mechanism is part of an extensive antioxidant response under stress conditions to restore redox homeostasis.

In this work, we observed a significant downregulation of PGC-1α in both HD-iPSC and HD-NSC. mRNA analysis of the PGC-1α downstream target, TFAM, showed a similar significant downregulation in HD-iPSC; however, in HD-NSC we observed a similar (although not significant) decrease tendency. These results corroborate previous findings of impaired PGC-1α transcriptional activity in HD, which we further explored by evaluating mitochondrial complexes subunits.

From all the mitochondrial complexes, the most consistent reports of impaired activity in caudate and the putamen from HD patients are complex II and III.(Gu et al. 1996, Bénit, Lebon, and Rustin 2009, Tabrizi et al. 1999, Mann et al. 1990). Complex II and III abnormalities have also been reported outside the nervous system, namely in skeletal muscle and lymphoblasts from HD patients (Turner, Cooper, and Schapira 2007, Sawa et al. 1999).The significant downregulation observed in the complex III subunits, CYC1, UQCR10 and MT-CYB, in the HD-iPSC model corroborates mitochondrial complex III impairment and is consistent with impaired complex III activity verified earlier in the same model (*Lopes C., PhD thesis, 2015*). The downregulation of mRNA levels from complex III subunits in HD-iPSC may explain, in part, the decreased activity of this complex. We suspect that the dowregulation of transcription factors, namely PGC-1α and TFAM, is responsible for the downregulation of complex III subunits.

Regarding complexes I and IV, there are contradictory reports in HD patients and animal models of the disease (Gu et al. 1996). The vast majority of the cases where there are alterations in complexes I and IV were determined in post-mortem HD patient's tissues in later stages of the disease; this stage is associated with advanced neurotrophy of striatum and mitochondria might be severely affected (Browne et al. 1997, Arenas et al. 1998, Brennan, Bird, and Aprille 1985). Another possible reason for the changes observed in complexes I and IV activities in late onset patients may arise from mitochondrial mutations associated with ageing. It has been shown that mtDNA is highly susceptible to mutations due to chronic exposure to ROS, which is further increased in HD patients (Tuppen et al. 2010). In presymptomatic HD brains no impairment in mitochondrial complexes I-IV activities were observed (Guidetti et al. 2001).
Interestingly, our observations indicate a significant increase in ND1 (a complex I subunit) mRNA levels in HD-iPSC. Although we did not observe compromised gene expression of ND1, we can not exclude the downregulation of complex I, since we only measured gene expression of one out of 46 subunits of this mitochondrial complex (39 nuclear- and 7 mitochondrial- encoded subunits); therefore, it is possible that other complex I subunits are compromised at early stages of the disease. In platelets from presymptomatic HD patients no alterations in complex II activity or subunit expression

levels were observed, while in symptomatic patients the authors identified correlation between decreased mitochondrial-encoded ND6 subunit and decreased activity of complex I (Silva et al. 2013), which can be a potential subunit to study in the future to further characterize mitochondrial complex I status in HD.

Moreover, the analysis of complex IV expression showed no alterations in mRNA levels between both iPSC and NSC lines. No alterations in COXIII complex IV subunit mRNA levels and unaltered complex IV activity verified in a previous study in HD-iPSC (*Lopes C., PhD thesis, 2015)* appears to indicate that complex IV is not compromised in early stages of the disease. Possibly, downregulation of complex I and IV may occur as late events in HD.

Part of the mechanism that allows the metabolic transition from iPSC into differentiated cells is regulated by PDH, a crucial enzyme that links multiple metabolic pathways such as the regulation of TCA cycle and OXPHOS, as well as glycolysis. In previous studies, hESC exhibited downregulated PDH, whereas hexokinase II was upregulated (Varum et al. 2011). Inactivation of PDH promoted by PDK1 impairs acetyl-CoA formation and decreases NADH levels, a by-product of TCA cycle, leading to a decrease in OXPHOS activity (Zhang, Hulver, et al. 2014). Additionally, several reports indicate alterations in PDH activity or expression in HD. Reduction of PDH activity was initially described in HD patients in the earlier 80s (Sorbi, Bird, and Blass 1983), which was later correlated with illness duration (Butterworth, Yates, and Reynolds 1985). Alterations in PDH mRNA were also reported in the striatum of R6/2 transgenic mice with a tendency for a decrease with ageing (Perluigi et al. 2005). Likewise, alterations in mitochondrial function related with decreased PDH complex activity were previously observed in HD by our group (Ferreira et al. 2011, Naia et al. 2017). Consistently, increased levels of phosphorylated PDH E1α at Ser292, 293 and 300 were detected in HD human cybrids (Ferreira et al. 2011) and in patient-derived iPSC (*Lopes C., PhD thesis, 2015*). Therefore, we analysed the mRNA expression of two of the enzymes responsible for PDH regulation at these 3 sites, PDK1 and PDP1, in HD- iPSC and NSC, in an attempt to establish a relationship between increased PDH complex phosphorylation and mRNA levels of PDK1 and PDP1.

Our data suggest that downregulation of PDH E1α activity is enhanced by PDK1, which mRNA levels were shown to be upregulated in HD-iPSC. mRNA levels of PDP1, PDH E1α activator, revealed to be decreased in HD-iPSC, which further corroborate the impairment in PDH activity in HD. Moreover, despite no significant changes in HD-NSC, we observed a tendency for increased PDK1 mRNA levels, a tendency also shared by PDP1. Apart from changes in PDK1 and PDP1 mRNA, other mechanisms may also be involved in altered PDH activity in HD, such as modulating of PDK1 and PDP1 activity

or a direct regulation of PDH activity by the remaining forms of PDK and by PDP2. Furthermore, NADH and acetyl-CoA are well-known PDK activators, while elevated pyruvate, ADP, CoA are responsible for the downregulation of PDK (Jeoung 2015). Overall, our results indicate that increased inactivation of PDH E1α subunit may be promoted by PDK1 upregulation and downregulation of PDP1 in HD-iPSC, indicating a possible increased dependency on glycolysis metabolic pathway.

Cellular redox homeostasis is dependent on ROS production and antioxidant response to stress. In agreement with previous reports showing elevated basal ROS levels, we observed elevated intracellular H_2O_2 levels in HD-iPSC and HD-NSC, when compared with control cells. Over-time, HD-iPSC also showed increased H_2O_2 production in a much faster rate than control cells (AMS-iPSC). Interestingly, HD-NSC presented a lower rate in H_2O_2 production than control cells. This contradictory result may be a consequence of compromised cell viability, which tends to decrease upon fluorimetric analysis. Indeed, the basal levels in HD-NSC are much higher than the control, which may increase cell vulnerability, leading to short-term degeneration upon stress induced during the assay.

UCP2 has been proposed as a cellular antioxidant defence mechanism (Mailloux and Harper 2011). In a recent study, increased UCP2 activation protected from increased ROS levels in a Parkinson's disease model (Hwang et al. 2014). The exact mechanism by which UCP2 decrease ROS levels is not yet fully understood; however, several studies using different cell lines suggested that UCP2 levels decreased ROS production by decreasing $\Delta\Psi m$, which reduces mitochondrial activity and elevates calcium levels in response to upregulated H_2O_2 levels (Mattiasson et al. 2003, Clavel et al. 2003, Teshima et al. 2003, Richard et al. 2001). *In vitro* and *in vivo* analysis demonstrated that overexpression UCP2 also protects from ischemia brain damage (Mattiasson et al. 2003). The neuroprotective role of UCP2 has also been proposed in neurodegenerative diseases. In HD, alteration in UCP2 gene expression have also been described in the literature. Chaturvedi and colleagues observed decreased UCP2 gene expression in the striatum of HD transgenic mice (Chaturvedi et al. 2010). Concordantly, despite increased basal ROS production observed in HD-iPSC and -NSC, we also observed a tendency to decrease in UCP2 mRNA levels in HD-iPSC and a significant downregulation of UCP2 mRNA expression in HD-NSC, when compared with control cells. Similar downregulation of UCP2 expression was observed in leukocytes from symptomatic patients; in cells from pre-symptomatic HD patients this effect on UCP2 mRNA levels was not observed, which might indicate that decreased UCP2 levels in leukocytes may occur as a late event in HD (Chang et al. 2012). These evidences support impairment of UCP2 antioxidant

response in HD. UCP2 has been also reported to participate in differentiation by regulating OXPHOS activity. In a previous study, it was demonstrated that, in early stages of differentiation, iPSCs had lower levels of UCP2 mRNA (Zhang et al. 2011). In the same study, overexpression of UCP2 supressed OXPHOS and restrained differentiation; however, it was also demonstrated that UCP2 knock-down did not affect stemness. Thus, upregulated ROS levels have a severe impact on iPSC and in some cases, such as glial precursor cells, regulate the differentiation process (Smith et al. 2000). Furthermore, ROS levels favours iPSC differentiation; to control this process, an upregulation of antioxidant stress genes, such as UCP2, was shown to be required (Saretzki et al. 2008, Zhang, Nuebel, et al. 2012). By contrast, in cancer cells, which present a similar metabolic profile to stem cells, increased ROS levels favoured cell proliferation without inducing differentiation (Weinberg et al. 2010). Additionally, a possible explanation for decreased UCP2 levels may arise from transcriptional dysregulation. PGC-1α was described in several studies to modulate UCP2 and UCP3 expression (St-Pierre et al. 2006, Zheng et al. 2009, Boudina et al. 2007). Concordantly, reduced mRNA levels of PGC-1α mRNA observed in both HD-iPSC and -NSC in this work are in line with previous observations, thus stablishing a possible link between transcriptional dysregulation and impairment in antioxidant defences. Overall, downregulation of UCP2 gene expression observed in HD-iPSC and HD-NSC further supports impaired redox homeostasis as an early event in HD, contributing to increased oxidative stress due to defects in cellular antioxidant defence mechanisms.

One of the first lines of defence activated against cellular oxidative status is held by ROS scavenging enzymes. SOD2 is part of the PGC-1α antioxidant response under stress signal such as hypoxia, starvation or oxidative stress (Itoh et al. 1997). Proteomic analysis in HD-iPSC revealed the downregulation of some antioxidant enzymes, such as SOD1, GST or GPx1, but not SOD2, as compared with hESC (Chae et al. 2012). Moreover, in our lab, previous analysis of SOD1 and SOD2 activity revealed unaltered enzyme activity in HD-iPSC. Concordantly, our protein analysis showed no alterations in SOD2 total levels (*Lopes, PhD thesis, 2015**)***. The activation of SOD2 is enhanced by sirtuin 3, a class III histone deacetylase responsible for the conversion of acetylated SOD2 at Lys68 into the active/non-acetylated form of SOD2 (Qiu et al. 2010). The literature is not consistent regarding sirtuin 3 levels in HD. Although our results, do not reveal major differences in acetylated SOD2 levels, a tendency for decrease was observed. Concordantly with this are the results showing an upregulation of sirtuin 3 in human HD-iPSC (C. Lopes and co-authors, *unpublished data)*.

Part of the antioxidant response includes the Nrf2 pathway, as mentioned above. The role of this pathway in HD is still not clear. Studies conducted *in vitro* and *in vivo* indicated an increase in expression of antioxidant Nrf2-dependent genes related to increased Nrf2 translocation into the nucleus in HD (Gao et al. 2015, van Roon-Mom et al. 2008). By opposition, another study observed increased Nrf2 nuclear translocation signaling, but decreased Nrf2/ARE transcriptional activity in striatal cells derived from HD knock-in mice (Ribeiro et al. 2014). In HD mice, transplantation of astrocytes overexpressing Nrf2 was shown to protect from striatal complex II inhibition (Calkins et al. 2005). Using HD-iPSC and HD-NSC we observed unaltered Nrf2 mRNA levels. Consistent with previous results showing unchanged Nrf2 or p-Nrf2 protein levels in total extracts from HD-iPSC (*Sahana, Master Thesis, 2016*), no significant changes in Nrf2 levels were detected in cytosolic or nuclear fractions from HD-iPSC. Interestingly, we observed a tendency for increase in p-Nrf2 levels in the cytosolic fraction of HD-iPSC, suggesting enhanced nuclear translocation. These results could indicate a partial response of Nrf2 following increased ROS levels, which were further confirmed through increased GCLc gene expression in HD-iPSC. Upregulation of GCLc, one of the rate-limiting enzymes for GSH biosynthesis, is in agreement with enhanced GSH levels observed previously in HD-iPSC (*Sahana, Master Thesis, 2016*).

Given that the antioxidant response mediated by UCP2 is downregulated and SOD2 remained unchanged, the elevated GSH levels mediated by the partial activation of Nrf2 pathway may be an attempt of the cell to restore cellular redox homeostasis in early stages of HD. Thus, further analysis will be required to fully understand the involvement of Nrf2 pathway in HD and the selective alterations in enzymatic levels and activity. Although our observations in HD-iPSC indicate an upregulation of GCLc gene expression, in HD-NSC no significant changes in mRNA expression were found. Additionally, HD-iPSC presented unaltered HO-1 mRNA. Even though these changes in mRNA levels do not implicate alterations in protein content, the variability in antioxidant phase II gene expression might indicate that other factors may be involved in the response to elevated ROS levels. hESC and iPSC present elevated proteasome activity, which upon differentiation tend to decrease; indeed, proteasome inhibition in these cells reduces pluripotency markers (Vilchez, Boyer, et al. 2012). As such, Nrf2 itself is regulated by the activity of proteasome system. The relation of Nrf2 and the proteasome seem to function as a positive feedback loop, restraining the Nrf2 response in stress conditions. This close relationship might explain the absence of increased Nrf2 levels in the nucleus of HD-iPSC following a stress signal. Moreover, Nrf2 fluxes in and out of the

nucleus, which may turn difficult to determine significantly enhanced levels in the organelle.

In summary, we showed that alterations in PGC-1α pathway occur as an early event in HD. These alterations at the transcriptional levels seem to affect mitochondrial biogenesis in HD-iPSC, especially mitochondrial complex III subunits, underlying the decrease in complex III activity. Observations of altered PDK1 and PDP1 mRNA expression in HD-iPSC further support previous observations of PDH inactivation by phosphorylation (C. *Lopes, PhD Thesis*), reducing OXPHOS activity and favouring glycolysis. Additionally, both HD-iPSC and HD-NSC face increased ROS levels at an early stage of HD pathogenesis. Downregulation of UCP2 mRNA expression in HD-iPSC and HD-NSC indicate an apparent mitochondrial-linked compromised antioxidant response, which might be linked to downregulation of PGC-1α pathway. Finally, analysis of antioxidant response mediated by Nrf2 pathway was inconclusive, since there was a tendency for an increase in phosphorylated Nrf2 in the cytosolic fraction of HD-iPSC, but unchanged levels in the nuclear fraction. Nevertheless, downstream enzyme of Nrf2, GCLc, was shown to be upregulated. Thus, future studies may include clarifying the role of Nrf2 in early antioxidant response, although evidences of this study point out to a partial activation of Nrf2 antioxidant response as an attempt to attenuate the redox imbalance. Overall, we provide one of the first reports using early disease models of HD where impairment of cellular redox homeostasis is observed. In agreement with previous studies, this work implicates mitochondrial dysfunction and redox changes in early stages of HD, which may contribute for HD progression.

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