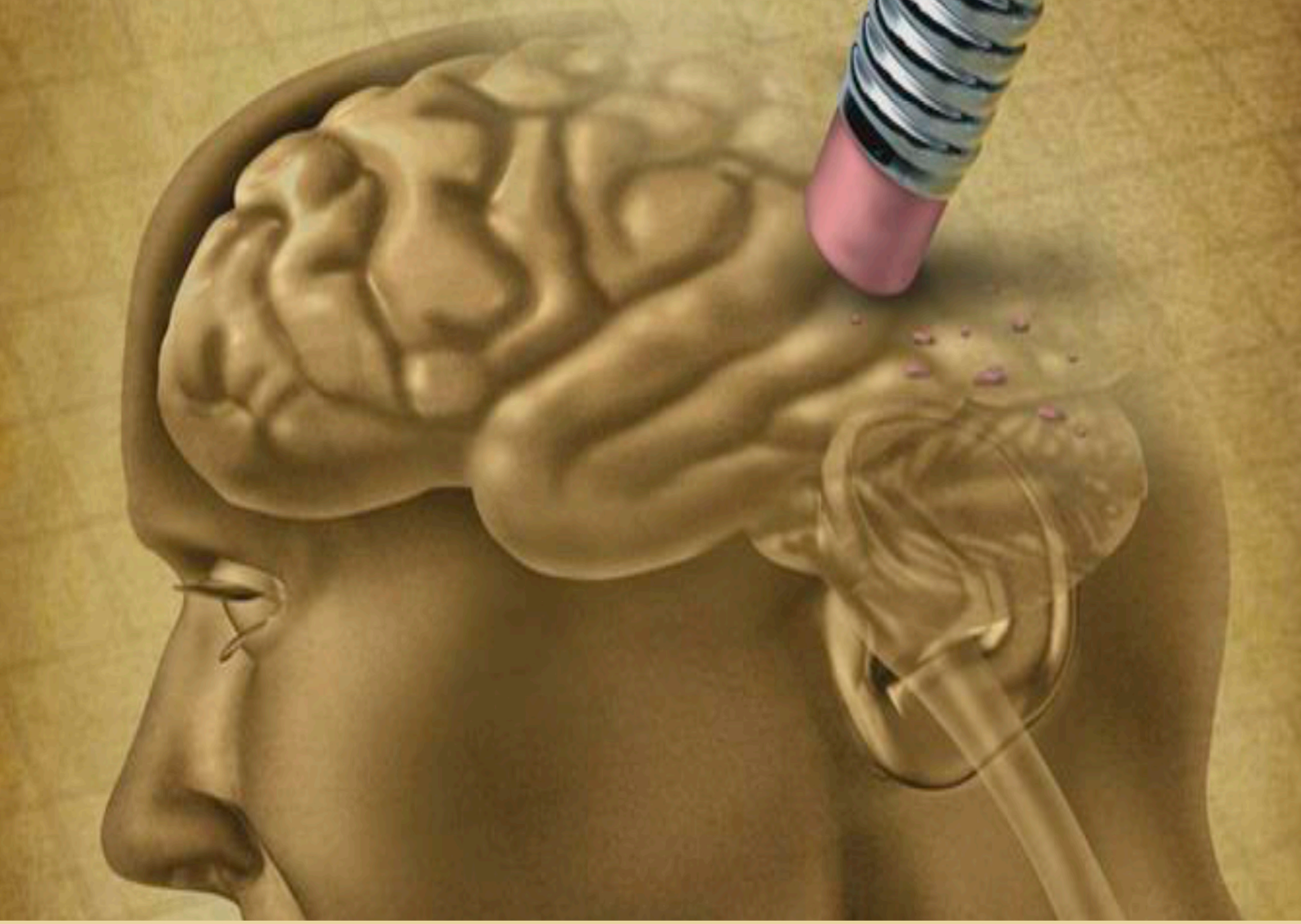

Ana Rita Monteiro

INVESTIGATION OF PLASMA ATP LEVELS IN FRONTOTEMPORAL LOBAR DEGENERATION

Dissertação apresentada na Faculdade de Farmácia da Universidade de Coimbra para a obtenção do grau de Mestre em Biotecnologia Farmacêutica sob a orientação científica da Professora Doutora Manuela Grazina

2013

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UNIVERSIDADE DE COIMBRA

Faculdade de Farmácia

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Investigation of plasma ATP levels in Frontotemporal Lobar Degeneration

Investigação do conteúdo plasmático de ATP na Degenerescência Lobar Frontotemporal

Dissertação apresentada à Faculdade de Farmácia da Universidade de Coimbra, para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia Farmacêutica, realizada sob a orientação científica da Professora Doutora Manuela Grazina (Faculdade de Medicina Universidade de Coimbra).

Rita Monteiro, 2013

Agradecimentos

A dissertação apresentada resulta do trabalho de um ano lectivo no Laboratório de Bioquímica Genética (LBG) da Faculdade de Medicina da Universidade de Coimbra. Esta dissertação não seria possível sem a ajuda de colegas, amigos e família, a quem gostaria de deixar o meu sincero agradecimento.

Em primeiro lugar, gostaria de agradecer à Professora Doutora Manuela Grazina, na qualidade de orientadora cientifica, pelo excelente acolhimento no LBG, por toda a motivação e por todo o valioso conhecimento que me transmitiu durante este ano.

Gostaria também de deixar o meu agradecimento ao Professor Doutor Luís Almeida, na qualidade de Orientador interno pela sua disponibilidade e apoio institucional.

Agradeço também aos coordenadores de mestrado, Doutor Sérgio Simões, Doutor João Nuno Moreira pela disponibilidade e ajuda.

À Dra. Marta Simões e à Mestre Cândida Mendes por toda disponibilidade, amizade e ajuda indispensável durante a realização do trabalho experimental no LBG. Muito obrigada pela inesgotável paciência que sempre tiveram comigo!

Aos meus colegas e amigos do LBG, Isabel Oliveira, Alexandre Nery, Mafalda Bacalhau, Rosa Quinta, Gabriela Andrade, Daniela Luís, Gonçalo Ferreira, José Albuquerque e Pedro Guiomar, pelo companheirismo, amizade e por tornarem os dias de trabalho em dias de alegria e boa disposição.

Gostaria também de agradecer a todos os técnicos do LBG, pelo agradável ambiente e convívio dentro deste laboratório.

Aos meus colegas de mestrado, pela amizade, partilha e entreajuda.

Aos meus colegas da licenciatura em Farmácia Biomédica da Faculdade de Farmácia da Universidade de Coimbra por todos os bons momentos, amizade e ajuda, nestes cinco anos de permanência em Coimbra.

A todos os meus amigos de Vila Franca de Xira, pela amizade e companheirismo.

À minha acarinhada tuna, "Mondeguinas", agradeço toda a amizade e compreensão que sempre tiveram comigo por não poder ter estado tão presente este ano. A vossa boa disposição foi, sem dúvida, indispensável nos momentos mais difíceis.

À minha família, por acreditarem sempre em mim e nunca me deixarem desistir dos meus sonhos.

A todos os doentes e seus familiares por tornarem este estudo possível.

A todos vocês, um sincero muito obrigada!

Levarei comigo todos os bons momentos e espero que a nossa amizade prevaleça por muitos anos.

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ABBREVIATIONS

λ	Wavelength
Δp	Proton-motive force across the inner membrane of mitochondria
ΔpH	pH variation
AD	Alzheimer's disease
ADL	Activities of daily living
ADP	Adenosine Di-phosphate
ALS	Amyotrophic lateral sclerosis
AMP	Adenosine monophosphate
AMPA	2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) proprionate
ATP	Adenosine Tri-phosphate
aβ	Amyloid beta
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium
CDR	Clinical Dementia Rating
CH	Correction for haemolysis
CHUC	Centro Hospitalar e Universitário de Coimbra
CNS	Central nervous system
COX	Cytochrome c oxidase
CS	Citrate synthase
EDTA	Ethylenediamine tetraacetic acid
$FADH_2$	Flavin adenine dinucleotide
FDA	Food and drug administration
FTLD	Frontotemporal lobar degeneration
FTLD-FUS	Frontotemporal lobar degeneration with fused-in-sarcoma proteins
FTLD-MND	Frontotemporal lobar degeneration with motor neuron disease
FTLD-tau	Frontotemporal lobar degeneration with inclusions of Tau protein
FTLD-TDP	Frontotemporal lobar degeneration with inclusions of TDP-43
FTLD-U	Frontotemporal lobar degeneration ubiquitin-positive
GABA	Gamma-aminobutyric acid
Hb	Haemoglobin
HD	Huntington´s disease
MIM	Mitochondrial Inner membrane
Ψm	Mitochondrial membrane potential

IMS	Intermembrane space
Mg^{2+}	Magnesium
MMSE	Mini mental state examination
MPT	Mitochondrial permeability transition
mtDNA	Mitochondrial DNA
mV	milivolts
NAD	Nicotinamide adenine dinucleotide
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
NO	Nitric oxide
OXPHOS	Oxidative phosphorylation
PBS	Phosphate-buffered saline
PD	Parkinson´s disease
Pi	Inorganic phosphate
PNFA	Progressive non-fluent aphasia
PPA	Primary progressive aphasia
RLU	Relative light units
ROS	Reactive Oxygen Species
sd	Standard deviation
SD	Semantic dementia
SEM	Standard error of mean
TCA	Tricarboxylic acid cycle
TNF	Tumour necrosis factor

This study was financed by Portuguese Foundation for Science and Technology (FCT), with the project PTDC/SAL-EPI/121811/2010 (FCT) and partially supported by FCT project PEst-C/SAU/LA0001/2011.

RESUMO

Nos últimos anos, a disfunção mitocondrial e o stresse oxidativo têm sido apontados como intervenientes principais no processo neurodegenerativo subjacente a doença de Alzheimer, Parkinson, Huntington, Esclerose amiotrófica lateral e mais recentemente, na Degenerescência lobar frontotemporal (FTLD).

A diminuição da actividade dos complexos da cadeia respiratória mitocondrial tem como principal consequência o aumento das espécies reactivas de oxigénio e a diminuição da produção de ATP pela fosforilação oxidativa, o que leva ao comprometimento de diversos processos metabólicos indispensáveis à sobrevivência celular. Deste modo, o conteúdo plasmático de ATP poderá ser um bom indicador do défice energético resultante da diminuição da função mitocondrial nos tecidos de maior aporte energético, nomeadamente no cérebro dos doentes com FTLD.

Usando um método de bioluminescência, foram analisados os níveis plasmáticos de ATP em 40 doentes com diagnóstico provável de FTLD, seguidos na Unidade de Neurologia do Centro Hospitalar e Universitário de Coimbra, e em 20 indivíduos saudáveis.

Os resultados obtidos mostram que a concentração plasmática de ATP nos doentes com FTLD está significativamente diminuída, relativamente ao grupo controlo, principalmente nos doentes com défice cognitivo de acordo com a escala MMSE. A actividade do complexo I da cadeia respiratória mitocondrial nos linfócitos destes doentes apresenta uma correlação positiva com a concentração plasmática de ATP, o que reflecte uma possível diminuição da actividade deste complexo associado a uma menor produção de ATP. Os nossos resultados mostram também uma correlação negativa entre o conteúdo plasmático de ATP e a atividade da ATP-sintetase nos linfócitos dos doentes com FTLD, reforçando a evidência de disfunção mitocondrial e consequente défice energético no processo neurodegenerativo que ocorre na FTLD.

Por outro lado, uma vez que o ATP pode atuar como sinalizador, nomeadamente na libertação de glutamato, pode desempenhar um papel importante na desregulação neuroquímica que ocorre na neurodegenerescência.

Os resultados do presente trabalho são originais e adicionam um conjunto significativo de dados ao conhecimento dos mecanismos envolvidos na patogénese da FTLD.

Palavras-chave: Degenerescência lobar frontotemporal, ATP, mitocôndria, cadeia respiratória mitocondrial, défice energético

BACKGROUND

1. Frontotemporal lobar degeneration

Frontotemporal lobar degeneration (FTDL) is the second most common type of primary degenerative dementia and comprises a spectrum of clinically, pathologically and genetically heterogeneous neurodegenerative disorders with often asymmetrical atrophy of the frontal and anterior temporal brain lobes [1–3].

In 1892, Arnold Pick described the first patient with progressive aphasia and lobar atrophy, and later in 1911, Alois Alzheimer reported the presence of argyrophilic neuronal inclusions at neuropathological examination, usually known as 'Pick bodies' [3].

Usually, FTLD occurs between 35-75 years and is more common in individuals with a positive family history of dementia, although the age of onset in familiar and sporadic cases does not differ significantly [4,5]. Several epidemiological studies suggest that men and women are equally affected and the duration of illness from onset to death is between 6-8 years, with a wide range of 2-20 years [5,6].

In spite of the difficulty in correlate all clinical symptoms with histopathological hallmarks and genetic mutations, some experts have tried to reach a consensus nomenclature for the better understanding for physicians and scientists [7,8]. Presently, there are two nomenclatures of Frontotemporal lobar degeneration. Some American authors designed FTD as a general term for the clinical syndrome (including the behavioural and language variants) and the term FTLD is used for all associated pathologies [7]. In other hand, some European authors use the term FTLD to both clinical and histopathological characterization, whereas they refer to FTD only for the clinical subtype with strong evidence of behavioural abnormalities [7]. In the present study, the term FTLD is used to designate all histopathological variants (e.g. FTLD-protein) and the term FTD is applied only to behavioural variant (bv-FTD) [9].

FTLD spectrum can be clinically categorized into three main frontotemporal dementia syndromes: (i) behavioural variant (bv-FTD), (ii) semantic dementia (SD), and (iii) progressive nonfluent aphasia (PNFA) [1,10]. Some authors include the SD and PNFA in the same language variant termed as primary progressive aphasia (PPA) [1].

The bv-FTD is mainly characterized by progressive impairment of behaviour, personality changes, disinhibition, apathy and diet modifications [11]. On the opposite, patients with PNFA have motor speech deficits, characterized by non-fluent and laboured language articulation. Other language features such as slow rate of speech, apraxia of speech, anomia, agrammatism and impaired word repetition are commonly observed in those patients [1].

Patients with SD have difficulties with the understanding of sentences' meaning and lose the ability to recognize the significance of faces, objects, and other sensory stimuli [5]. In contrast to SD, patients with PNFA have usually preserved social and behavioral function as other language features like single-word and object comprehension in the early stage of the disease [1]. Despite the first diagnosis of a FTLD patient, it is important to note that, with disease evolution, other distinct clinical phenotypes usually start to show overlap, possibly reflecting the progressive involvement of other brain regions [12].

The most common histopathological hallmarks of FTLD are mainly accumulation of microtubule-associated protein tau or/and accumulation of TAR DNA-binding protein (TDP-43) [7,13]. Tau protein plays a central role in maintaining neuronal integrity and axoplasmic transport [7]. In Alzheimer's disease (AD) the presence of accumulated tau protein in affected tissues has also been detected, and are the major component of the neurofribrillary tangles [7]. Tau protein abnormalities result from mutations in *MAPT* gene, located at chromosome 17p21, and are usually associated with FTLD clinical subtypes, such as bv-FTD, PNFA, FTLD with Pick's bodies, progressive supranuclear palsy (PSP), corticobasal degeneration (CBD) and argyrophilic grain disease (AgD) [7,9,13].

In the last years, most cases of tau-negative FTLD were thought to have no inclusions, but later several studies concluded that some of those cases were immunoreactive for ubiquitin and became known as FTLD with ubiquitinated inclusions (FTLD-U). Because almost all ubiquitin-positive cases have the presence of mutant TDP-43 protein, FTLD-U has become known also as FTLD-TDP [7]. This histopathological phenotype is also associated with mutations in progranulin, encoded by *PGRN* gene, located at chromosome 17q21.32, and in valosin-containing protein, encoded by *VCP* gene, located at chromosome 9p13.3, [7,13] (Figure 1). Nevertheless, some patients with immunoreactive inclusions of ubiquitin but TDP-43-negative, have inclusions of fused-in-sarcoma protein (FUS), associated to *FUS* gene (chromosome 16p11.2) mutations, referred as FTLD-FUS [10] (Figre1). Recent studies have been demonstrated that a number of patients without TDP-43 pathology, have detectable abnormalities in ubiquitin proteasome system (FTLD-UPS) [7]. Additionally, there is also a clinical overlap between the FTLD-TDP and mutations in chromosome-9p-linked to Motor Neuron Disease, known as FTD-MND [3,5,7] (Figure 1).

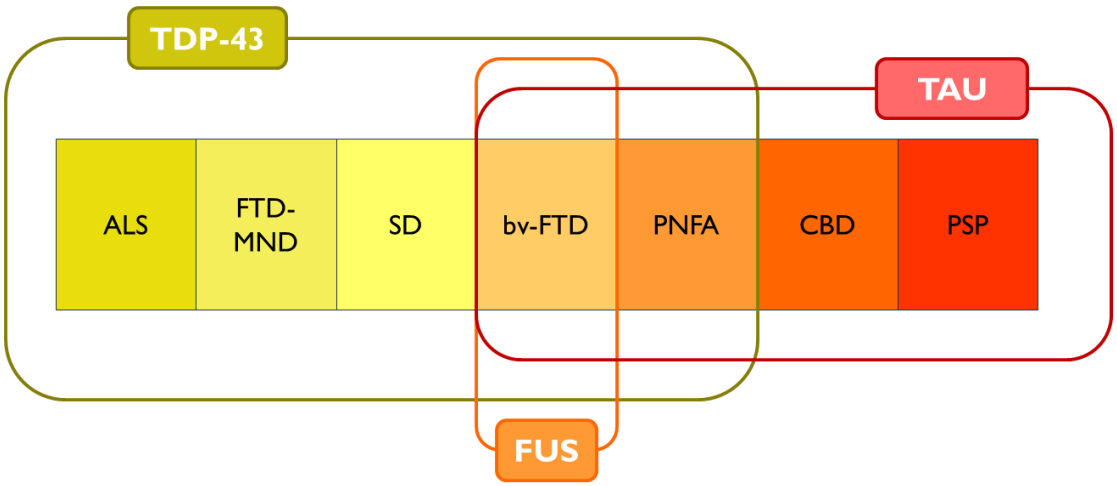

Figure 1 - Clinical and histopathological overlap in FTLD. Adapted from [3]. ALS – Lateral amyotrophic sclerosis; FTD-MND – Frontotemporal lobar degeneration with Motor Neuron Disease; SD – Semantic Dementia; bv-FTD – behavioral variant of FTLD; PNFA – Progressive Non-fluent aphasia; CBD – Corticobasal degeneration; PSP – Progressive supranuclear palsy; TDP-43 - DNA-binding protein; TAU – microtubule-associated protein tau; FUS – fused-in-sarcoma protein.

However, despite the advances of immunochemistry techniques, there are several tau-negative and ubiquitin-negative cases that were considered as dementia lacking distinctive histopathology (DLDH), currently referred as FTLD with no known inclusions (FTLD-ni) [1,7,9,14]. The presence of neurological abnormalities are usually associated with shorter survival of FTLD patients [5].

One important source of information about histopathology of associated clinical FTLD diagnosis is an inherited mutation in a gene coding for a protein with specific abnormality. Recently, the presence of abnormal proteins in biological fluids has conducted to the identification of genetic alterations, favoring genotype-phenotype correlation. Accordingly, genetic characterization has become an important tool in diagnosis and treatment approach, particularly in familial variants of FTLD [15–18]. Approximately 40% of FTLD patients have a positive family history of dementia, suggesting a strong genetic hereditary contributing factor for this disease; in most cases, there is an autosomal dominant inheritance pattern [1,6]. The first-degree relatives of a FTLD patient have 3.5 times more probability to develop dementia before 80 years of age when compared to subjects without family history of disease [1].

The FTLD patients usually show deficiencies in serotonin and dopamine neurotransmitter systems, while there is no evidence of impairment of acetylcholinergic and GABAergic system [5,19]. However, there has been an increased interest in the glutamatergic system in FTLD, since the FDA approval of Memantine, a low to moderate affinity noncompetitive N-

methyl-D-aspartate (NMDA) receptor antagonist, for treatment of other neurodegenerative disease, such as AD and recently also for FTLD [1,19]. Because of the neuroprotective effect of Memantine in slowing disease progression of FTLD, the glutamatergic system seems to have an important role also in this disease [19].

Currently, there is no plausible explanation for selective vulnerability of individual neurons and particular populations of neurons, but several factors, such as metabolic activity, excitatory amino acid input, numbers and types of excitatory amino acid receptors, Ca^{2+} homeostasis and presence of free-radical scavengers can be crucial to determine the onset of disease development [20].

Because mitochondria play a central role in so many fundamental metabolic processes for cell survival, mitochondrial dysfunction has long been associated as an important marker of the pathogenesis of many common age-related neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), Amyotrophic lateral sclerosis (ALS) and recently in FTDL [21–24].

2. The mitochondrion

Mitocondria are membrane bound organelles present in all cells of the body (except in erythrocytes) and are usually designated as the "powerhouse of the cell" because they are responsible for almost all adenosine 5'-triphosphate (ATP) production [25,26]. Mitochondria are maternally inherited and essential to maintaining tissues with high ATP demand healthy, as brain and muscle [27]. They are dynamic organelles, which trafficking is critical to their strategic intracellular distribution, presumably according to local energy demands [25,28]. In neurons, presynaptic terminals, at the ends of axons, and at postsynaptic terminals, at the ends of dendrites, where bioenergetic demand is particularly high, are enriched in mitochondria [25].

The main energy fuel for cellular metabolism is glucose, which is catabolized in three processes: glycolysis, tricarboxylic acid cycle (TCA or Krebs cycle) and oxidative phosphorylation (OXPHOS) [29,30]. To iniate the glycolysis, mitochondrion needs four molecules of ATP and two of NADH, per molecule of glucose [30]. Then, the glucose is partially converted through a series of enzyme-catalyzed reactions into two pyruvate molecules [30]. Then, Pyruvate is catalyzed by pyruvate dehydrogenase complex into Acetyl-CoA in the matrix of mitochondrion [29,30]. Later, Acetyl-CoA is catalyzed into NADH, $FADH_2$ and CO_2 in TCA, that also occurs in the mitochondrion matrix providing reduced equivalents to drive OXPHOS in the mitochondrial respiratory chain (MRC) system [30,31].

The MRC consists in five complexes (complexes I–V, NADH-coenzyme Q reductase, succinate-coenzyme Q reductase, ubiquinol-cytochrome c reductase, cytochrome oxidase and ATP synthase, respectively), two mobile electron carriers (ubiquinone/coenzyme Q and cytochrome c), all located in the mitochondrial inner membrane (MIM) of mitochondria [26,32] (Figure 2). The energy released by NADH and $FADH_2$ oxidation are used to generate a proton gradient across MIM that will be used by ATP-synthase to phosphorylate ADP and produce ATP, at the same time that protons return to the mitochondrial matrix through a channel in complex V [26,33,34].

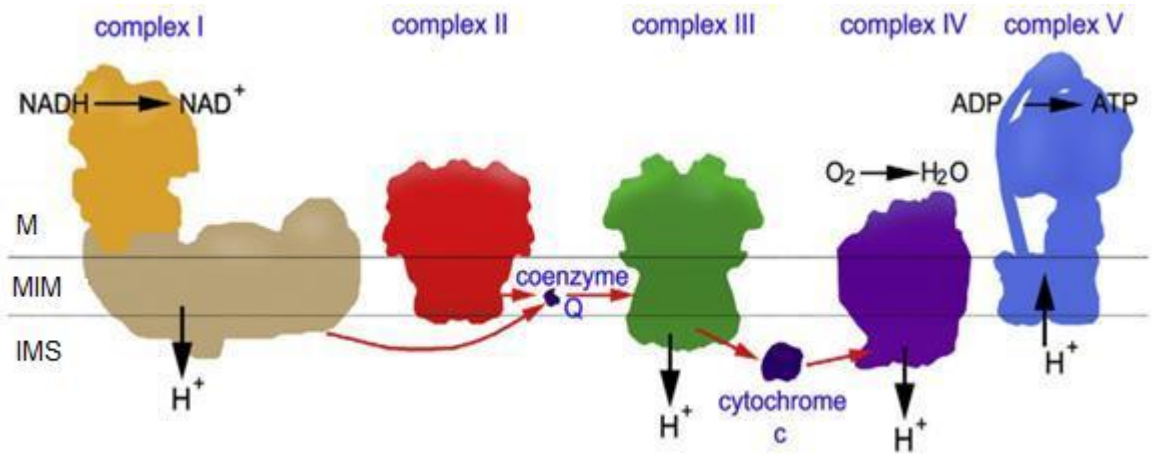

Figure II – Mitochondrial respiratory chain complexes. Adapted from Dudkina *et al.,* 2010 [35]. M- matrix; MIM- mitochondrial inner membranne; IMS - intermembrane space.

The regulation of ATP production by ATP-synthase is determinated by cellular energy demand and by other factors, such as intramitochondrial substrate concentration (eg. ADP) or Ca^{2+} levels [36]. An important key parameter for determining energy status of mitochondria is the proton-motive force across the inner membrane (Δp) [29]. In the presence of physiological concentrations of P_i and Mg^{2+}, brain mitochondria maintain a total Δp of 220mV with a membrane potential between 150-180 mV [37]. All MRC complexes have subunits encoded by nuclear and mitochondrial DNA (mtDNA), except complex II, wich is exclusively encoded by the nuclear genome [26].

In addition to ATP synthesis, mitochondria are also responsible for other important regulatory metabolic pathways, such as steroid synthesis, modulation of Ca^{2+} signaling, amino acid biosynthesis, fatty acid oxidation, insulin regulation and cell death [26,28,38,39].

2.1 Mitochondria and cell death

Mitochondria are also involved in the regulatory process of cell death by necrosis and apoptosis [23,34]. In neurons, both of these conditions can either coexist or be sequential events depending of the severity of the initiating insult and ATP levels, among other factors[23].

There are two main apoptotic pathways described in literature: the extrinsic pathway, involving the activation of death receptors, and the intrinsic mitochondrial pathway, both involving the activation of a caspase cascade [40]. Some stimulus, such as growth factor deprivation, ionizing radiation, and several chemical agents can activate the mitochondrial pathway of apoptosis inducing cytochrome c release [40]. Characteristic features of

apoptosis are condensation of nuclear chromatin and regulated action of catabolic enzymes (proteases and nucleases) [41,42]. In contrast, necrosis does not involve any regular DNA and protein degradation pattern and is followed by cytoplasm swelling, which occur shortly before cell membrane rupture [43].

If cell death can occur in two different pathways, depending of the intensity of the initiating insult, this suggests the possibility of a downstream event that could control and decide the progression of cell death to apoptosis or necrosis [44,45]. Accordingly, individual cell death can be decided by available ATP stores originated from OXPHOS and glycolysis. In apoptosis, the maintenance of a certain ATP level is needed to initiate cell death, because many of these subsequent events are ATP-dependent processes [44]. By contrast, cell death by necrosis is associated with low ATP levels and could be initiated by toxic insults, such as glutamate excitotoxicity [23]. Thus, ATP levels seem to be an important switch for decision between cell death by apoptosis and necrosis in different cellular tissues, including central nervous system (CNS) cells (Figure 3) and may contribute to the neurodegeneration process in diseases like FTLD.

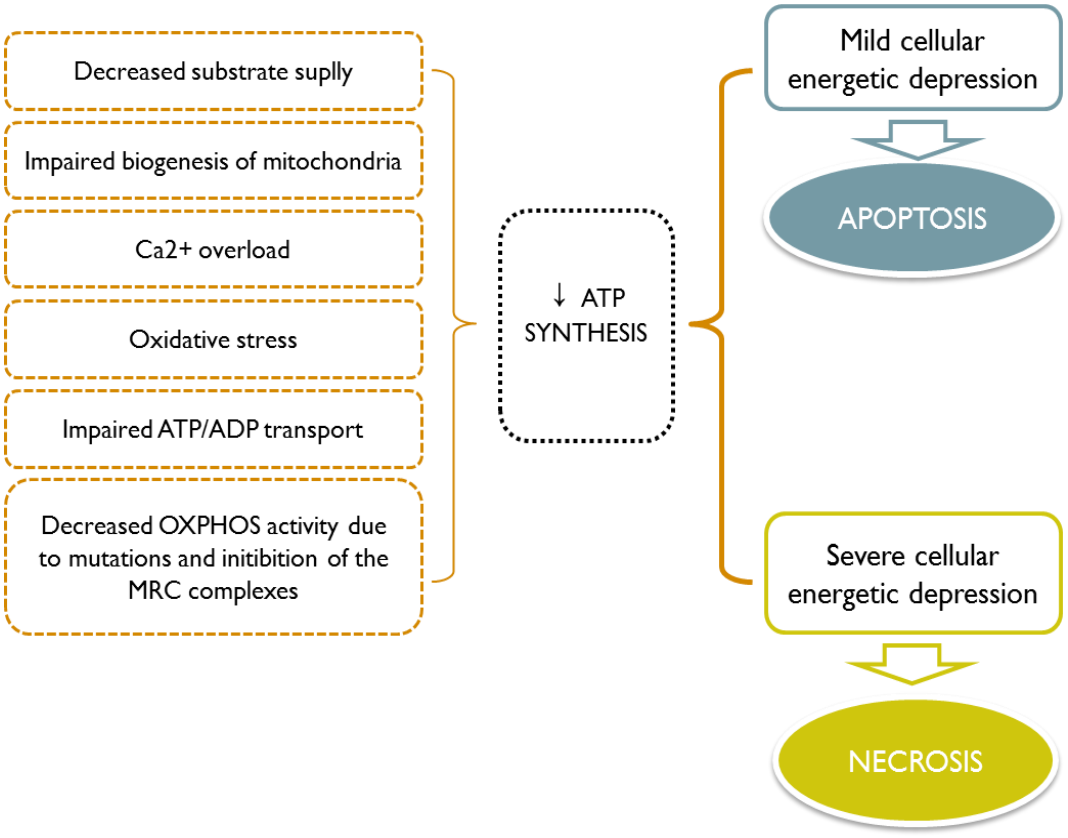

Figure III – Influence of ATP synthesis in type of cell death. In mild cellular energy decrease, there is sufficient ATP to induce cascade of apoptosis pathway. When energy impairment is severe, the cell cannot initiate cell death through apoptosis and switch to necrosis. From Seppet *et al.* (2009) [45].

3. Mitochondrial cascade hypothesis for neurodegenerative diseases

The first hypothesis explaining the development of AD derived from the studies that emphasize the importance of mutant *β*-amyloid protein (a*β*) deposition, as a consequence of mutations in *APP* gene that codifies the precursor of amyloid protein. This theory became known as "amyloid cascade hypothesis" [46]. In Tauopathies (as FTLD-tau and some forms of AD), the presence of tangles with aggregated protein tau were thought to be intimately related with disease development [47]. However, this hypothesis does not specify what initiates the common sporadic form of AD because in almost all cases there is no identifiable mutation responsible for the disease development [46,47].

To fill this gap, Swerdlow and Khan proposed, in 2004, the "mitochondrial cascade hypothesis", with the intention to build a bridge between the "amyloid cascade hypothesis" and the central role of mitochondria in aging and in neurodegenerative process [46,47]. In fact, they suggested that inheritance of mitochondrial baseline function can influence how mitochondria may change with aging, and AD histopathology and symptoms emerge when mitochondria reaches a pathologic threshold [47]. An interaction between a primary genetic defect involving energy metabolism and the impairment of mitochondrial function that accompanies normal aging and structural changes in MRC complexes, would subsequently lead to a decrease in ATP production and possibly to electron leakage, accumulation of toxic ROS, and release of apoptotic-inducing factors that would ultimately lead to neuronal loss [26,28,48]. According to this idea, Yao et al. showed that mitochondrial dysfunction and bioenergetics impairment can occur early in pathogenesis of AD and precede the development of observable plaque formation, in a mouse AD model [20].

During normal embryogenesis, reduced cell energy promotes physiologically tau phosphorylation. Repeated cell division in normal embryogenesis uses considerable high quantities of cell energy and energy storage is not possible during this phase. Mitochondrial cascade hypothesis also suggest that a similar mechanism can occur in neurodegenerative diseases with presence of histopathological hallmark of tau protein [47].

Thus, impaired mitochondrial function is one possible mechanism responsible for bioenergetic failure and some cell demise consequences in neurodegenerative diseases as FTLD [2].

4. Mitochondrial dysfunction in neurodegenerative diseases

It is widely recognized that there is a decline of basal metabolic function of mitochondria and MRC activity becomes less eficient during aging. There are evidences that old mitochondria are morphologically altered and functionally produce more ROS and less ATP [49]. The complexes I and IV activity seems to be the most affected by aging process; in contrast, the activity of complexes II and III appear to be the most preserved [49].

There are few studies evidentiating an impairment of MRC activity in neurons and other periphery tissues in FTLD [50]. However, FTLD pathology is similar in some points with AD and other neurodegenerative diseases, such as PD, HD and ALS [51].

In AD, there are previous studies showing decreased activity of MRC complexes I, III and IV in neurons, platelets and lymphocytes from blood samples and posmorten brain tissue [24,26,49,52]. Cardoso et al. (2004) showed a deficiency in cytochrome c oxidase (COX, complex IV) activity in AD platelets [53]. These results were consistent with the study of Bosetti et al. (2002) that have previously reported a decreased COX activity in human platelets and in brain tissue samples from hippocampus [54].

In tauopathies, Schulz et al. (2012) demonstrated a substancial complex I deficiency followed by a decrease in mitochondrial ATP production [50,55]. Complex I is an important target for ROS and abnormal activity of this complex usually leads to more ROS production and disturbance of the mitochondrial membrane potential, $m\Delta\Psi$ [56]. In fact, there are evidences in literature that reduced activity of one or more OXPHOS enzymes can compromise ATP synthesis and further increase ROS production, which often causes structural and functional cell membrane alterations and, in the limit, leading to neuronal death [53].

Although, MRC is not the only system in mitochondria that is affected in neurodegenerative diseases, an altered activity of OXPHOS enzymes had also been reported [45]. For example, there are evidences of reduced activity of pyruvate dehydrogenase and ketoglutarate dehydrogenase complexes in AD neurons [47].

Moreover, published data support the idea that mitochondrial enzyme impairments in AD brain are reflected in peripheral tissues, particularly in platelets, fibroblasts, as well as in lymphocytes [52–54]. Additionally, a complex I reduced activity has been reported in a FTLD patient [2].

5. Energy impairment and excitotoxicity

In the last decades, a close association between the energy state of nervous cells and glutamate neurotoxicity has been suggested [57]. As previously mentioned, the glutamatergic system also seems to have an important role in FTLD [19].

Glutamate is the most abundant excitatory amino acid neurotransmitter in the brain and plays a proeminet role in fast synaptic plasticity and in important cognitive functions like learning and memory [58]. Moreover, glutamate can serve as an alternative energy supplier in the absence of glucose in neurons [58,59].

Neurotoxicity-evoked by glutamate, designed as "excitotoxicity", has been implicated in neurodegeneration linked to pathological conditions, associated with failure of energy metabolism, such as brain ischemia, hypoglycemia and cerebral trauma, as well as, with some chronic neurodegenerative diseases such as Alzheimer's disease, Huntington's disease, Parkinson's disease and amyotrophic lateral sclerosis [57,58,60,61].

The toxic effects of glutamate was first observed at 1957 by Lucas and Newhouse in degeneration of retinal cells after exposure to noxious concentrations of glutamate [62]. Olney and co-workers in 1969, coined the term "excitotoxicity" when described the intracranial brain lesions in response to subcutaneous injections of glutamate in infant and adult mice [63].

Glutamate receptors can be divided in two major groups: metabotropic and ionotropic receptors [58]. Ionotropic receptors include N-methyl-D-aspartate (NMDA) receptors, 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) propionate (AMPA) receptors and kainate receptors [64]. Under physiological conditions, glutamate is released from glutamatergic nerve terminals and causes depolarization of post-synaptic receptors, leading to Mg^{2+} release from NMDA receptor channel and allowing Ca^{2+} influx into cytosol [58,65].

The excitotoxic neuron injury can be the consequence of direct or indirect pathways, and both have the same finishing course to induce cell death. In the direct pathway, glutamate transporters are also altered in both quantity and structure, and there is a less effective process in removing glutamate from the extracellular space [66]. This results in increased concentration of glutamate in postsynaptic receptors and it remains for longer periods [66]. Prolonged activation of NMDA receptors leads to a number of deleterious consequences, such as impairment of intracelular calcium buffering, ROS production and activation of the mitochondrial permeability transition pore (MPTP) and ultimately in neuronal death [39,58]. In indirect excitotoxic pathway, a primary bioenergetic impairment causes non-toxic

glutamate levels to become lethal and causing membrane depolarization of the pos-synaptic terminals and excessive Ca^{2+} influx through NMDA receptors [58]. According to this hypothesis, Del Rio et al. (2007) proved that an increase of extracellular concentration of glutamate does not cause neuronal death, unless impairment of energy metabolism occurs simultaneously with a severe decrease in cytosolic ATP levels [57].

One of the primary physiologic functions of Ca^{2+} in mitochondria is stimulation of OXPHOS and ATP production [39]. Neurons can control intracellular Ca^{2+} levels through a complex interplay between Ca^{2+} influx, Ca^{2+} efflux, Ca^{2+} buffering, and internal Ca^{2+} storage [64]. Moreover, Ca^{2+} inside mitochondria regulates several important biological mechanisms, such like allosteric activation of pyruvate dehydrogenase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase, as well as stimulation of ATP synthase (complex V) activity [39]. When intracellular Ca^{2+} concentrations increase, mitochondria can buffer the excess of Ca^{2+} through a sodium-calcium exchanger into mitochondrial stores, that results in decrease of mitochondrial membrane permeability, increasing ROS generation and, consequently, enhance mtDNA mutations that results in a decline of OXPHOS activity [59].

This excitotoxic process can be divided into three phases of cytoplasmatic Ca^{2+} elevation: the initial peak after exposure of glutamate, a subsequent plateau and after the begining of the "delayed Ca^{2+} deregulation" phase, when the continued presence of glutamate is not obligatory and which culminates in the irreversible failure of cytoplasmic Ca^{2+} homeostasis [67]. These excessive influx of Ca^{2+}, via NMDA receptors, attenuates the $m\Delta\Psi$, and leads to the opening of the MPTP and initiates cell death cascade by apoptosis [39,68].

From these evidences, mitochondrial Ca^{2+} loading seems to be one critical step in acute glutamate excitotoxicity and several experiments have been done for therapeutic aproaches in blocking NMDA recptors or in removing Ca^{2+} from the cytosol of the affected neurons [64,65,68]. Presently, there are some NMDA receptors antagonists that are intended to modulate glutamatergic function and reduce neuronal death induced by excitotoxicity in some neurodegenerative disorders, such as AD [60,65]. Nonetheless, NMDA receptors antagonists had not proven clinically the most wanted treatment effects, and new approaches have been designed to avoid glutamate exposure by reduce circulating glutamate levels in the blood and consequently lowering glutamate concentrations in the brain [58].

In conclusion, mitochondrial dysfunction, energy impairment and excitotoxic neuronal injury seems to be one common point among different neurodegenerative diseases, such as AD, PD, ALS and FTLD, that, in the end, share the same cell fate, with different but similar implicated neuropathological mechanisms.

Massive extracellular release of ATP has been associated with metabolic stress, brain ischemia and trauma, which make purinergic system an important mechanism in neurodegeneration aetiopathology [69].

6. Extracellular ATP

Adenosine 5'-triphosphate (ATP) is a nucleotide found in cytosol of every cell, and is the major energy source for maintenance of cell survival, especially in the brain, because neurons are cells wih a high energy demand [24].

On the other hand, some properties of ATP make it an ideal molecule for cell–cell signaling: it is a small, rapidly diffusing molecule, highly unstable and not abundant in the extracellular environment [69,70]. In fact, extracellular ATP has been involved in a wide range of physiological processes, such as neurotransmission, smooth muscle contraction, endocrine secretion, vasodilation and more complex processes, such as immune cell regulation and neuroinflammation, pain, modulation of cell proliferation, differentiation and death, regeneration, male reproduction, fertilization and embryonic development [69].

Virtually, every type of eukaryotic cell can release ATP, and in most of them the intracellular ATP concentration are in the milimolar range (3-10 mM) [71,72]. Although, in the extracellular environment, ATP concentration is considerably lower (20-100nM) [72].

Extracellular ATP acts alone as a neurotransmitter, neuromodulator or growth factor, or is co-released with acetylcholine, noradrenaline, GABA and glutamate in CNS cells [73,74]. It has also been identified as an autocrine/paracrine signal modulator by activation of purinergic receptors in the plasma membrane of the exocrine cell and neighborhood cells [69,70,75,76].

There are several purinergic receptors with different actions (Table 1). The P1 receptors also belong to the purinergic receptor family but its main agonist is adenosine, not ATP [73]. The P2 receptors were divided into P2X and P2Y receptors due to their pharmacologic and molecular cloning properties. Currently, there are 7 subtypes of ionotropic P2X receptors (P2X1, P2X2, P2X3, P2X4, P2X5, P2X6 and P2X7) and 8 subtypes of metabotropic P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 and P2Y14) known [72,77].

Table A

Endogenous agonist of purinergic P2X and P2Y receptors and its distribution in the human body. Adapted from Burnstock *et al.* (2006) and Abbracchio *et al.* (2009) [73,74].

P2X	**Endogenous agonist: ATP**	
	P2X1	Smooth muscle, platelets, cerebellum, dorsal horn spinal neurons
	P2X2	Smooth muscle, CNS, retina, chromaffin cells, autonomic and
	P2X3	Sensory neurons, NTS, some sympathetic neurons
	P2X4	CNS, testis, colon
	P2X5	Proliferating cells in skin, gut, bladder, thymus, spinal cord
	P2X6	CNS, motor neurons in spinal cord
	P2X7	Apoptotic cells in, for example, immune cells, pancreas, skin
P2Y	**Endogenous agonists: ATP, ADP, UTP, UDP, NAD^+**	
	P2Y1	Epithelial and endothelial cells, platelets, immune cells, osteoclasts
	P2Y2	Immune cells, epithelial and endothelial cells, kidney tubules, osteoblasts
	P2Y4	Endothelial cells
	P2Y6	Some epithelial cells, placenta, T cells, thymus
	P2Y11	Spleen, intestine, granulocytes
	P2Y12	Platelets, glial cells
	P2Y13	Spleen, brain, lymph nodes, bone marrow
	P2Y14	Placenta, adipose tissue, stomach, intestine, discrete brain regions

The P2X receptors are called "fast receptors" because are transmitter-gated cation channels and act within milliseconds when ligand bind and consequently open the ion channel. As a consequence of their properties and relatively low affinity for ATP (in the μM range), P2X receptors mediate fast ATP signaling over short distances, for example, in response to the release of ATP from a synaptic vesicle [76]. By contrast, P2Y receptors are stimulated at low concentrations of ATP (nM range), and ATP binding triggers second-messenger cascades that amplify and prolong signal duration [76]. These receptors have neuromodulatory functions because they can detect lower ATP concentrations over longer distances from the release site [76]. In nervous system, P2 receptors are widely expressed both in neurons and in cells involved in neuroinflammatory responses, such as astrocytes and microglial cells [69].

Moreover, a number of physiological and pathophysiological stimuli including ischemia, hypoxia, platelet aggregation, sympathetic nerve stimulation and cell damage, can lead to an increase in extracellular ATP concentration, despite the strict control exerted by ectonucleotidases, which act to maintain ATP and its metabolites (AMP and ADP) in low physiological concentrations [69,78,79]. The extracellular ATP is hydrolyzed by ectonucleotidases CD39, which converts ATP and ADP to adenosine monophosphate (AMP), and CD73, which converts AMP to adenosine [71,72]. The nucleotide-degrading system also plays an essential role in the purinergic signaling because ATP hydrolysis generates adenosine, which is also a powerful modulator of cell functions by activation of P1 receptors [72]. Then, extracellular ATP concentration can change as a consequence of enhanced ATP release, as well as of reduced ATP hydrolysis [72].

6.1 Extracellular ATP and neurodegeneration

The existence of important physiological extracellular functions of ATP, such as cell differentiation and growth, embryonic development and neurogenesis, suggests that an imbalance of this homeostasis may be associated with several human diseases, like neurodegenerative disorders, immune-mediated inflammatory dysfunction and tumors [69].
In brain, ATP can be massively released from damaged cells during some pathological conditions. Consequently, high extracellular levels of ATP can promote the inflammatory process mediated by microglial cells in consequence of a pathological insult. Microglial activation results in release of immunoregulatory substances, including cytokines or chemokines, or even neurotoxic molecules, such as nitric oxide (NO) and ROS, which will contribute to the development of an acute or chronic associated inflammatory reaction [69].
In addition, Feuvre et al. (2002) showed that extracellular ATP induces the processing and release of interleukin-1β by activation of P2X7 receptors [80]. Cells with exacerbated inflammatory mediators are particularly susceptible to the toxic actions of excessive ATP, because activation of P2X7 receptors leads to selective activation of nuclear factor kB (NF-kB), in microglial cells, that enhance expression of NF-kB dependent genes, such as interferons and citokines, and activation of other cell death related genes [80]. These results have been proposed P2X7 receptors as important mediators in inflammation during neurodegeneration.

There are also some evidence of a synergism between P2 receptor modulation and glutamate-induced excitotoxicity and apoptotic cell death. Some P2 receptors are associated with exacerbation of neurotoxic effect induced by glutamate exposure in neurons [69]. For all this, P2Y receptors antagonists have been proposed as potential neuroprotective agents for neuronal death associated with several neurodegenerative conditions [77].

SCIENTIFIC PAPER

To be submitted to BBA- Bioenergetics

Investigation of plasma ATP levels in Frontotemporal Lobar Degeneration

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ABSTRACT

In the last years, mitochondrial dysfunction and oxidative damage have been pointed as major contributors to neuronal loss in several neurodegenerative disorders, such as Alzheimer's, Parkinson's and Huntington's diseases, Amyotrophic Lateral Sclerosis and, more recently, in Frontotemporal Lobar Degeneration (FTLD). The decreased activity of mitochondrial respiratory chain complexes will enhance ROS production and leads to a decline in ATP production, compromising important dependent processes to cell maintenance and survival. Accordingly, we hypothesize that plasma ATP levels may be an indicator of the mitochondrial activity disturbance in tissues with high ATP demand, such as brain, possibly reflecting the energy impairment in the FTLD associated pathology.

The plasma ATP concentrations of 40 patients with probable diagnosis of FTLD followed in the Neurology Unit of the Centro Hospitalar e Universitário de Coimbra were determined using a bioluminescence technique and compared to an age-matched control group of 20 healthy subjects. Our results showed that plasma ATP concentrations in FTLD patients are significantly decreased compared to controls, particularly in patients with cognitive impairment, according to MMSE scale evaluation. The activity of complex I in lymphocytes had a positive correlation with plasma ATP concentration in FTLD patients. Moreover, our results showed a negative correlation between the activity of ATP-synthase in lymphocytes and plasma ATP levels. These findings provide more evidence of low ATP production due to mitochondrial impaired activity in FTLD neurodegeneration. Additionally, since ATP may act as a signaling molecule, namely in glutamate release, it may play a role in neurochemical impairment occurring in neurodegeneration. These results are original and add a significant amount of data to the knowledge of mechanisms involved in FTLD pathogenesis.

Keywords: Frontotemporal lobar degeneration, ATP, mitochondria, mitochondrial respiratory chain, energy impairment

1. Introduction

Frontotemporal lobar degeneration (FTLD) is the second most common form of early onset-dementia, after Alzheimer disease (AD) [2,10]. This neurodegenerative condition usually occurs between 35-75 years of age and is characterized by gradual and progressive changes in behaviour, personality and different types of language impairment with concomitant atrophy of the frontal and anterior temporal brain lobes [1,4,8,14]. The FTLD spectrum can be clinically divided into three main subtypes: behavioural variant (bv-FTD), Semantic Dementia (SD) and Progressive Nonfluent Aphasia (PNFA) [1,11,12]. Some authors include the SD and PNFA in the same language variant termed as primary progressive aphasia (PPA) [1]. There is also a significant clinical overlap with motor neuron disease (FTD-MND), as well as, with parkinsonian syndromes, progressive supranuclear palsy and corticobasal degeneration [3,5]. The principal hallmarks of FTLD histopathology are inclusions of abnormal protein Tau and disturbances in ubiquitin system, whereas there are some patients without abnormal protein inclusions and identified gene mutations [7,12,13]. Some evidence from epidemiological studies revealed that presence of neurological abnormalities are usually associated with shorter survival of FTLD patients [5]. An important source of information about histopathology of associated clinical FTLD diagnosis is an inherited mutation in a chromosome coding for a protein with the specific abnormality, favouring the genotype-phenotype correlation, particularly in familiar forms of FTLD [6].

The FTLD aetiology is complex and the absence of information about an associated pathologic mutation in some sporadic cases, raises other possible explanations for the primary event capable of triggering the neuropathologic process. In the last years, some of the proposed explanations considered mitochondria as a central player in neurodegenerative process and in 2004, Swerdlow and Khan proposed the "mitochondrial cascade hypothesis" suggesting that an inheritance of mitochondrial baseline function can influence how mitochondria may change with aging and when they will reach a pathologic threshold [22,46,52,81]. A possible interaction between a primary genetic defect involving energy metabolism and a decline in mitochondrial function that accompanies normal aging, can consequently leads to abnormal activity of OXPHOS complexes, a decline in ATP production, accumulation of toxic ROS that would ultimately lead to neuronal loss [47,82]. In accordance, some previous studies demonstrated that mitochondrial dysfunction accompanied by energy impairment can precede plaque formation in AD neurons [20]. Then, impairment of mitochondrial activity is associated with the pathogenesis of many common

age-related neurodegenerative diseases, such as AD, Parkinson's disease, Huntington's diseases, Amyotrophic lateral sclerosis and, recently in FTLD [26,48,49,83,84].

The activity of mitochondrial respiratory chain (MRC) complexes I, III and IV seems to be the most affected in neurons, platelets and lymphocytes of AD patients [53,54]. In tauopathies (as FTLD-tau and some variants of AD), a substantial complex I deficiency with concomitant decrease of ATP production has also been reported [50].

Furthermore, mitochondria are involved in the regulatory process of cell death by necrosis and apoptosis. In neurons both these conditions can either coexist or be sequential events depending of the initiating insult [23,40,45]. If cell death can occur in two different pathways, this suggests that a downstream event could control and decide the evolution of cell death [23,45]. Some studies evidentiate that bioenergetic failure resulted by impairment of OXPHOS activity seems to be an important switch in decision between these two mechanisms of cell death, since in apoptosis, several subsequent events are ATP-dependent processes [44,45]. Moreover, this energy deficit can leads to other deleterious consequences and compromise some metabolic pathways indispensable to cell survival, particularly in neurons, which are cells with high ATP demands [67,85]. In addition, energy deficit has also been associated with an exacerbation of glutamate-induced neurotoxicity [37,57,59,65].

Extracellular ATP has been involved in a wide range of physiological processes, such as neurotransmission. Massive extracellular release of ATP has been associated with metabolic stress, brain ischemia and trauma, which make purinergic system an important mechanism in neurodegeneration aetiopathology [69]. Extracellular ATP may also act as a neurotransmitter, neuromodulator or growth factor, or being co-released with acetylcholine, noradrenaline, GABA and glutamate in CNS cells [73,74]. It has also been identified as an autocrine/paracrine signal modulator by activation of purinergic receptors in the plasma membrane of the exocrine cell and neighborhood cells [69,70,75,76].

For all this, mitochondrial dysfunction and impaired energy metabolism seems to be one common point among several neurodegenerative disorders, including FTLD, that in the end shares the same cell fate, with different, but similar implicated neuropathological mechanisms [58,60]. An increased body of evidence supports the idea that these mitochondrial enzyme impairments in neurons are reflected in periphery tissues, particularly in platelets and fibroblasts, as well as, in lymphocytes of these patients [52–54]. Accordingly, in this study we aimed to investigate if plasma ATP concentrations can traduce the neuronal energetic depression that possibly may be present in FTLD and if it is related with mitochondrial activity in lymphocytes of these patients.

Materials and methods

2.1 Patients and control subjects

Participation of patients and control subjects was approved by the Institutional Review Board of the Centro Hospitalar e Universitário de Coimbra and all patients or their legal representatives signed the informed consent to participate. The 40 included patients (17 females and 23 males; age range: 38-84 years old, mean 67 ± 10) with probable diagnosis of FTLD according to the standard criteria of DMS-IV, had been followed at the Neurology Unit of the Centro Hospitalar e Universitário de Coimbra [86]. All patients was submitted to an evaluation of cognitive impairment by Mini Mental State Examination (MMSE) and global dementia severity by Clinical Dementia Rating (CDR) scale, among other clinical batteries [87–92]. The MMSE scale (range 0-30) indicates the current severity of cognitive impairment [93]. The results obtained with MMSE scale was adjusted according to years of scholarity for a total score of 0 and 1 (MMSE 0 – absence of cognitive impairment; MMSE 1 – presence of cognitive impairment). Patients' group concerns 18 patients with MMSE 0 and 21 patients with MMSE 1 evaluation (Table 1).

The CDR scale is between 0 and 3, where "CDR 0" indicates no impairment and CDR 0.5, 1, 2 and 3 indicate questionable, mild, moderate and severe dementia, respectively [89]. Our patient group included 6 patients with "CDR 0.5", 22 with "CDR 1", 8 with "CDR 2" and 3 with "CDR 3" (Table 1). For statistical analysis, we considered early-onset patient group when FTLD diagnosis was performed before 65 years and the late-onset after 65 years according published references [10,94–97].

Twenty healthy age-matched control subjects free of progressive neurological disorders (14 female and 6 males; age range: 37-80 years old, mean 63 ± 13), were randomly recruited also at Neurology Unit of the Centro Hospitalar e Universitário de Coimbra.

2.2 Isolation of lymphocytes and plasma

Venous blood from FTLD patients and controls was collected into tubes containing K_3EDTA as anticoagulant. First, the blood sample was diluted (1:1) with Phosphate-Buffered Saline (PBS), pH 7.4 and carefully layered on Ficoll-Paque™ before centrifugation. The lymphocytes interface layer was collected and washed with PBS and centrifuged again. The cellular pellet was suspended into 50-200 µl of PBS and stored in liquid nitrogen, for further MRC

enzymatic activities evaluation. Plasma was collected from upper layer after centrifugation and was stored at -80°C until analysis of ATP concentration.

2.3 Measurement of MRC complexes activities

The MRC complexes I, II, III, IV and V activities were evaluated as previously described [98,99], at 37°C and using a dual wavelength spectrophotometer (SLM AMINCO DW-2000_{TM}, SLM Instruments). The results were expressed as nmol/min/mg of protein normalized to Citrate Synthase (CS) activity, used as a reference of mitochondrial number [53,54,98].

2.4 Analysis of plasma ATP concentration

Plasma ATP was measured using a firefly bioluminescence assay (ATP Bioluminescence Assay Kit CLS II: Roche Diagnostics) by luminometry (Berthold FB12 Luminometer), and following the manufacturer's instructions. In the presence of substrate D-luciferin, Mg^{2+}, molecular oxygen and ATP, Luciferase catalyses a multistep reaction [72,100], according to the reaction:

$$\text{ATP} + \text{D-luciferin} + O_2 \rightarrow \text{oxyluciferin} + \text{PPi} + \text{AMP} + CO_2 + \text{light} \ (\lambda_{max}=560 \text{ nm})$$

The ATP concentration was determined by measuring the light produced in the reaction and the results were expressed in Relative Light Units (RLU's) [79]. Every ATP measurement of each sample was performed at least in triplicate.

2.5 Correction for haemolysis in plasma samples

There is evidence in the literature that haemoglobin (Hb) content can influence the measurement of plasma adenine nucleotides, including ATP, even at low concentrations [79,101]. Accordingly, we had determined the Hb concentrations for all plasma samples of the FTLD patients and control subjects, used to estimate the degree of sample haemolysis. Plasma Hb concentration was measured using Drabkin's method, by spectrophotometry (λ max= 540 nm) [102] and every sample was analysed in triplicates. In order to determine the correlation between Hb concentration and RLU's values, we evaluated the Hb content and

ATP level in seven lysates of blood samples enriched in erythrocytes, as previously described by Gorman et al. (2007) [79,101] with slight modifications (appropriated dilutions and without stabilizing solution).

2.6 Determination of protein content

Plasma protein concentration was determined by the Bradford method, as previously described [103]. The protein content was measured by spectrophotometry (λ_{max}= 595 nm) at least in triplicate for each sample [103].

2.7 Statistical analysis

The different data groups analysed were compared by a nonparametric Mann–Whitney test (two-tailed). To quantify the degree of MRC complexes activities were related to the plasma ATP concentrations, we have used a correlation analysis of Spearman. The best-fit line was achieved by linear regression. All the statistical tests were evaluated using Software GraphPad Prism® 5 considering the p-value < 0.05 as statistically significant [104].

2. Results

3.1 Adjust of plasma ATP concentration with correction for haemolysis

To determine the ATP concentration attributable to haemolysis we performed the evaluation of haemoglobin content in seven aliquots with different solutions of the same supernatant used for ATP determination. The obtained values were correlated by a linear regression (y=1,315x) and extrapolated to all ATP and Hb plasma concentration measurements of each patient and control subject (Figure1). All the values of plasma ATP concentration are presented with correction for haemolysis.

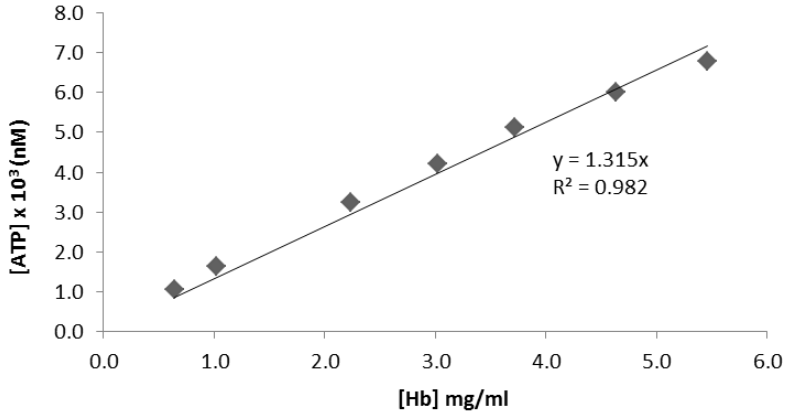

Figure1– Correlation between ATP concentration and haemoglobin content in plasma.
[Hb] – Haemoglobin concentration

3.2 Plasma ATP levels and probable diagnosis

Our results show decreased plasma ATP levels in FTLD patients (n=40) comparatively to an aged-matched control group (p=0.0124) (Figure 2). The majority of FTLD patients had probable diagnosis of bv-FTD (n=35) and the statistical analysis showed significant difference between these group (p=0.0161) and controls (Figure 2). Patients with other FTLD variants (APP and CBD) were not included in the statistical analysis, because the number was too small to conclude a correct interpretation (Table I).

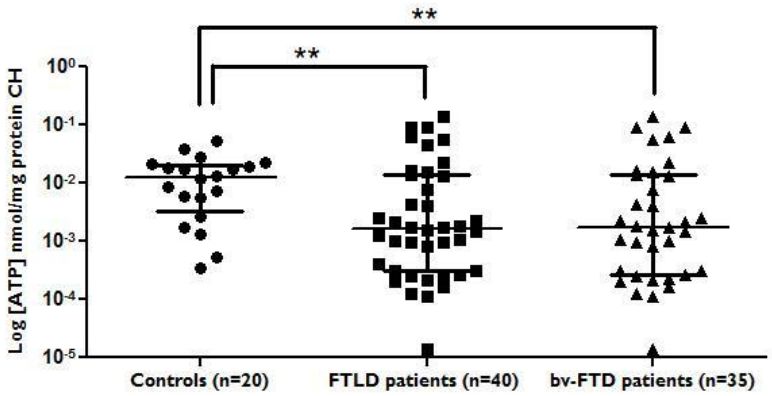

Figure 2 – Extracellular ATP levels in FTLD and healthy age-matched control subjects. The bv-FTD patient group include the familiar variant. The differences between control group and FTLD patients or bv-FTD patients are both statistically significant (p=0.0124 and p=0.0161, respectively). The traces represent from top to the bottom, upper quartile (Q_3), median and lower quartile (Q_1) values for each group. CH - corrected for haemolysis.

3.3 Plasma ATP levels and MMSE scale evaluation

All FTLD patients were organized in two groups categorized with absence or presence of cognitive deficit, corresponding to total scores of 0 and 1 of the MMSE adjusted scale, respectively. The results show that patients' group with cognitive impairment have significantly lower ATP plasma levels, compared to controls (p=0.0026) (Figure 3) (Table 1). There are no significant differences in ATP levels between control group and patients with MMSE 0 (p=0.1248) and between both patients' groups (p=0.1468) (Figure 3).

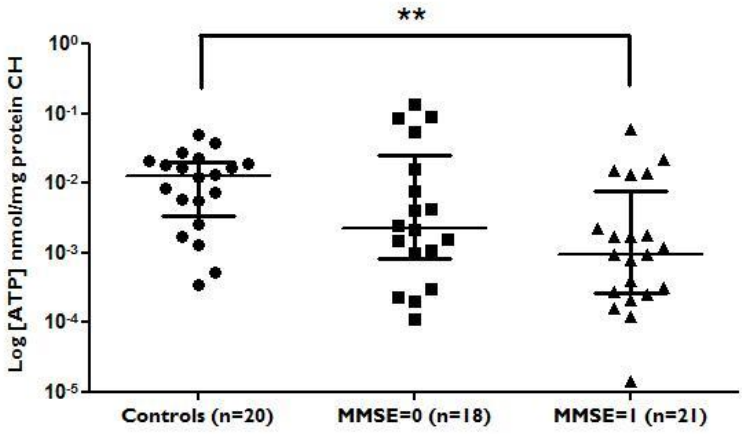

Figure 3 – Plasma ATP concentration according to cognitive impairment score. The difference between the control group and MMSE 1 patients is statistically significant (p=0.0026). The traces represent from top to the bottom, upper quartile (Q_3), median and lower quartile (Q_1) values for each group. CH- corrected for haemolysis.

3.4 Plasma ATP levels and CDR scale evaluation

The analysis of ATP levels according dementia severity using the CDR evaluation for each FTLD patient was also performed. The FTLD patients with "CDR 1" (mild dementia) and "CDR 2" (moderate dementia) were the lowest ATP values (p=0.0043 and p=0.0307, respectively) compared to control group (Table 1). However, patients with "CDR 3" (severe dementia) were not included in the statistical analysis because the number was too small for a correct interpretation (Table 1).

3.5 Plasma ATP content according to disease duration

Considering duration of disease (years), there is a statistical significant difference between each of the two defined groups of patients and the control group (p=0.0037 and p=0.0225 respectively) (Figure 4). However, there is no significant difference between both patients' groups (Figure 4).

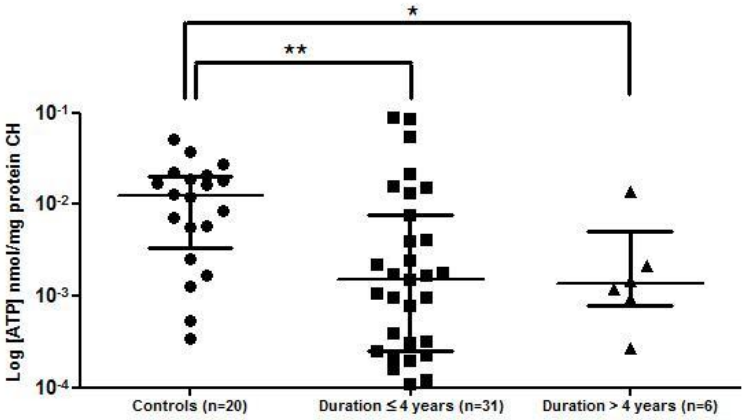

Figure 4 – Plasma ATP concentration according to duration of disease (years).The difference between both patients' groups and controls is statistically significant (p=0.0037 for disease duration ≤ 4 years; p=0.0225 for disease duration > 4 years). The traces represent from top to the bottom, upper quartile (Q_3), median and lower quartile (Q_1) values for each group. CH - corrected for haemolysis.

3.6 Influence of age of onset in plasma ATP content

The ATP levels of all patients were also evaluated in order to age of onset, and the results obtained showed a statistical significant difference between patients with age of onset before 65 years (p=0.0098) and after 65 years (p=0.0044), versus the control group (Table 1). However, there is no significant difference between both patients' groups (p=0.3001).

3.7 Plasma ATP levels and gender

The gender distribution, showed that FTLD female group had the lowest ATP values compared to the control female group (p=0.0276). However, there is no difference for male gender (Figure 5).

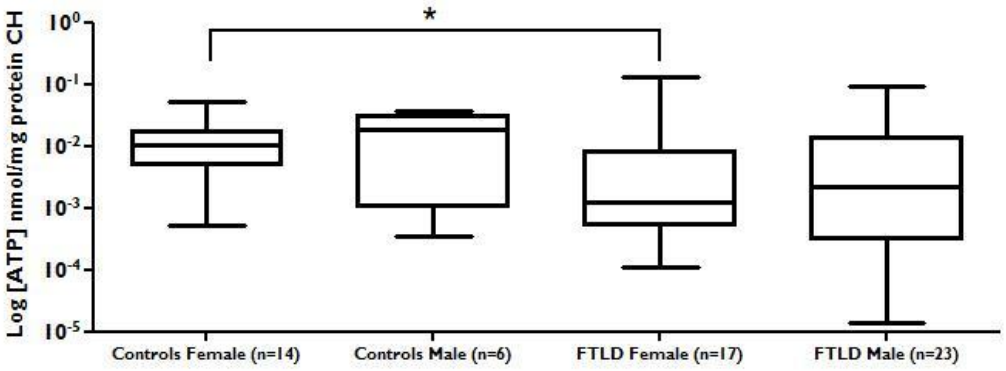

Figure 5- Extracellular ATP levels in FTLD patients and healthy age-matched control subjects according to gender. The difference between the female patient and control groups is statistically significant (p=0.0276). The traces represent from top to the bottom, upper quartile (Q_3), median and lower quartile (Q_1) values for each group. CH- corrected for haemolysis.

Table I

Plasma ATP values in controls, according to gender, and in patients according to gender, clinical variant, MMSE and CDR scores, age of onset and disease duration.

Controls			20	1.442	1.283	1.246	5.070	0.034
	Gender							
		Female	14	1.317	1.240	1.016	5.070	0.053
		Male	6	1.734	1.334	1.850	3.747	0.034
FTLD patients			40	1.472	2.949	0.160	13.340	0.001
	Gender							
		Female	17	1.808	3.722	0.121	13.340	0.011
		Male	23	1.223	2.177	0.219	9.011	0.001
	Diagnosis							
		bv-FTD[a]	27	1.566	3.163	0.149	13.340	0.001
		Familiar bv-FTD[b]	8	1.460	2.765	0.230	8.663	0.016
		Other variants	4	0.108	0.048	0.108	0.174	0.040
	MMSE evaluation							
		MMSE 0	18	2.262	3.916	0.229	13.340	0.011
		MMSE 1	21	0.651	1.338	0.096	5.960	0.001
	CDR evaluation							
		CDR 0.5	6	3.276	4.902	0.317	13.340	0.023
		CDR 1	22	1.094	2.506	0.114	9.011	0.011
		CDR 2	8	0.555	0.663	0.097	1.520	0.001
		CDR 3	3	2.076	2.747	0.174	5.960	0.094
	Disease duration							
		≤ 4 years	31	1.068	2.298	0.152	9.011	0.001
		> 4 years	6	0.330	0.470	0.135	1.373	0.027
	Age of onset							
		≤ 65 years	22	1.013	2.108	0.161	9.011	0.016
		> 65 years	15	0.855	2.157	0.107	8.663	0.001

Some patients' clinical information was not available: 1 FTLD variant diagnosis, 1 for MMSE and CDR criteria, 3 for age of onset and disease duration. [a] include the familial variant of bv-FTD. [b] Other variants include APP and CDB.

3.8 Plasma ATP content versus MRC activity in lymphocytes

Our results showed a positive correlation between ATP values and activities of complexes I (p=0.0149, Spearman r = 0.3822) in lymphocytes of FTLD patients (Figure 6, A). However, the statistical analysis showed no correlation in activities of complexes II, III and IV and plasma ATP content of these patients (Figure 6, B-D). On the other hand, a diminished activity of complex V correlated with plasma ATP content of each patient was also observed (p=0.0002, Spearman r = -0.5544) (Figure 6, E).

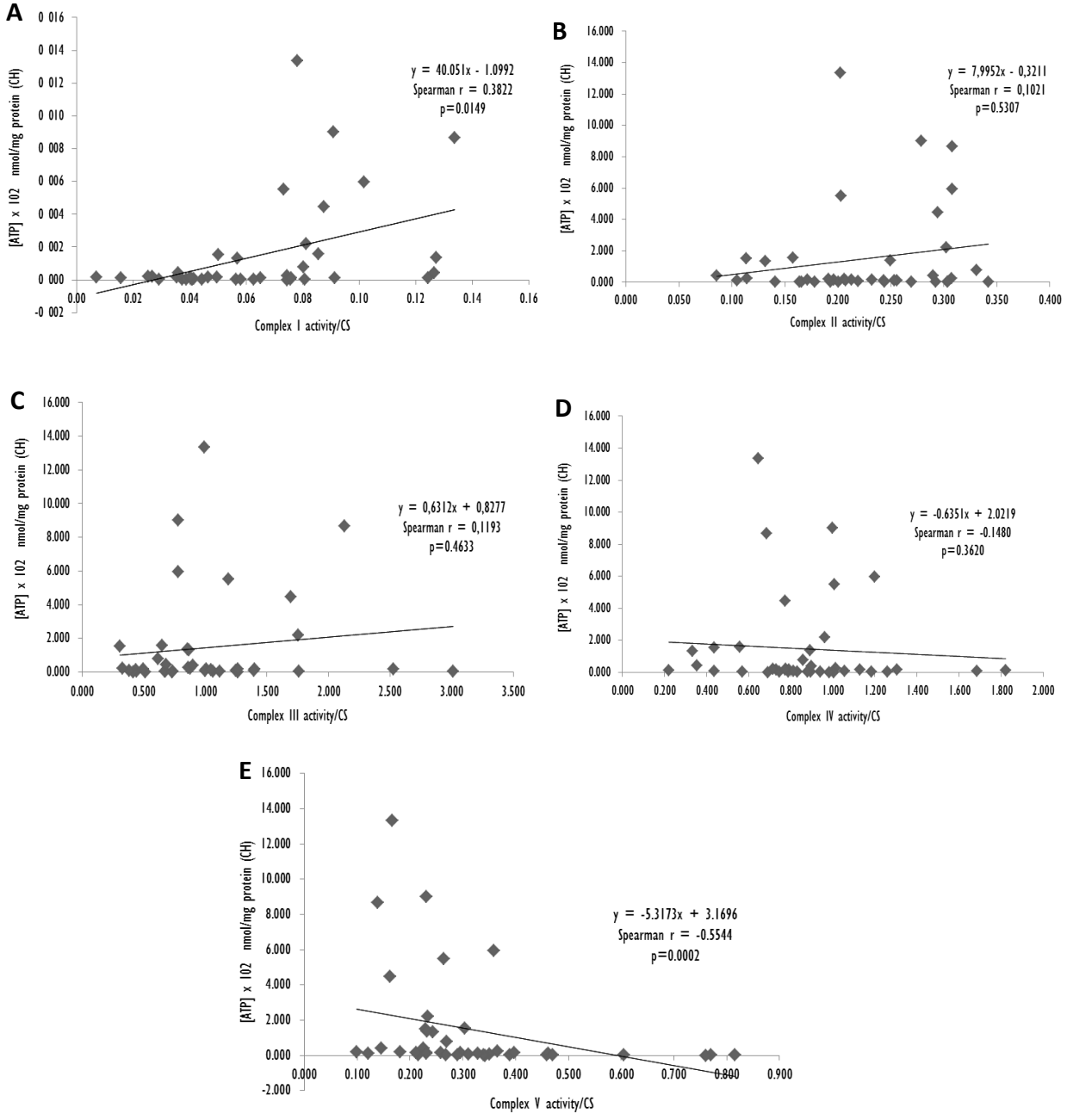

Figure 6 – Correlation between plasma ATP content and MRC complexes activities of FTLD patients. A – Complex I activity/CS; B – Complex II activity/CS; C- Complex III activity/CS; D- Complex IV activity/CS; E – Complex V activity/CS. CH- corrected for haemolysis; CS – citrate synthase.

3. Discussion

In the present study, it was aimed to evaluate the involvement of ATP content and its correlation with MRC activity in neurodegeneration occurring in Frontotemporal lobar degeneration. We measured the plasma ATP content in 40 patients with probable diagnosis of FTLD and in 20 healthy age-matched controls.

As previously described by Gorman et al. (2007) [79,101], haemolysis could play an important influence on plasma ATP concentration that must be taken into account for ATP measurement. Accordingly, this aspect has been analysed in the present study and the obtained mean value of haemolysis percentage was 3.5 ± 1.1 (mean ± SEM) for the population in study, indicating that ATP concentration attributable to erythrocytes lysis in plasma samples is a minor variable. However, in order to avoid associated error, all ATP concentration values were corrected for haemolysis.

The ATP values were studied according to patients' diagnosis, either for FTLD patient group and bv-FTD variant group (Figure 2). Present findings indicate that plasma ATP levels in FTLD patients are significantly lower, compared to the control group ($p=0.0124$) and the same observation is valid if patients are affected with the bv-FTD variant ($p=0.0161$) (Figure 2). These low extracellular ATP concentrations may reflect mitochondrial dysfunction in brain cells of FTLD patients.

In spite of the limited reported data concerning mitochondrial dysfunction in FTLD, the implicated neuropathological mechanisms have similarities with other neurodegenerative disorders. In fact, different studies have also been demonstrated mitochondrial impairment activity in related diseases, such as AD, PD and ALS [24,26,52,84,105–110]. Additionally, our group has previously reported a complex I decrease in a patient with FTLD [2]. It is widely recognized that there is a decline of basal metabolic function and MRC activity in neurodegeneration [46]. This process can be potentiated by accumulation of abnormal intracellular or/and extracellular proteins in the affected tissues [21]. When mitochondrial MRC activity is impaired, ATP production is mainly derived from glycolysis [83]. However, if glycolytic pathway is not sufficient to provide all ATP needed to maintain cell survival, cytosolic ATP levels drop, which leads to a chronic bioenergetic failure [58,111]. This can be observed in diminished plasmatic ATP concentrations of FTLD patients in the present study (Figure 2).

Moreover, the "mitochondrial cascade hypothesis" proposed for AD [46] and later in FTLD [2] suggests that low ATP reserves would promote tau phosphorylation in tauopathies. The

overexpressed and hyperphosphorylated tau, appears to impair axonal transport of organelles causing synapse starvation, enhance ATP depletion, and ultimately leading to neuronal damage in tauopathies [112].

In addition, intracellular ATP stores can modulate the type of cell death through apoptosis or necrosis [23,39]. Both these molecular pathways of cell death can occur simultaneously during neurodegeneration, but depending on the extent of the bioenergetics' failure, cell death will show an apoptotic or a necrotic phenotype [57]. In these situations, ATP levels can act as an important switch between necrosis and apoptosis and may influence the subsequent involved mechanisms.

Another possible consequence of a decline in ATP production during neurodegeneration is the exacerbation of glutamate-induced neuroexcitotoxicity [57,67]. In fact, Del Río et al. (2007) [57] reported that a pathological energetic failure associated with mitochondrial dysfunction can exacerbate the excitotoxic neuronal injury after glutamate exposure, even at low concentrations. The overactivation of NMDA receptors leads to an excessive influx of Ca^{2+} to cytosol of post-synaptic neurons [40,65]. The increased cytosolic Ca^{2+} concentration will be buffered by mitochondria and endoplasmatic reticulum [113]. Consequently, high concentrations of Ca^{2+} inside mitochondrial matrix will in turn promote disturbances in mitochondrial membrane permeability, decreased MRC activity and ATP production that will enhance the bioenergetics' disruption, contributing to the neurodegeneration process [40,58,64,65]. In addition, when intracellular ATP levels drop, the mitochondria cannot eliminate the excess of cytosolic Ca^{2+} because these processes are mainly ATP-dependent [67]. Then, it will be acting as a positive feedback mechanism that results in mitochondrial Ca^{2+} overload and enhanced neuronal cell death.

Although almost all FTLD patients have lower plasma ATP values compared to controls, some patients have extremely high ATP values. These could be a possible consequence of the neuroinflammation process stimulated by accumulation of dysfunctional proteins or by a chronic inflammation associated with other comorbidities [114–116]. Furthermore, the intensification of ATP release to the extracellular space have been usually related with a number of physiological and pathophysiological stimuli including ischemia, hypoxia, platelet aggregation, sympathetic nerve stimulation and cell damage that may possible interfere with plasma ATP content [69,78,79].

The evaluation of cognitive function was achieved by MMSE scale [91,92,117]. The MMSE criteria does not define a clinical or pathological diagnostic category, but gives some information about the disease severity and dementia progression of an patient [92,117]. The

items of the MMSE test include evaluation of orientation, registration, recall, calculation and attention, naming, repetition, comprehension, reading, writing and drawing [87,118]. However, it is important to note that MMSE evaluation for tracking disease-related cognitive decline in the later stages of the illness may be compromised because the scores dropped when patients manifested symptoms of apathy and mutism [117]. Patients' cognitive decline is usually related with an intensification of FTLD symptoms and with worse prognosis, which is consistent with lowered ATP values observed in patients with MMSE score "1" compared to control group ($p=0.0026$) (Figure 3). It is now generally accepted that cognitive impairment and language changes are due to cellular alterations, such as oxidative stress and low ATP production in neurons, particularly at synapses [119].

The evaluation of dementia severity using CDR scale was also performed in 39 FTLD patients. The CDR scale is a clinical staging instrument that combines 6 domains of cognitive and functional performance: memory, orientation, judgment and problem solving, community affairs, home and hobbies, and personal care [90]. An algorithm allows the calculation of a total score, when a total score of zero reflects no dementia and higher scores denote higher impairment [90]. The analysis of ATP levels according to CDR score of each FTLD patient, showed differences between all groups (Table 1). The lowest ATP values are presented in patients with CDR "1" ($p=0.0043$) and CDR "2" ($p=0.0307$) comparatively to the control group. The ATP levels in patients with CDR "3" ($p=0.7493$) were higher compared to values observed in CDR "1" and CDR "2", but it is important to note the limited number of cases with CDR "3" score. This suggests that, in the first stages of illness associated with mild stages of dementia (CDR "0.5", $p=0.6481$), the ATP content is similar to the observed in controls. However, with disease progression (CDR "1" and CDR "2"), there is an exacerbated bioenergetics failure associated with a prominent mitochondrial dysfunction, probably associated to decreased ATP. Moreover, with disease progression, a subsequent inflammatory process can be triggered by the intracellular and extracellular accumulation of aggregated proteins, by signals released from the injured neurons, or by imbalances between pro- and anti-inflammatory processes [116,120,121]. In addition, if cell death occurs by apoptosis, there is no evidence of associated inflammation, but the necrotic pathway triggers a exacerbated inflammatory reaction [116]. Accordingly, Piguet et al. (2013) [114], reported that plasma levels of tumour necrosis factor (TNF), a marker of systemic inflammation, were significantly elevated in SD patients and in FTLD patients harbouring mutations in PGRN gene. The TNF expression, will in turn, induce ROS production and MRC activity decay [122].

The disease duration analysis was performed using the cut-off at 4 years of illness, which correspond to half of mean disease duration reported in several epidemiological studies [5,95,96,123]. It is important to note that, that disease duration refereed in the present study corresponds to years of survival with illness until the moment of the blood collection. Patients with shorter duration of illness have the lowest ATP concentrations (Figure 4) (Table I). These results suggest that the pathological trigger that initiates the neurodegenerative process in FTLD may be associated with energy impairment translated into low ATP concentrations, but further research is needed to confirm this hypothesis. The higher ATP values presented in patients with longer duration of disease may be a consequence of an increased number of injured neurons and an augment of microglial activation with disease progression. When released in excess and/or for longer periods of time, ATP may become toxic and could contribute to neurodegeneration due to an activation of P2X and P2Y receptors, that will promote neuroinflammation via activation of P2 receptors on neurons or, indirectly, via release of pro-inflammatory cytokines from macrophages and/or microglia [74]. However, the analysis of ATP in cerebral spinal fluid would help to clarify this point.

Patients' distribution according to age of onset revealed a significant difference between the two patients' groups and controls ($p=0.0098$ before 65 years; $p=0.0044$ after 65 years) (Table 1). This suggests that age of onset is not related ATP content.

The female FTLD patients had significantly decreased ATP values ($p=0.0276$) compared to healthy female group (Figure 5). The vast majority of women included in patient and control group are in the menopause phase (patients mean age: 70±9 years; controls mean age: 63±13 years). In menopausal women, ovarian production of estradiol is significantly diminished and plasma oestrogen concentration will be consequently reduced [124]. At high levels, oestrogens promote the expression of antioxidant molecules, and prevent initial ROS production induced by toxic insults [124]. In contrast, at low concentrations, oestrogens can stimulate disease process and will facilitate the deposition of abnormal proteins [124]. Although the female gender are associated with less risk of mortality in FTLD than male [125], the cessation of ovarian oestrogen production in postmenopausal FTLD women, might possibly aggravate the pathological consequences of an inherited genetic defect or a metabolic imbalance, contributing to disease progression [125].

Concerning the MRC complexes' activities, no significant differences were observed between all patients and controls. However, diminished activity of complexes I, III and IV were identified in 6 FTLD patients, according to previously established criteria [98]. These

findings are consistent with other published data showing decreased activity of complexes I, III and IV in neurons and platelets of AD patients [24,26,49,52]. Plasma ATP values and MRC activities in lymphocytes were matched for each patient. A positive correlation between ATP values and activity of complex I ($p=0.0149$, Spearman $r=0.3822$) and a negative correlation between the same ATP values and the ATP synthase (Complex V) activity ($p=0.0002$, Spearman $r=-0.5544$) (Figure 6). These results suggest that high ATP concentrations correspond to an increased activity of complex I, and low ATP concentrations, correspond to a decreased activity of this complex. In fact, these results are in accordance with diminished activity of complex I and low ATP levels observed in 3 patients of the sample in study, according previously published criteria [98]. In addition, impairment of complex I activity together with decreased ATP levels are also related with presence of abnormal tau protein *in vitro* and *in vivo* models of tauopathies [50,126]. The abnormal function of complex I could enhance ROS production and upturn mtDNA mutations in the affected cells, contributing to the neurodegenerative process [43]. The ATP synthase activity in FTLD lymphocytes was similar to values obtained in control group, which are in accordance with data reported for AD [54].

Nevertheless, it is quite remarkable the presence of a negative correlation between plasma ATP levels and ATP synthase activity in lymphocytes ($p=0.0002$) (Figure 6, E).

When the MRC is inhibited, $\Delta\Psi m$ would decrease and prevent OXPHOS collapse, the ATP synthase starts to function as a proton pump and hydrolyses ATP present in the mitochondrial matrix [127]. Other authors reported that partial inhibition of complex I by rotenone can maintain $\Delta\Psi m$, but collapses in the presence of oligomycin (ATP synthase inhibitor), clearly demonstrating the role of ATP synthase in the maintenance of $\Delta\Psi m$ in mitochondria of synaptosomes [37,67,127]. Moreover, the same effect is observed in the presence of the protonophore FCCP (MRC uncoupler), that collapses cellular ATP by reversal of ATP synthase activity. As it occurs in PD, partial inhibition of complex I is sufficient to reverse the activity of ATP synthase in order to stabilize $\Delta\Psi m$ with ATP hydrolysis. We hypothesize that a similar mechanism can also occurs in neuronal cells of FTLD patients, translated by an exacerbated activity of ATP synthase with correspondent plasmatic low ATP levels. In this case, glycolysis will be the major source of ATP and, as long as it is sufficient to maintain $\Delta\Psi m$ at a suboptimal level and several ATP-dependent functions, the cellular ATP pool is not depleted and cell survives. However, survival of cells with impaired MRC activity depends of their glycolytic capacity and energy demands. Furthermore, it has been demonstrated that ATP consumption increases dramatically when

cells are exposed to glutamate. In these conditions, an excess of Ca^{2+} influx through NMDA receptors would also result in collapse of the $\Delta\Psi m$, that will in turn, interrupt ATP synthesis and causing ATP synthase reversal activity [37,67,85]. While Ca^{2+} accumulation by the mitochondria may affect ATP synthesis, alterations in this process will in turn affect the Ca^{2+} extrusion and contribute to this pathologic mechanism and enhance the associated bioenergetics' failure [57,58].

Our findings are original and may contribute to elucidate the important role of mitochondrial impairment in the complex aetiology of FTLD. Moreover, the extracellular ATP evaluation can also give some information about the bioenergetics' failure associated with neurodegenerative process occurring in FTLD. However, further research is required in order to evaluate the correlation between the plasma and cytosolic ATP concentrations in the compromised neurons and peripheral tissues. A more detailed characterization of the neuropathological mechanisms involved in FTLD would also contribute to develop future treatment approaches.

4. Conclusions

Currently, the prevalence of neurodegenerative diseases is rising exponentially due to the increased lifespan, representing one of the major health problems in modern society.

In the last years, mitochondria have been pointed as a central player in the neurodegeneration process that occurs in AD, PD, HD, ALS, and recently in FTLD. Mitochondria are responsible for producing the majority of the energy required for metabolic processes, essential to cell survival, and are of crucial importance in regulating cell death.

The present results suggest that mitochondrial impairment in FTLD leads to a decline in ATP production by OXPHOS pathway, which will in turn, be reflected in decreased plasma ATP levels. Moreover, there is a negative correlation between plasma ATP levels and activity of ATP-synthase in lymphocytes of FTLD patients, due to a possible disturbance in MPTP that results in ion imbalance, Ca^{2+} overload and ultimately in ATP depletion. These findings support the idea that mitochondrial dysfunction and bioenergetics failure may be both the cause and the consequence of the neurodegeneration process that occurs in brain cells of FTLD patients. Additionally, since ATP may act as a signaling molecule, namely in glutamate release, it may play a role in neurochemical impairment occurring in neurodegeneration. However, more research is required to support this hypothesis and reinforce the important role of ATP in the neuropathological mechanisms involved in FTLD aetiology.

These results are original and add a significant amount of data to the knowledge of mechanisms involved in FTLD pathogenesis, providing clues to the development of future therapies.

Acknowledgments

The authors would like to thank all patients and families for participating and makes this study possible.

This study was financed by Portuguese Foundation for Science and Technology (FCT), with the project PTDC/SAL-EPI/121811/2010 (FCT) and partially supported by FCT project PEst-C/SAU/LA0001/2011.

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APPENDIX

BBA - BIOENERGETICS

AUTHOR INFORMATION PACK

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BBA Bioenergetics covers the area of biological membranes involved in energy transfer and conversion. In particular, it focuses on the structures obtained by X-ray crystallography and other approaches, and molecular mechanisms of the components of photosynthesis, mitochondrial and bacterial respiration, oxidative phosphorylation, motility and transport. It spans applications of structural biology, molecular modelling, spectroscopy and biophysics in these systems, through bioenergetic aspects of mitochondrial biology including biomedicine aspects of energy metabolism in mitochondrial disorders, neurodegenerative diseases like Parkinson's and Alzheimer's, aging, diabetes and even cancer.

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