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Review

Genetic basis of Alzheimer's dementia: role of mtDNA mutations

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Alzheimer's disease (AD) is the most common neurodegenerative disorder associated to dementia in late adulthood. *Amyloid precursor protein, presenilin 1* and *presenilin 2* genes have been identified as causative genes for familial AD, whereas apolipoprotein E *ε4* allele has been associated to the risk for late onset AD. However, mutations on these genes do not explain the majority of cases. Mitochondrial respiratory chain (MRC) impairment has been detected in brain, muscle, fibroblasts and platelets of Alzheimer's patients, indicating a possible involvement of mitochondrial DNA (mtDNA) in the aetiology of the disease. Several reports have identified mtDNA mutations in Alzheimer's patients, suggesting the existence of related causal factors probably of mtDNA origin, thus pointing to the involvement of mtDNA in the risk contributing to dementia, but there is no consensual opinion in finding the cause for impairment. However, mtDNA mutations might modify age of onset, contributing to the neurodegenerative process, probably due to an impairment of MRC and/or translation mechanisms.

Keywords: *16S rRNA*, age of onset, Alzheimer's disease, amyloid, gene, mitochondria, mtDNA, *ND1*, oxidative stress, point mutation

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Alzheimer's disease (AD) (Alzheimer 1907) is the most common form of dementia, presenting a broad and heterogeneous range of symptoms, including progressive loss of memory, cognitive/language impairment and behaviour disturbances. Diagnosis can only be achieved by autopsy, comproving neuron loss and the presence of neurofibrillary tangles (NFTs) and senile plaques (SPs) in brain tissue (Terry & Katzman 1983), considered as the neuropathological hallmarks of the disease. Nevertheless, it is clear that the cellular pathways responsible for Alzheimer's pathology are complex in nature and extend beyond the production of extracellular plaques of β-amyloid (Aβ) aggregates and intracellular NFTs (Chong *et al*. 2005).

Pathological mechanisms of AD

The exact mechanism underlying the aetiology of the disease remains unknown. However, several hypotheses have been raised to explain the pathological alterations observed (recently reviewed by Pereira *et al*. 2005).

Loss of neurons and brain-impaired function occur in the early phase of the disease and cause synaptic dysfunction, leading to memory loss and cognitive impairment, two main features of AD presentation (Walsh & Selkoe 2004a).

Amyloid-β (A-β) peptides aggregate in the extracellular space forming the SPs.

Insoluble A-β is the major component of SPs, resulting from the proteolysis of amyloid precursor protein (APP) by γ and β secretases. APP is a transmembrane glycoprotein that undergoes proteolytic cleavage by γ and α or β secretases, respectively (Mattson 1997). A-β is a spontaneous aggregating peptide variably comprised 37–43 amino acids, the peptides 42–43 being the most toxic component of SPs (Walker *et al*. 2005). According to the amyloid hypothesis, it has been suggested that the accumulation of A-β peptides results from the dysregulation of the production/processing pathways, leading to AD-related neurodegeneration (Hardy & Selkoe 2002). But, it is also possible that A-β toxicity occurs in consequence of more fundamental pathological changes and may even play a protective role in the diseased brain (Lee *et al.* 2004), raising some controversial points to this issue. Some recent data contributed to the revision of the amyloid cascade hypothesis (Golde 2003; Hardy & Selkoe 2002), suggesting that soluble forms of A-β (Kuo *et al*.

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1996), such as oligomers (Walsh & Selkoe 2004b), may play a pivotal role in the neurodegenerative process leading to AD.

Another mechanism contributing to AD pathology involves the hyperphosphorylation of tau, a microtubule-associated protein. Aggregation of hyperphosphorylated tau occurs in several neurodegenerative disorders, including AD, affecting microtubule stability and polymerization, due to conformational changes (see Iqbal *et al*. 2005 for review).

One of the most comprehensively studied systems currently accepted to be implicated in AD phenotype involves mitochondria dysfunction and oxidative stress. It has been shown that mitochondria degeneration occurs early in the course of the disease (Hirai *et al*. 2001). Additionally, mitochondrial energy metabolism key enzymes, with special attention to cytochrome c oxidase (Castellani *et al*. 2002), have been shown to be severely affected in AD (Blass 2001; Eckert *et al*. 2003). More recently, it has also been reported that genes coding for subunits of the oxidative phosphorylation system are differentially expressed in patients with AD, suggesting that mtDNA defects may be responsible for the heterogeneity of the phenotype observed in AD patients (Manczak *et al*. 2004).

Several lines of evidence from the literature point towards the fact that the proteolytic processing of A-β is synergistically related with impaired energy metabolism (reviewed in Pereira *et al*. 2005). It has also been shown that accumulation of APP in mitochondria, in a transmembrane-arrested form, causes mitochondrial dysfunction and impairment of energy metabolism, demonstrating that APP is targeted to neuronal mitochondria under some physiological and pathological conditions (Anandatheerthavarada *et al*. 2003), including AD. The mechanism by which A-β induces mitochondrial dysfunction appears to involve abnormal reactive oxygen species (ROS) production, emphasized by the fact that most of the MRC complexes are susceptible to either A-β or ROS (Arias *et al*. 2002; Cardoso *et al*. 2004b; Casley *et al*. 2002; Pereira *et al*. 1998). Evidence has been presented suggesting a role for mitochondrial dysfunction in upstream events predisposing to neurodegeneration, and in downstream events accelerating cell dysfunction and loss (Byrne 2002), with implications to Alzheimer's pathology. The defective energy production and increased oxygen radicals may induce mitochondria-dependent cell death because damaged mitochondria are unable to maintain the energy demands of the cell (Zhu *et al*. 2004).

Moreover, recent data propose that mitochondrial energy metabolism is impaired by the expression of mutant APP and/or A-β and that the up-regulation of mitochondrial genes is a compensatory response, with important implications for the understanding of the mechanism of A-β toxicity in AD and for developing therapeutic strategies for the disease (Reddy *et al*. 2004). Recent studies in transgenic mice overexpressing mitochondrial Aβ-binding alcohol dehydrogenase (ABAD), ordinarily a contributor to metabolic homeostasis, provide additional evidence for ABAD–A-β interaction leading to oxidative stress and mitochondrial dysfunction (Lustbader *et al*. 2004) and that ABAD has the capacity to become a pathogenic factor in an A-β–rich environment (Yan & Stern 2005).

Because mitochondria are the major site of free radical production in cells, they are also a primary target for oxidative damage and subsequent dysfunction (Sullivan & Brown 2005). Furthermore, mitochondrial genome (mtDNA) appears to be more susceptible to accumulating oxidative damage (compared with nuclear DNA) due to its proximity to ROS production (Zhu *et al*. 2004), and abnormalities in mtDNA may be a major cause of abnormal ROS production in AD (Migliore *et al*. 2005) due to malfunction of respiratory chain subunits.

Mitochondria are the primary source of cellular oxidants, taking into account that 2–5% of O_2 is not completely reduced to H_2O at the electron transport chain and, therefore, a prime target of cumulative oxidative damage. Damage to mitochondrial proteins and mtDNA would be expected to decrease mitochondrial bioenergetics and efficiency (Sullivan & Brown 2005). The underlying mechanism of abnormal mitochondrial ROS production is the altered redox potential of MRC carriers (due to hyperpolarization of mitochondrial membrane) and an increase in ubisemiquinone anion half-life time, resulting in slower electron transport, producing intermediates that stay reduced longer, thus increasing the chance that the electrons can escape to molecular oxygen, originating ROS (Sullivan & Brown 2005).

When the antioxidant defences in the cell are not able to withdraw its effects, oxidative stress occurs and damage to bio-molecules (proteins, lipids, nucleic acids) take place (Boveris *et al*. 1972; Pereira *et al*. 2001). Several oxidative stress markers have been found in different types of samples (including brain, urine, plasma or CSF) from AD patients (Abe *et al*. 2002; Butterfield *et al*. 2001; Gibson & Huang 2002; Migliore *et al*. 2005; Praticò *et al*. 2000, 2002), and data have been reported demonstrating that antioxidants' treatment delays the progression of the disease (Kontush & Schekatolina 2004; Sano *et al*. 1997; Viña *et al*. 2004, for reviews).

The fact that abnormal mitochondria contribute to oxidative stress in AD brain is supported by the correlation found between mtDNA alterations and the extent of nucleic acid injury in cytoplasm (Hirai *et al*. 2001; Zhu *et al*. 2004), suggesting a temporal relationship between neuronal oxidative damage and mitochondrial abnormalities (Hirai *et al*. 2001).

On the contrary, oxidative stress may be directly involved in abnormal APP processing. In fact, it has been recently demonstrated that A-β accumulation is increased in transgenic mice harbouring alterations in brain oxidative status (Apelt *et al*. 2004). Moreover, A-β peptides have been suggested to be a source of oxidative stress in AD (Pereira *et al*. 2005), and it has been shown that A-β inhibits MRC activity and induces increased ROS production (Mark *et al*. 1997; Casley *et al*. 2002).

Data reported by Sung and colleagues (Sung *et al*. 2004), showing that vitamin E may reduce A-β levels and amyloid deposition in a transgenic model of AD, support the hypothesis that oxidative stress is an important early event in AD pathogenesis, and antioxidant therapy may be beneficial only at this stage of the disease process.

Recently, Praticò (2005) predicted the two possible scenarios concerning the relevance of oxidative stress hypothesis in AD pathology: (1) neuronal degeneration is the consequence of an oxidative stress response to SPs and NFTs rather than to generate these lesions *per se*; (2) oxidative stress is an early event triggering the formation of SPs and NFTs, which in turn activate more oxidative reactions that drive a self-sustained 'autodestructive' process and the relentless progression of AD. However, the question whether oxidative stress is an early component in the pathogenesis of the disease or a common final step of the neurodegenerative process in AD remains unclear.

The pathways thought to be involved in AD pathology also include alterations in metal ion and calcium homeostasis. It has been shown that copper, iron and zinc have the capacity to bind A-β, accelerating its aggregation and enhancing toxicity, possibly by increasing oxidative stress (Castellani *et al*. 2004; Mattson 2004a, 2004b; Moreira *et al*. 2000; Rogers & Lahiri 2004). The accumulation of the redox-active metals, iron and copper, may be a source of ROS, which are in turn responsible for oxidative stress observed in AD (Pereira *et al*. 2005).

It is currently accepted that calcium homeostasis disruption plays an important role in synaptic dysfunction and neurodegeneration occurring in AD (LaFerla 2002; Mattson & Chan 2003). Recent data suggest that A-β is capable of being directly incorporated into cell membranes, forming calcium-permeable ion channels, and causing abnormal intracellular calcium levels (Kawahara 2004), compromising the activity of calcium-dependent proteins (O'Day & Myre 2004). However, amyloid peptides have been described as being able to both alter the dynamics of intracellular calcium ion signals or have no effect at all, and it is almost impossible to extract an intelligible picture from which to draw conclusions, probably due to the variety of preparations used (Canevari *et al*. 2004).

The fact that the main genetic risk factor associated to AD codes for apolipoprotein E (APO E), the main cholesterol carrier in the brain, called the attention to the involvement of cholesterol dyshomeostasis, playing a role in the disease. APO E regulates cholesterol distribution in the brain; in addition, both APO E and cholesterol, are co-localized in mature SPs, further supporting their involvement in plaque formation (Burns *et al*. 2003; Mori *et al*. 2001). Even without unifying results, most studies suggest that an increased content of cholesterol in the brain correlates with an increased risk of developing AD (Vance *et al*. 2005). Moreover, transgenic mice experiments revealed that a high cholesterol diet increased brain cholesterol levels and accelerated deposition of A-β (Howland *et al*. 1998; Shie *et al*. 2002).

There is considerable evidence that endoplasmic reticulum (ER) dysregulation is involved in neuronal death occurring in AD, through the inhibition of protein glycosylation, perturbation of calcium homeostasis and reduction of disulphide bonds that provoke accumulation of unfolded protein in the ER, called 'ER stress' (Katayama *et al*. 2004). Dynamic calcium changes within the ER are implicated in the regulation of many cellular functions, from rapid signalling to long-lasting adaptive responses, determining the cell fate (Verkhratsky & Toescu 2003). It has been also proposed that A-β can directly mediate ER stress responses and apoptosis (Pereira *et al*. 2004). Additionally, ER stress and mitochondrial dysfunction might cooperatively regulate apoptotic-signalling cascades, pointing to ER stress as a key event in triggering and mediating neuronal death in AD (Takuma *et al*. 2005).

It has been observed that the neurotransmission systems impairment, particularly affecting cholinergic neurons, is one of the earliest pathological events in AD. In fact, it has been shown that acetylcholinesterase (AChE) activity, responsible for acethylcholine hydrolysis, was largely reduced, together with neuronal loss occurring in the brain of AD patients (Candy *et al*. 1983). Additionally, it has been demonstrated that A-β may increase AChE activity (Fodero *et al*. 2004), and conversely, it has been suggested that this enzyme can induce A-β aggregation (recently reviewed by Inestrosa *et al*. 2005). It has been recently found that increased AChE expression may impair glutamatergic neurotransmission (Dong *et al*. 2004). Several authors have provided further evidences that other neurotransmission pathways may be involved in AD, namely alterations in glutamatergic function and serotonin system. Evidence exists showing that glutamate-mediated excitotoxicity may contribute to the synaptic impairment observed in AD, particularly the altered expression and pharmacology of glutamate receptors and transporters in pathologically susceptible brain regions (see Tannenberg *et al*. 2004 for review). The role of serotonin in ageing and related disorders, such as AD (Meltzer *et al*. 1998), shows that serotoninergic dysfunction (Terry *et al*. 1991) is potentially responsible for a significant portion of the behavioural aspects of the disease, reinforcing the earlier observation that 5HTR2A-binding sites are diminished in post-mortem brain of AD patients. Furthermore, recent *in vitro* and *in vivo* pharmacological and biochemical studies, showing the involvement of 5-HT4 receptors in cognitive processes and the APP processing, have been presented (Maillet *et al*. 2004), stressing out the importance of serotoninergic neurotransmission dysfunction in AD.

Epidemiological data show that sex-specific incidence of AD is higher in women than men, suggesting the involvement of oestrogen and other hormones in the pathophysiological aspects of the disease. Accordingly, oestrogen has become the primary focus of research in this field. Both oestrogen concentrations, ranging from physiological to pharmacological, are neuroprotective in a variety of *in vivo*

and *in vitro* models of AD, as well as in reducing key neuropathologic features of the disease (Simpkins *et al*. 2005). However, inconclusive data regarding oestrogen-replacement therapy (recently reviewed by Gleason *et al*. 2005) have led to further investigate the role of other hormones in AD pathogenesis (Webber *et al*. 2004). It has been recently proposed that the increase in gonadotropin hormones levels, and not the decrease in steroid hormones production following menopause/andropause, may be a primary causative factor for AD development (Casadesus *et al*. 2005; Webber *et al*. 2005).

Studies demonstrating that non-steroidal anti-inflammatory drugs significantly reduce the risk of developing AD suggest the involvement of inflammatory response in AD pathogenesis (Stewart *et al*. 1997; reviewed by Tuppo & Árias 2005).

Damaged neuronal cells release cytokines, chemokines and acute phase/complement proteins, possibly activating microglia cells and astrocytes for an inflammatory response, with up-regulation of inflammatory mediators (recently reviewed by Tuppo & Árias 2005). Data available from literature point to the involvement of ROS and reactive nitrogen species in the microglia response during the neuroinflammation process (reviewed in Emerit *et al*. 2004). On the contrary, chronically activated glia associated with amyloid plaques might contribute to neuronal dysfunction in AD through the generation of neuroinflammatory molecules. APO E, associated with amyloid plaques, has been hypothesized to serve an anti-inflammatory role in the CNS through its ability to modulate Aβ-induced glial activation. Guo *et al*. (2004) provided evidence supporting that A-β stimulation of glial APO E limits neuroinflammation, but overproduction of APO E by activated glia might exacerbate inflammation. In addition, the observation that apo e4 isoform has more robust pro-inflammatory activity than apo e3 provides a mechanistic link between the *APO E ε4* allele and AD, and suggests potential apo E-based therapeutic strategies (Guo *et al*. 2004).

Furthermore, A-β itself may act as a pro-inflammatory agent (Franciosi *et al*. 2005), and recent evidence suggests that A-β oligomers induce a profound, early inflammatory response, whereas fibrillar A-β shows a slighter increase of pro-inflammatory response, consistent with a more chronic form of inflammation (White *et al*. 2005).

However, the role that neuroinflammation plays in AD is not fully clarified, and a better understanding of inflammatory response occurring in disease progression is needed to improve research in designing therapy specifically targeted against the inflammatory processes (Tuppo & Arias 2005).

Finally, an extensive research in the last few years has been focusing on cell death occurring in AD, particularly by apoptosis. A large amount of data support that A-β may lead to neuronal apoptosis (reviews in Pereira *et al*. 2004; Takuma *et al*. 2005). Several lines of evidence demonstrate that A-β peptides activate intracellular apoptosis pathways leading to neuronal cell death, through mitochondrial dysfunction and ER stress (Takuma *et al*. 2005) and also mediated by cell membrane death receptor activation (Dickson 2004; Li *et al*. 2004).

Mitochondrial dysfunction is thought to be relevant in AD not only through the abnormal generation of ROS but also through the release of molecules that may initiate apoptotic cell death pathways, such as cytochrome c (Morais Cardoso *et al*. 2002). However, the precise molecular mechanisms underlying mitochondrial dysfunction and ER stress in neuronal cell death and neurodegeneration remain unknown (Takuma *et al*. 2005).

Genetics of AD and APP

Several genetic factors have been associated to AD (Lendon *et al*. 1997). It has been demonstrated that the presence of the *APO E ε4* allele accounts for the risk of developing the disease. APO E is a polymorphic protein with three common isoforms – apo e2, apo e3 and apo e4 – coded by three alleles – *ε2*, *ε3* and *ε4* – at chromosome 19. *APO E ε4* appears to confer substantial risk for late-onset AD, either sporadic or familial (Saunders *et al*. 1993). Furthermore, mutations in the *APP* (St George-Hyslop *et al*. 1987) and presenilin (*PS1* and *PS2*) (St George-Hyslop *et al*. 1992; van Broeckhoven *et al*. 1992) genes were shown to be responsible for a fraction of the familial forms of AD (Levy-Lahad *et al*. 1995), with a mutation frequency of 18–50% in autosomal dominant early onset AD (Rademakers *et al*. 2003). The number of pathogenic mutations identified in the genes associated to the familial forms of AD is now over 170 (144 for *PS1*; 18 for *APP* and 10 for *PS2*) (AD Mutation Database http://www.molgen.ua.ac.be/ADMutations/default.cfm). Extensive cell biology research in the last decade raised the hypothesis that alterations of these genes cause AD through a common biological pathway, resulting in an abnormal APP metabolic processing (Rademakers *et al*. 2003).

Recently, transgenic mice have been investigated for studying the influence of the above-mentioned mutations *in vivo*. Mice overexpressing mutant AD-related proteins exhibit many of the neuropathological and behavioural features of the human disease. Transgenic animals have been created that express mutations in the *APP*, *PS1* and *PS2* genes, and in *APP* mouse models, an age-related accumulation of A-β-containing neuritic plaques have been described in the hippocampus and cerebral cortex, the activation of astrocytes and microglial cells (German & Eisch 2004). On the contrary, dramatic changes in A-β 42/40 levels have been observed in animals carrying PS2 mutations (Walker *et al*. 2005). The animal models of AD have also been used to develop and test treatments that reduce brain levels of the A-β 42 peptide (German & Eisch 2004), leading to the development of novel therapeutic strategies (Selkoe 2004; Walsh & Selkoe 2004a).

Despite the high number of pathogenic mutations of the above-mentioned genes identified in AD patients, which have been associated with an alteration of APP/A-β metabolic processing, they do not explain all AD cases.

A substantial body of evidence, indicating that disturbances of energy metabolism may play a fundamental role in AD, suggests that a mitochondrial location for a 'mechanistic bottleneck' in the neurodegenerative cascade of the disorder should be considered (Davis *et al*. 1995; Lee & Wei 1997).

APP metabolic processing in AD

Altered metabolic processing of APP, including the aggregation of A-β peptide (Hardy & Selkoe 2002) and, more recently, soluble protein aggregates – oligomers (Walsh & Selkoe 2004b), have been considered the central mechanisms underlying neuronal degeneration occurring in AD [modified A-β cascade hypothesis, reviewed in (Pereira *et al*. 2005; Wirths *et al*. 2004)]. Nevertheless, the direct link between A-β production and neuronal death has not been clearly established. However, there is evidence that oxidative stress promotes A-β generation and that hydrogen peroxide is likely to mediate A-β toxicity (Pereira *et al*. 2001).

Several lines of evidence support the hypothesis that A-β toxicity is mediated by ROS (Pereira *et al*. 2001); the first evidence of this mechanism/pathway has been demonstrated by the rescue of PC12 cells from A-β injury with antioxidants, including vitamin E (Behl *et al*. 1992). Furthermore, it was shown that A-β might induce membrane lipid peroxidation and damage of intracellular membrane systems (Behl *et al*. 1994a), and the toxic effects were prevented by antioxidants (Behl *et al*. 1994b). A direct evidence of oxidative damage conducted using A-β toxicity, in cultured neurons, was later obtained (Harris *et al*. 1995). The involvement of oxidative stress in AD has been recently reviewed, suggesting that A-β can also induce oxidative changes on its way to brain deposition, rendering nerve cells more vulnerable to additional insults (Behl 2005). Nevertheless, the precise molecular mechanisms and the pathophysiological impact of A-β-mediated oxidative stress on the development of AD require further investigation.

Data from literature point towards the involvement of mitochondrial dysfunction in A-β-induced toxicity and the role of mitochondria in A-β-triggered apoptosis (Cardoso *et al*. 2001; Mark *et al*. 1996; Pereira *et al*. 2004).

A reduced cytochrome c oxidase (COX) activity in AD patients' platelets has been observed (Cardoso *et al*. 2004a), and it was demonstrated that functional mitochondria are required for A-β toxicity (Cardoso *et al*. 2001). In contrast to NT2 rho+ (normal content of mitochondria, mtDNA), rho-0 cell (devoided of mtDNA, see King & Attardi 1989) viability was not affected by exposure to A-β, suggesting that a functional mitochondrial respiratory chain (MRC) is required for A-β toxicity. NT2 cells exposed to A-β were shown to release cytochrome c with subsequent activation of caspase 3, whereas rho-0 cells, which maintained mitochondrial membrane potential, demonstrated no cytochrome c release or caspase activation upon A-β exposure. A reduction of COX activity and a decrease in ATP levels have been found in AD cybrids, obtained by the fusion of rho-0 cells with AD patients' platelets, pointing to mtDNA involvement in AD pathogenesis. When these cell lines were exposed to A-β 1-40, a significant mitochondrial membrane depolarization, an increase in cytochrome c release and an elevated caspase 3 activity were also observed (Cardoso *et al*. 2001). Mitochondria seem to play a central role as a target for A-β peptides (Moreira *et al*. 2001), either as a cause or as an enhancing A-β toxicity (Cardoso *et al*. 2005). Furthermore, increased levels of 8-hydroxy-2′deoxyguanosine (8-OHdG), evaluated as a marker of mtDNA oxidation, were found in the brain of AD patients (Mecocci *et al*. 1994).

Hirai *et al*. (1991) have elegantly shown that damaged mtDNA in AD brain is restricted to neurons vulnerable to death, meaning that, although the increase is striking on a per-cell basis, when seen in the context of brain tissue, the change is very selective. This selective neuronal vulnerability is also observed for oxidative damage, suggesting that a relationship exists between the two phenomena. However, several studies have demonstrated mitochondrial abnormalities in non-neuronal tissues of AD patients (for example Blass & Gibson 1991; Trimmer *et al*. 2000).

The particular characteristics of mtDNA, including an increased susceptibility to oxidative damage and a higher rate of ROS-induced mutations (Schon 2000), render this genome as a potential target for oxidative injury. Accordingly, the pathological pathway triggered by A-β might include the increase in mtDNA mutations, possibly causing MRC impairment, which could be used as a marker of AD. Mitochondrial dysfunction has been reported to alter APP processing, leading to increased intracellular A-β accumulation (Beal 2004). mtDNA somatic mutations may confer increased sensitivity to A-β toxicity or may contribute to alterations in APP/A-β normal metabolic processing. In addition, Khan *et al*. (2000), in a study evaluating A-β peptides' secretion by cybrid cells containing 'AD mitochondrial genes', demonstrated that mtDNA genes from AD patients are responsible for cellular oversecretion of A-β peptides. These findings implicate mitochondrial genetic abnormalities as contributing to pathogenic amyloid metabolism in sporadic AD, and A-β oversecretion would serve a critical secondary role in the pathogenesis of the disease.

Accordingly, it can be hypothesized that cells may survive to a certain level of A-β buildup but then succumb as A-β further inhibits COX and possibly increases ROS production. Finally, those authors propose that mtDNA defects and A-β overproduction are etiologically intertwined, and that both would be necessary for the cell death and cognitive impairment that define this disease.

Mitochondrial DNA mutations and risk for AD

Mutations of mtDNA may cause a progressive decline in tissue output that could precipitate the onset of degenerative diseases in individuals harbouring inherited deleterious mutations. Thus, bioenergetic defects resulting from mtDNA mutations may be a common cause of human degenerative diseases, including AD (Brown & Wallace 1994; DeVivo 1993; Graeber *et al*. 1998; Hutchin *et al*. 1997; Wallace 1992). Moreover, impaired mitochondrial function caused by mtDNA damage may render brain cells more susceptible to oxidative injury and thereby provide a mechanism by which systemic or environmental factors could influence the course of the disease (de la Monte *et al*. 2000).

In 1992, Lin and colleagues identified a G-A transition and a G-T transversion, both at nucleotide 5460 of mtDNA NADH dehydrogenase subunit 2 gene, in AD patients, which were not confirmed by Edland *et al*. (2002), Janetzky *et al*. (1996), Kösel *et al*. (1994) and Petruzzella *et al*. 1992). Later, Shoffner *et al*. (1993) reported four variants (nucleotides 3196, 3397, 4336 and 956-965 insertion), having special importance the tRNA(Gln) alteration at position 4336 that modifies a moderately conserved nucleotide. Hutchin and Cortopassi (1995), Egensperger *et al*. (1997) and Edland *et al*. (2002) confirmed the presence of this tRNA variant in AD patients, whereas Wragg *et al*. (1995) and Tysoe *et al*. (1996) did not observe this association. Furthermore, Davis *et al*. (1997) found specific missense mutations in mitochondrial cytochrome oxidase subunit 1 and subunit 2 genes that appeared to be associated with late-onset AD. However, Hirano *et al*. (1997) provided evidence that the method employed by Davis *et al*. resulted in the coamplification of nuclear mtDNA pseudogenes.

A point mutation 16390 (C-T), located at the control region of the mtDNA, was reported to be two- to threefold more frequent in AD brain compared with normal brain (Chang *et al*. 2000). The same authors provided evidence for no significant changes in 4977-kb deletion in the same samples but observed regional variance in the distribution frequency of this alteration. Two other mtDNA variants (G8206T and A8224T) have been reported in one AD patient's blood cells, located at the gene coding for subunit 2 of complex IV (Qiu *et al*. 2001), reinforcing complex IV impairment, but COX activity has not been evaluated. In 2002, Lin and colleagues reported for the first time a quantification of the aggregate burden of point mutations in AD brain mtDNA. They have found that although the level of any one individual mutation is low, the aggregate burden of somatic mtDNA point mutations in AD brains is high, and the accumulation of these mutations correlates with a decrease in COX activity (Lin *et al*. 2002). The mutations/polymorphisms found so far, associated to AD, are summarized in Table 1.

mtDNA deletions have also been investigated in AD patients, but there is no consensus concerning this issue. Blanchard *et al*. (1993) reported no significant differences in deleted/undeleted mtDNA (common deletion, 4977 bp) content comparing AD cases with age-matched controls, consistent with findings from other authors (Chang *et al*. 2000). On the contrary, other authors have found significantly increased levels of the same deletion in AD brain (Corral-Debrinski *et al*. 1994; Hamblet & Castora 1997).

We have recently reported mtDNA variants in two AD patients: in one patient two already known mtDNA modifications (3197 T-C and 3338 T-C) were found and a novel mutation (3199 T-C) was identified (Grazina *et al*. 2005). A significantly higher frequency of the T3197C polymorphism was also detected in AD patients (Table 2) as well as an elevated frequency of *ε4* allele. Most of the cases having the 3197C variant, presented the *ε3ε3* genotype. We have identified three homoplasmic mtDNA variants in 14 AD patients (3197C, 3199C and 3338C) and three control subjects (3197C), by studying 129 AD patients and 125 normal subjects, affecting both *16S rRNA* (3197C, 3199C) and mtDNA *ND1* (3338C) genes. No positive cases were found for 3196 position, in both patients and control group.

The 3196A variant was found by Shoffner *et al*. (1993) in one late-onset AD patient, in a population of 73 individuals, and it was also described in an Asian control (homoplasmic). The fact that it was heteroplasmic in the patient suggested a new mutation, but by the fact of being found in only one patient precludes its pathological significance (Shoffner *et al*. 1993). The search for this mutation in Japanese AD patients did not show any positive case among 92 sporadic AD subjects (Tanno *et al*. 1998), similar to what occurred in our 129 AD patient subset. This variant is also absent in patients suffering from inclusion-body myositis (Kok *et al*. 2000). Another alteration at this position (G-T) has been recently described in a patient suffering from dilated cardiomyopathy (Ruppert *et al*. 2004).

The T-C 3197 transition at the *16S rRNA* mtDNA gene, reported earlier in one of our AD patients (Grazina *et al*. 2005), had been previously described (Hess *et al*. 1995) in a patient with ischaemic colitis and in AD, PD, AD + PD patients and Caucasian controls (Brown *et al*. 1996) as a polymorphism. This polymorphism was also described by Sternberg *et al*. (1998), Arbustini *et al*. (1998), Klemm *et al*. (2001) and Vives-Bauza *et al*. (2002) in control subjects and in patients with mitochondrial disorders, dilated cardiomyopathy, maternal diabetes mellitus and PD patients, respectively. The T-C 3199 transition was described for the first time by our group (Grazina *et al*. 2005). The two patients having the variants 3197C + 3338C and 3199C (Grazina *et al*. 2005) presented an *ε3ε3* genotype at *APO E* gene, as the majority of the cases having the 3197C variant, in accordance to what had been described by Egensperger *et al*. (1997) in an AD patient harbouring the A4336G mtDNA mutation.

If the 3197 T-C alteration is weakly conserved among hominoid primates (Fig. 1) and consensually considered a polymorphism, reported as associated to haplogroup U5,

Table 1: Summary of mutations/polymorphisms found in AD (modified from www.mitomap.org)

mtDNA variant	Mutation type	Affected gene	Amino acid change	References
146 (T-C)	SM	D-LOOP	NC	Unpublished (www.mitmap.org)
414 (T-C)	SM	D-LOOP	NC	Unpublished (www.mitmap.org)
477 (T-C)	SM	D-LOOP	NC	Unpublished (www.mitmap.org)
956-965 (insertion)	PM	12S RNA	NC	Shoffner *et al.* 1993
3196 (G-A; G-C)	PM	16S rRNA	NC	Shoffner *et al.* 1993; Tanno *et al.* 1998; Wallace *et al.* 1992;
3197 (T-C)	P	16S rRNA	NC	Grazina *et al.* 2005
3199 (T-C)	P	16S rRNA	NC	Grazina *et al.* 2005
3338 (T-C)	P	ND1	Valine-Alanine	Grazina *et al.* 2005
3397 (A-G)	PM/P	ND1	Methionine-Valine	Brown *et al.* 1996; Shoffner *et al.* 1993; Tanno *et al.* 1998; Wallace *et al.* 1992; 1995; Brown *et al.*1996;
4336 (T-C)	PM	tRNA Gln	NC	Cortopassi & Hutchin 1994; Coskun *et al.* 2004; Edland *et al.* 2002; Egensperger *et al.* 1997; Hutchin & Cortopassi 1995; Kok *et al.* 2000; Shoffner *et al.* 1993; Tanno *et al* 1998; Tysoe *et al.* 1996; Wallace *et al.* 1992; Wragg *et al.* 1995;
4580 (G-A)	P	ND2	Silent (methionine)	Kok *et al.* 2000;
5460 (G-A; G-T)	PM/P	ND2	Alanine-Threonine; Alanine-Serine	Edland *et al.* 2002; Janetzky *et al.* 1996; Kösel *et al.* 1994; Lin *et al.* 1992; Petruzzella *et al.* 1992
8206 (G-T)	PM?	CO2	Methionine-Isoleucine	Qiu *et al.* 2001
8224 (A-T)	PM?	CO2	Leucine-Phenylalanine	Qiu *et al.* 2001
16390 (C-T)	PM	D-LOOP	NC	Chang *et al.* 2000

SM, somatic mutation; PM, point mutation; P, polymorphism; NC, non-coding; ND, gene(s) coding for mitochondrial respiratory chain complex I subunits; CO, gene coding for cytochrome oxidase (COX, complex IV) subunit.

present in European populations (Herrnstadt *et al.* 2002), this assumption may not be true for the nucleotide change at position 3338. This modification was found by sequencing analysis only in one hypertrophic cardiomyopathy patient (Obayashi *et al.* 1992; Ozawa *et al.* 1991), in one multiple sclerosis control subject (Chalmers *et al.* 1995), in one Parkinson's patient (Kösel *et al.* 1998) and in our Alzheimer's patient (Grazina *et al.* 2005). Meanwhile, an absence of Rsa I cleavage site was found in several samples at nucleotide 3337 (Macaulay *et al.* 1999). This may be due to a change at nucleotide 3338 but, as Rsa I recognizes the same nucleotide sequence as Csp 6I, any of the four nucleotides (3337, 3338, 3339 and 3340) may have a change that causes the absence of cleavage, because there was no sequencing confirmation of those results. Besides, this is a much-conserved nucleotide among the hominoid primates (Fig. 1) as well as nucleotides 3339 and 3340, which does not occur with nucleotide 3337 because in *Pongo pygmaeus* (orangutan) there is an A instead of a G in the DNA sequence and a methionine instead of a valine in the ND1 protein (Fig. 1). It is quite clear that, even with a group of controls that does not cover all the population, we are facing an alteration at nucleotide 3338 with a lower frequency. The nucleotide change at position 3199 of the *16S rRNA* mtDNA gene found in another patient occurs in a moderately conserved nucleotide in hominoid primates because in *Pan paniscus* (bonobo) and *P. pygmaeus* (orangutan), there is a C instead of a T (Fig. 1). On the contrary, at position 3196, there is a G in *Homo Sapiens*, but there is an A in the other species analysed. It is possible that 3196A variant may represent some evolutionary advantage or that it has some protective role. Moreover, we cannot exclude that there is an interaction between *APO E* genotype and mtDNA alterations, or that in patients with an *ε3/ε3* genotype, there is a

Table 2: Frequencies of the mtDNA variants A3196G and T3197C and apolipoprotein E (APO E) alleles in Alzheimer's disease (AD) and controls

	APO E alleles			mtDNA variants	
	ε2	*ε3*	*ε4*	G3196A	T3197C
AD patients ($n = 129$†)	0.028	0.732	0.240**	0.000 (0/129)	0.101* (13/129)
Control subjects ($n = 125$)	0.060	0.880	0.060	0.000 (0/125)	0.024 (3/125)

AD, Alzheimer's disease; n, number of individuals; $*P < 0.050$ $**P < 0.0001$.

†125 for *APO E* genotyping.

chance of the mtDNA changes to be a genetic risk factor comparable with the risk associated to *ε4* allele, but additional studies are needed to elucidate this hypothesis. Nevertheless, Carrieri *et al*. (2001) have presented a possible association between mtDNA variations and *APO E* genotypes.

It is quite remarkable that the majority of AD patients presented alterations in the same region of the *16S rRNA* mtDNA gene, suggesting a possible contribution of mitochondrial *16S rRNA* to AD. The functional relevance of the modifications at rRNA mtDNA genes is still unclear, and additional studies are needed to elucidate the possible pathogenic role of rRNA mutations and to clarify the significance of our finding. As translation mechanisms occur at ribosomes, rRNA mutations may cause major assembly defects or may interfere with tRNA protection activity (Noller 1991).

Concerning age of onset, sex, MMSE and CDR data of patients carrying mtDNA mutations, it was not possible to establish a correlation with mtDNA analysis. However, it was possible to observe that AD patients carrying 3197C polymorphism presented, in majority of cases, with severe dementia (54%), as indicated by CDR, and/or cognitive impairment (69%), scored by MMSE. Apparently, such alterations in mtDNA do not interfere with cognitive functions. However, in patients with mild cognitive impairment and/or dementia, it is possible that the patients might have been evaluated at the beginning of the disease and being barely unaffected at the time, or other risk factors were absent in those cases, allowing a slower impairment progress.

Moreover, both polymorphisms in nucleotide 3197 and in nucleotide 3338, present in one AD patient, are considered harmless alone, but the synergistic effect of these alterations or the association to other mitochondrial or nuclear mutations and polymorphisms is still unknown. It is logical

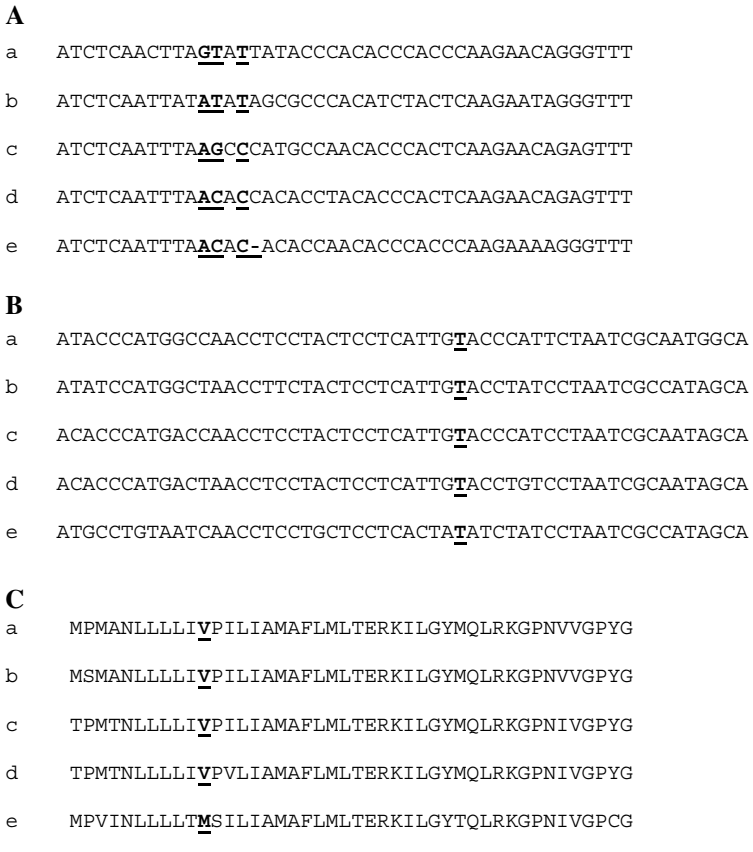

Figure 1: Evolutionary conservation among hominoid primates [*Homo sapiens* (a), *Gorilla gorilla* (b), *Pan troglodytes* (c), *Pan paniscus* (d) and *Pongo pygmaeus* (e)]. (a) mtDNA sequence surrounding nucleotides 3196, 3197 and 3199, all underline and bold. (b) mtDNA sequence surrounding nucleotide 3338, underline and bold. (c) *N*-terminal amino acid sequence of NADH dehydrogenase subunit 1 protein with amino acid coded by nucleotides 3337–3339, underline and bold. *Homo sapiens* mtDNA sequence obtained from Anderson *et al*. (1981) and Andrews *et al*. (1999). *G. gorilla*, *P. troglodytes*, *P. paniscus* and *P. pygmaeus* mtDNA sequences obtained from Horai *et al*. (1995).

to think that, as more nucleotide mutations are present in the mtDNA, higher should be the probability to develop an energy failure.

It is also possible that distinct combinations of non-mutated electron transport chain components do not function identically; being so, non-pathogenic mtDNA variations could contribute to AD risk while explaining why mtDNA polymorphism–AD associations are difficult to establish (Swerdlow & Kish 2002). This idea is supported by a recent study conducted with PD patients, demonstrating a significant decrease in risk of PD patients carrying haplogroup J (van der Walt *et al*. 2003). Additionally, Ross *et al*. (2003) have also demonstrated that mtDNA variant 4216C (segregates with JT haplogroups cluster) may influence mitochondrial dysfunction, affecting MRC complex I activity and increasing the risk for the disease (Ross *et al*. 2003). In Alzheimer's pathology, an association between mtDNA haplogroups and *APOE ε4* allele has been shown ; in particular, haplogroups K and U seem to be protective relatively to the harmful effect of the *ε4* allele (Carrieri *et al*. 2001).

We have recently reported for the first time, mtDNA alterations, one already known (3316 G-to-A) and another unreported (3337 G-to-A), and MRC complex I activity reduction in a frontotemporal dementia patient (Grazina *et al*. 2004), reinforcing the involvement of mtDNA alterations in dementia.

Conclusion

The study of peripheral tissues may be a step forward in identifying possible genetic risk factors in earlier stages of AD, allowing earlier therapeutic approaches. Additionally, *in vitro* studies performed in fibroblasts (Gibson *et al*. 1998) and cybrids (Cardoso *et al*. 2004b) of AD patients (Kumar *et al*. 1994) may contribute to clarify the mechanisms underlying the disease related to mitochondrial genome. It is important to take into consideration that analysis of mtDNA alterations should avoid false positives due to nuclear pseudogenes' detection (Wallace *et al*. 1997), which might be achieved by testing polymerase chain reaction primers with rho-0 cells' DNA.

Additionally, caution is necessary when dealing with mtDNA, because it has a high mutation rate due to a lack of a histone coat and an inadequate repair system (Schapira 1994). Given this vulnerability, free radical mtDNA damage may occur, particularly in ageing and neurodegenerative disorders, causing additional mtDNA injury, possibly initiating a vicious circle, making the interpretation of these results even more difficult, and no familial studies to evaluate the maternal heredity are possible in majority of cases.

It has been shown that elevated oxidative damage can be found in some AD patients, but no direct cause-effect relationship with mtDNA alterations, including 'common deletion', could be established (Bonilla *et al*. 1999). However, it has been recently shown that a higher degree of A-β deposition, overexpression of oxidative stress markers, mtDNA deletion and mitochondrial structural abnormalities were present in the vascular walls of the human AD,YAC and C57B6/SJLTg(+) mice compared with age-matched controls (Aliyev *et al*. 2005), as well as increased RNA oxidation and COX immunoreactivity (Aliev *et al*. 2003).

Although differences in mtDNA heredity may be important to interpret mtDNA alterations, the restriction of changes to vulnerable neurons only in cases of AD (shown by Hirai *et al*. 2001), rather than a more generalized defect involving all cell types, indicates that mitochondrial inheritance alone is not the only factor, but probably differences in mitochondrial turnover and metabolism, as well as antioxidant defences between different cells, are involved in Alzheimer's pathology (Hirai *et al*. 2001; Ito *et al*. 1999).

mtDNA mutations associated to AD may induce different phenotypic alterations, including functional impairment of the MRC activity, depending on the metabolic characteristics of the subject, the individual antioxidant defence panel or the environment exposure to toxic agents. Accordingly, oxidative stress resulting from mitochondrial abnormalities, including in mtDNA, seems to play an important role in AD pathological cascade.

Data reported in literature are controversial, and some discrepancy exists in the results from different groups, concerning mtDNA alterations. This might be due to several reasons, going from heterogeneity in the populations and tissues analysed by different research groups to age of onset of the disease or mtDNA regions investigated.

Taking into account mitotic segregation, the threshold effect and individual metabolic status, the moment when the samples are taken for analysis might influence the results obtained. Furthermore, different mtDNA regions have been considered for analysis, using different techniques (for example RFLP vs. sequencing), according to diverse reports. In addition, the biochemical results of MRC analysis in AD patients have been mainly directed to complex IV deficiency (Cardoso *et al*. 2004a; Parker *et al*. 1990), but the involvement of other MRC complexes, namely complex I, cannot be ruled out. The data reported on Table 1 strongly suggest that mtDNA genes coding for the subunits of complex I should be further investigated. Actually, the study of the expression of several mitochondrial and nuclear genes encoding the subunits of electron transport chain enzyme complexes showed a coordinated decrease of ND4 mRNA in AD hippocampus and inferior parietal lobule, probably leading to the inhibition of normal ubiquinone oxidoreductase activity of complex I (Aksenov *et al*. 1999).

It seems unlikely that mtDNA mutations found so far in AD patients are a genetic risk factor comparable with *APO E ε4* allele in sporadic AD or *APP/PS*1,2 mutations in familial cases. However, the fact that many of the identified mtDNA mutations are found in patients with neurodegenerative conditions implies that they may contribute to AD (Zhu *et al*. 2004).

The results reported in literature, including those recently obtained in our laboratory, suggest the contribution of mtDNA alterations to Alzheimer's pathology and that mtDNA alterations in 16S rRNA gene may also account for some risk in AD.

Furthermore, polymorphic variations in MRC subunits encoding genes (mtDNA and nDNA) determine MRC efficiency and basal mitochondrial ROS production, which correlates with mtDNA damage. Additionally, somatic mtDNA mutation decreases MRC efficiency leading to reduced OXPHOS and/or increased ROS production. Moreover, mtDNA mutations possibly modify age of onset, contributing to neurodegeneration process, probably due to an impairment of MRC and/or translation mechanisms.

Moreover, it is difficult to demonstrate a direct link between the mutant mtDNA genotype and a disease phenotype because of incomplete penetrance and the influence of environmental triggers (Ghosh *et al*. 1999).

It is likely that mitochondria play a proximal role in the pathogenesis of the disease and, despite the many known factors that can contribute to mitochondrial dysfunction at some time during the disease progression, it still remains to be determined what initiates the mitochondrial abnormalities in the disease (Zhu *et al*. 2004). Nevertheless, some authors have suggested that the possibility of mitochondrial involvement in AD may be secondary to other pathological events (Byrne 2002; Orth & Schapira 2001). Currently, it is not clear whether oxidative damage to mitochondria leads to a decreased efficiency of the MRC or results in excessive electron release and ROS formation; in any case, the result would be a vicious feed-forward cycle where increased oxidative stress would continually reduce mitochondrial bioenergetics (Sullivan & Brown 2005).

It has been suggested, in studies with cybrids, that complex IV deficiency described in a majority of AD cases found in literature may be a primary defect related to the pathology, rather than a secondary insult that is associated with disease morbidity (Ghosh *et al*. 1999). Nevertheless, this issue remains unclear, and further analyses are required to clarify the exact role of MRC defects in AD.

The role of mitochondria failure in the pathogenesis of AD has been widely accepted. However, the exact cellular mechanisms behind vascular lesions and their relation to oxidative stress markers identified by RNA/DNA oxidation, lipid peroxidation or mitochondrial DNA (mtDNA) alterations remain unknown. Future studies comparing the spectrum of mitochondrial damage and the relationship to oxidative stress-induced damage during the maturation of AD pathology are warranted (Zhu *et al*. 2004).

Aiming to reach a comprehensive role of mitochondria in AD, Swerdlow and Khan (2004) have recently proposed the 'mitochondrial cascade hypothesis' for sporadic AD. The authors suggest that inherited, gene-determined make-up of an individual's MRC sets basal rates of ROS production, potentially relevant to the rate at which mitochondrial oxidative damage accumulates in an individual over time. Additionally, oxidative mitochondrial nucleic acid, protein and lipid damage amplifies ROS production and triggers events that result in A-β overproduction, further perturbation of MRC function, abnormal apoptotic responses by the cells, aneuploidy, tau hyperphosphorylation and NFTs' formation (Swerdlow & Khan 2004). Other authors have also suggested that abnormalities in mtDNA may be a major cause of abnormal ROS production in AD (Migliore *et al*. 2005), and recent data provide additional evidence that energy failure may cause significant changes in both APP expression and APP metabolism (Hoyer *et al*. 2005).

Moreover, Swerdlow and Khan (2004) state that this hypothesis also allows and accounts for histopathologic overlap, mediated by bioenergetic dysfunction and mitochondrial ROS overproduction, between the sporadic, late-onset and autosomal dominant, early-onset forms of the disease. Epidemiological studies of AD cases showing a maternal overrepresentation among the affected parents (Duara *et al*. 1993; Edland *et al*. 1996) suggest a maternally inherited genetic factor (mtDNA) that helps to determine life expectancy, as well as contributing to AD risk (Swerdlow & Khan 2004).

In conclusion, controversial data from literature point to both directions: one stating that mtDNA mutations are more likely to be a by-product, secondary to other pathological features during the development of the disease; the other being consistent with the 'mitochondrial cascade hypothesis', claiming that mtDNA modifications (both pathogenic and/or polymorphic) are a cause of the disease.

However, the exact role of mtDNA mutations in AD remains as a piece of the puzzle on the AD pathological mechanisms' enigma, and effective translation of knowledge of the cellular mechanisms responsible for the disease into novel therapeutic modalities that may not conform to current consensus may require a level of dedication and enthusiasm that allowed Alois Alzheimer to bring forth his invaluable observations and identification of AD as a defined clinical entity (Chong *et al*. 2005).

More to the point, perhaps a key difficulty in the majority of the experiments published lies in the extent to which one can ever hope to mimic a disease that might take 70 years to develop in a tissue-culture dish in an afternoon's experiment (Canevari *et al*. 2004).

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