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Nutraceutical approaches against amyloid-β induced neuropathology: an *in vivo* and *in vitro* study

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ABSTRACT

Introduction:

Mounting evidence supports the beneficial effects of the Mediterranean diet (MD) and the Asian diet in delaying ageing and in preventing age-related dysfunctions, cancer, diabetes and neurodegenerative diseases. The beneficial effects of the MD and Asian diets in reducing age-related dysfunctions, including Alzheimer's disease (AD), could be the consequence of the presence in specific foods of substantial amounts of specific polyphenols whose beneficial properties include the ability to interfere with amyloid aggregation. Our previous data have highlighted the beneficial effects of oleuropein aglycone (OLE) against protein/peptide aggregation *in vitro* and in TgCRND8 (Tg) mice, a model of Aβ deposition.

Aim:

To check in Tg mice 1) the effects of OLE or a mix of polyphenol extracts or hydrohytyrosol (HT) on cognitive functions and neuropathology and 2) the effect of andrographolide administration on cognitive functions and its ability to cross the blood brain barrier (BBB) conjugating it with fluorescent albumin nanoparticles. In addition, in rats we investigated 1) the ability of intravenous (i.v.) administered human fetal cholinergic neurons (hfCNs), isolated from the nucleus basalis of Meynert (NBM) of 12-week old fetuses, to cross the BBB and to improve memory functions of quisqualic acid NBM-lesioned rats and 2) the ability of intravenously administered solid lipid nanoparticles (SLNs) to cross the BBB and to penetrate the central nervous system of adult rats.

Methods:

In vivo experiments were carried out using: 1) Tg and wild type (wt) mice treated for 8 weeks with a modified low-fat (5.0%) AIN-76A diet (10 g/day/ mouse) as such, supplemented with OLE (50mg/kg of diet) or with a mix of polyphenol extracts (50 mg/kg of diet) found in olive mill waste water or with HT (50 mg/kg of diet), 2) Tg and wt mice injected intraperitoneally (i.p.) for 4 weeks (3 i.p. for week) with andrographolide (ANDRO) (4 mg/kg) conjugated with fluorescent albumin nanoparticles (NPs-ANDRO), 3) male Wistar rats divided into the following groups: Group I: injected into the NBM with 0.5 μL of quisqualic acid and subsequent intravenous (i.v.) administration by the tail vein of 300 μL of 1.5ML hfCNs. Group II: injected into the NBM with 0.5 μL of quisqualic acid. Group III: injected into the NBM

with 0.5 μL saline and subsequent i.v. administration by the tail vein of 300 μL of 1.5M hfCNs. Group IV: injected into the NBM with 0.5 μL saline; Group V: un-injected rats and 4) male Wistar rats divided into the following groups Group I: injected i.v. with 200 μL of nanoparticles (SLN 28.57 mg/ml plus FITC 1.43 g/ml) and sacrificed after 3h; Group II: injected i.v. with 200 μL of nanoparticles and sacrificed after 24h; Group III: injected i.v. with 200 μL of nanoparticles and sacrificed after 72h; Group IV: injected i.v. with 200 μL of 0.9% of saline.

For *in vitro* experiments were used neuroblastoma cell line (N2a) maintained in a 5.0% CO2 humidified atmosphere at 37°C.

Results and Conclusion:

Our results show that OLE supplementation induces epigenetic modifications as previously reported, favoring the expression of SIRT1 and reducing that of PARP1 confirming the existence of a functional link between PARP1 and SIRT1. Furthermore, diet supplementation with a mix of polyphenols or HT strongly improved mouse cognitive performances and reduced ß-amyloid deposits. An intense activation of autophagy was found in the cortex of all treated groups. HT administration also affected the inflammatory response in the hippocampal areas, as shown by the reduced astrocytes activation and TNF-α mRNA levels.

These results show that diet supplementation with a mix of polyphenols or HT at the same dose as that of pure OLE previously administered ameliorate cognitive functions and indicate that either a mix of polyphenols or HT treatment is useful to treat neurodegenerative diseases.

In addition, we found that i.v. administration of hfCNs migrated to the injected NBM and along the needle tract. In the quisqualic acid NBM-injected rats the cognitive impairments were significantly improved by hfCNs. Finally, we found that both SLNs and NPs were able to cross the BBB and that NPs-ANDRO were able to cross the BBB and to improve cognitive functions in Tg mice, supporting that the developed nanocarriers represent new potential brain delivery system to increase the efficacy of andrographolide treatment in neurodegenerative diseases.

INDRODUCTION

1 Alzheimer's disease

1.1 The discovery of Alzheimer's disease

On Nov 4, 1906, Alois Alzheimer gave a remarkable lecture (Alzheimer A., 1906), in which he described for the first time a form of dementia that subsequently, at the suggestion of Emil Kraepelin, became known as Alzheimer's disease (AD). In his lecture, at the 37th Conference of South-West German Psychiatrists in Tübingen, Alzheimer described a patient called Auguste D., a 51-year-old woman from Frankfurt who had shown progressive cognitive impairment, focal symptoms, hallucinations, delusions, and psychosocial incompetence. At necropsy, there were plaques, neurofibrillary tangles, and arteriosclerotic changes. The eponym Alzheimer, originally used to refer to presenile dementia, came into later use for the largest cause of primary dementia-senile dementia of the Alzheimer type.

1.2 Auguste Deter: *the case*

''She sits on the bed with a helpless expression.
What is your name?
Auguste.
Last name?
Auguste.
What is your husband's name?
Auguste, I think.
Your husband?
Ah, my husband. She looks as if she didn't understand the question.
Are you married?
To Auguste.
Mrs D?
Yes, yes, Auguste D.
How long have you been here? She seems to be trying to remember.
Three weeks.
What is this? I show her a pencil.
A pen.
A purse and key, diary, cigar are identified correctly.
At lunch she eats cauliflower and pork.
Asked what she is eating she answers spinach.''
Dr. Alzheimer's notes: Nov 26, 1901.

This is an excerpt taken from Dr. Alois Alzheimer's note on conversation between him and Auguste D.. Auguste Deter was a 51-years old German houswife who became a patient of Dr. Alzheimer's in 1901.

On Nov 25, 1901, she was admitted to the Frankfurt hospital, where she was examined by Alzheimer. She had a striking cluster of symptoms that included reduced comprehension and memory, as well as aphasia, disorientation, unpredictable behaviour, paranoia, auditory hallucinations, and pronounced psychosocial impairment.

Deter died in April 1906, aged 55. Hearing of Deter's death, he requested from Dr. Sioli, the director of the Frankfurt institution where Deter had been, that her medical records be sent to him.

When he received the material, Bielschowsky has already published, in 1903, a silver impregnation method that revealed the neurofibrils (Berchtold N. C. and Cotman C. W., 1998). Together with two visiting Italian physicians, Gaetano Perusini and Francesco Bonfiglio, Alzheimer meticulously examined the histological sections. They showed a massive loss of cells throughout the brain.

In addition to the atrophy, Alzheimer and his colleagues observed peculiar thick and strongly staining fibrils in the remaining neurons. They also discovered deposits of an unidentified substance in the form of plaques throughout the cerebral cortex. The brain of Auguste D. thus displayed what today are considered the histopathological hallmarks of AD: a loss of neurons as well as the accumulation of neurofibrillary tangles and amyloid plaques (Fig. 1). To Alzheimer and his colleagues though, the findings on Auguste D.'s brain represented a novel and as yet uncharacterised pathology.

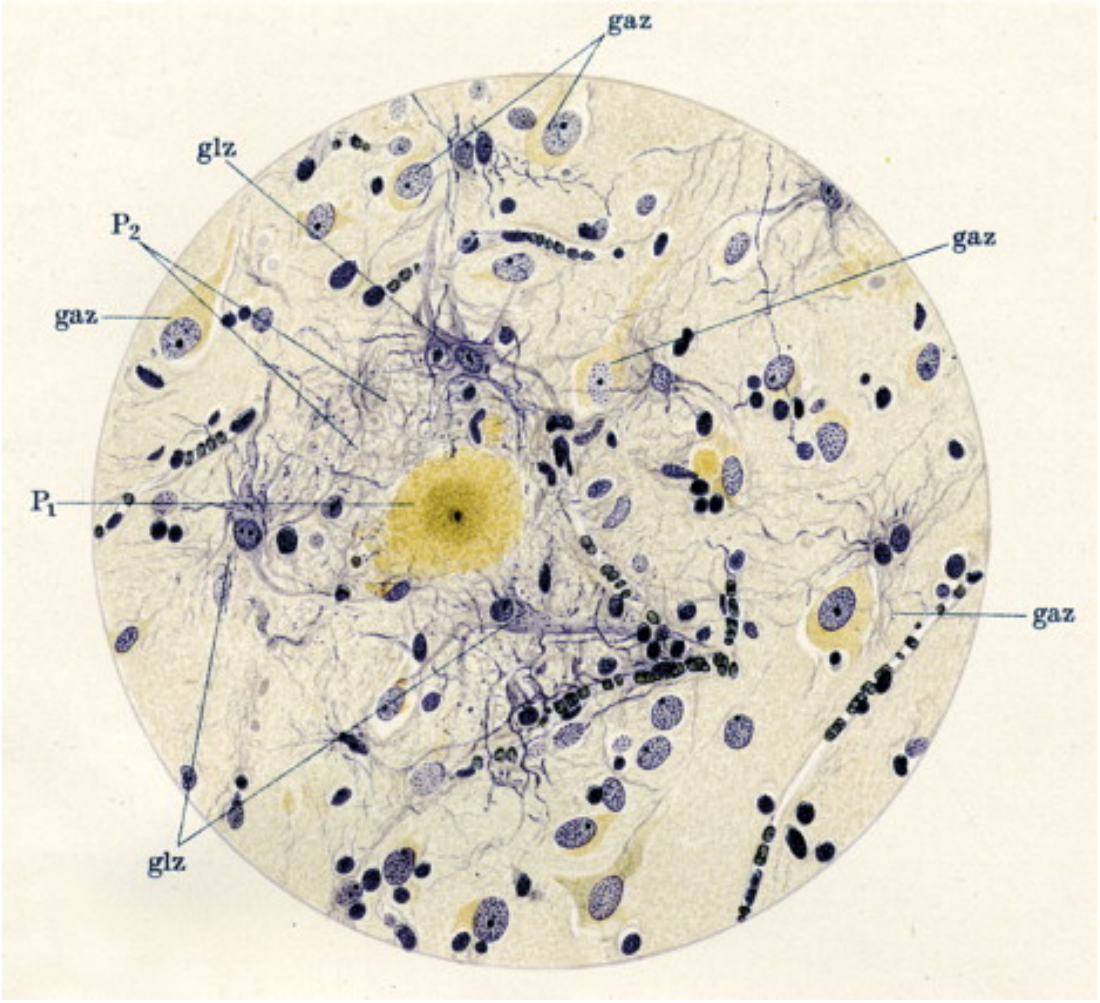

Fig. 1 *Illustrations by Alzheimer showing neurofibrillary tangles and amyloid plaques. The images were included in Alzheimer's 1911 article describing the cases of Auguste D. and Johann F.*

On November 3rd 1906, Alzheimer presented his results of the case of Auguste D. for the first time. The occasion was the 37th meeting of South-West German psychiatrists in Tübingen, Germany. Alzheimer's talk was entitled '*On a peculiar disease of the cerebral cortex'*.

Between 1907 and 1908, three additional patients of his, deceased after having displayed symptoms very similar to those of Auguste D. Together with Perusini, Alzheimer studied their brains and compared them to the changes observed in Auguste D. They discovered that the four cases shared key characteristics, including the neurofibrillary tangles and the formation of plaques throughout the cerebral cortex. Their results, including the first pictures of the histopathological changes of Auguste D.'s brain, were published by Perusini in 1909.

Alzheimer himself published the first comprehensive account of Auguste D.'s case only in 1911 (Fig. 2).

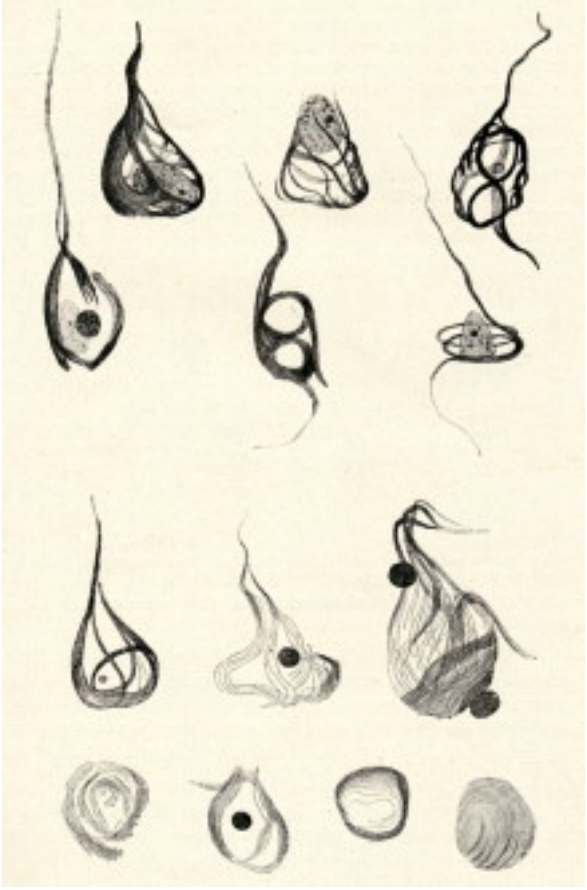

Fig. 2 *Drawings of neurofibrillary tangles by Alzheimer and published in his 1911 paper.*

This was the first documented case of a new presenile dementia subtype, soon named after him, AD, by Kraepelin.

1.3 Epidemiology

The World Health Organization (WHO) projected that in 2005, 0.379% of people worldwide had dementia, and that the prevalence would increase to 0.441% in 2015 and to 0.556% in 2030. A more recent WHO study estimates that in 2010, 47.5 million people had dementia, 35 million people of whom had AD (WHO, 2010, 2015). In line with the previous, another study estimates that by 2050, the prevalence of AD will quadruple, by which time 1 in 85 persons worldwide will be living with the disease (Brookmeyer et al., 2007). The death attributable to AD and other dementias, seems to be correlated to both yearly income (World Bank income categories) and living region (WHO region). Where the low-income category contributes to 0.41% of deaths as

compared to 2.84% for the high-income category. Correspondingly, the Eurozone contributes to 2.04% of AD and other dementias related deaths as compared to 0.1% for Africa, 1.47 for the Americas and 0.42% for Eastern Mediterranean countries (WHO, 2006).

1.4 Genetic epidemiology of AD

Following advanced age, family history is the second greatest risk factor for AD. AD is considered to be a genetically dichotomous disease presenting in two forms: early-onset familial cases usually characterized by Mendelian inheritance (EOAD), and late-onset (> 60 years), with no consistent mode of transmission (LOAD; Tanzi R. E. and Bertram L., 2005). Familial clustering of AD cases is more obvious for the early-onset form that strikes under the age of 60 years. However, it is estimated that up to 80% of AD involves the inheritance of genetic factors, based on twin and family studies (Gatz M. et al. 2006). So-called "sporadic" AD is strongly influenced by genetic variants combined with life exposure factors. EOAD is most often caused by rare, fully penetrant mutations in three different genes.

1.4.1 Early onset Alzheimer's disease

EOAD is most often caused by rare, highly penetrant mutations in three genes encoding proteins involved in amyloid precursor protein (APP) breakdown and A generation, namely, the APP (Goate A. et al., 1991), presenilin 1 (PSEN1) (Sherrington R. et al., 1995), and presenilin 2 (PSEN2) (Levy-Lahad E. et al., 1995) genes (Zhangyu Z. et al., 2014).

- ***APP gene:*** The APP gene has 18 exons and encodes a ubiquitously expressed single-pass transmembrane polypeptide of 770 aminoacids. Aβ is generated from APP by two endoproteolytic cleavage events catalyzed by β-secretase and γ-secretase, generating aβ peptide of 39–43 amino acids in length. The most common form of Aβ is 40 amino acids in length and is called Aβ40; the longer form, Aβ42, is less abundant than Aβ40 but is deposited first and is more amyloidogenic. It has been identified 33 different mutations, most of them dominantly inherited, in the APP gene, including 23 missense mutations nine duplications, and one deletion (Crutz M. et al., 2012). These are mostly located in the vicinity of the β-secretase cleavage sites (exons 16 and 17 of APP) and they influence APP proteolytic processing and/or aggregation (Zhangyu Z. et al.,

2014). Swedish APP Mutation (KM670/671NL) is a double point mutation, that determine increase of total Aβ load, located at the N-terminus of the Aβ domain adjacent to the β-secretase site in which lysine–methionine is replaced by asparagine–leucine (Mullan et al., 1992). There are others mutation such as Tottori mutation (Asp678Asn) and Leuven mutations (Glu682Lys) located at the N-terminus of the Aβ domain adjacent to the β-secretase site, London mutation (V717I) (Giri et al., 2016), Arctic mutation (E693G), Flemish mutation (A692G).

- ***PSEN1 and PSEN2 Gene***

  They encodes for presenilin 1 and presenilin 2 proteins, respectively. They are required for γ-secretase to produce Aβ from APP, and were identified in EOAD families (Levy-Lahad E. et al., 1995) (Sherrington R. et al., 1995) (Rogaev E. I. et al., 1995). PSEN1 gene is located on chromosome 14q24.3, while PSEN2 gene is located on chromosome 1q31-q42.

  PSEN1 is localized primarily to the endoplasmic reticulum and helps in protein processing. The function of PSEN2 is related to PSEN1. It has been identified 13 pathogenic PSEN2 mutations in 29 families (Cruts M. et al., 2012).

  Mutations, majority missense mutations, in PSEN1 and PSEN2 cause aminoacid sequence changes throughout the protein with some clustering within the transmembrane domains and the hydrophilic loops. Mutations in exons 5, 6, 7, and 8 account for > 70% of all identified mutations in PSEN1 (http://www.molgen.vib-ua.be/ADMutations/) (Crutz M. et al., 2012). The presenilins act as aspartyl proteases that carry out γ-secretase cleavage of APP to produce Aβ. Therefore, mutations in the PSEN1 and PSEN2 genes increase the ratio of Aβ42 to Aβ40. Studies have shown that Aβ42 is more amyloidogenic and more prone to aggregate in brain than Aβ40 (Tanzi R. E. et al., 2005; Esteller M. et al., 2001; Steiner H., 2004).

  The majority (78%) of the EOAD mutations are mostly PSEN1 mutations, followed by APP (18%), with PSEN2 mutations found in only a few families (4%). The mutations are responsible for 30–50% of autosomal dominant AD cases and about 0.5% of AD cases in general (Crutz M. et al., 2012) (Zhangyu Z. et al., 2014).

1.4.2 Genetics of Late-onset AD

LOAD is the most common form of the disease and is defined by onset age 65 years which genetic risk factors work together with environmental factors and life exposure events to determine lifetime risk for AD. Consequently, it is much more difficult to reliably identify novel LOAD loci, especially because efforts at replication are often plagued by a mixture of replications and refutations (Tanzi R. E., 2012).

ε4 allele of the APOE gene was the only well-established risk factor for the pathogenesis of LOAD, but with technological advances, researchers have identified a number of regions of interest in the genome that may increase a person's risk for LOAD to varying degrees.

It has been discover several genes in this form of disease:

- **APOE:** *APOE* gene, located on chromosome 19q13.2, is a major cholesterol carrier that supports lipid transport and injury repair in the brain. *APOE* polymorphic alleles are the main genetic determinants of AD risk: individuals carrying the ε4 allele are at increased risk of AD compared with those carrying the more common ε3 allele, whereas the ε2 allele decreases risk. Presence of the *APOE* ε4 allele is also associated with increased risk for cerebral amyloid angiopathy and age-related cognitive decline during normal ageing. ApoE–lipoproteins bind to several cell-surface receptors to deliver lipids and also to hydrophobic Aβ peptide, which is thought to initiate toxic events that lead to synaptic dysfunction and neurodegeneration in AD. ApoE isoforms differentially regulate Aβ aggregation and clearance in the brain, and have distinct functions in regulating brain lipid transport, glucose metabolism, neuronal signalling, neuroinflammation, and mitochondrial function (Chia-Chen L. et al., 2013): ApoE4, infact, inhibits Aβ clearance and is less efficient in mediating Aβ clearance compared with ApoE3 and APOE2. ApoE4 also contributes to AD pathogenesis by Aβ-independent mechanisms that involve synaptic plasticity, cholesterol homeostasis, neurovascular functions, and neuroinflammation.
- **SORL1:** (also known as LR11, SORLA or SORLA1) located on chromosome 11q23, produces a receptor for neuronal APOE, low density lipoprotein receptor class A. SORL1 binds APP and regulates its sorting into endocytic or recycling-pathways (Rogaeva E. et al. 2007). High levels of this receptor have been associated with lower Aβ peptide production due to SORL1 promoting recycling

of APP, instead of transporting it to endosomes or lysosomes where Aβ peptide is produced (Rogaeva E. et al., 2007). Many SORL1 SNPs have been associated with AD risk (Bertram L. et al., 2007), however further factors, both genetic and non-, are also thought to affect expression of this receptor, although the implications of these are not fully understood.

- **CLU** or clusterin, found on chromosome 8, also known as APOJ, and as suspected, encodes for a lipid transport molecule able to bind Aβ peptide like APOE (Jomary J., 2002). Recent sudies reported that it can binds, in animal models of disease, the soluble form of Aβ in a specific and reversible manner, forming aggregates able to cross the blood-brain barrier. It acts as a molecular chaperone for Aβ influencing conformation and toxicity of Aβ such as clusterin, that may regulate both the toxicity of Aβ and its conversion into insoluble forms. These results can suggest a protective role for clusterin in the amyloidogenic pathway.
- **PICALM**, found on chromosome 11, encodes the phosphatidylinositol. It can bind clathrin assembly protein and is thought to be involved in synaptic neurotransmitter release and intracellular trafficking (Yao P. J. et al. 2005). Researchers suggest its involvement in AD relates to PICALM's location in endothelial cells and is most likely associated with transporting Aβ peptide through the BEE (Baig S. et al., 2010).

Many more genes have been associated with AD risk, however the risk effects are small. Many studies suggest it is most likely a combination of multiple genes, as well as environmental effects that impact on an individual's AD risk, however studies revealing associations do reveal pathways that may be involved in the aetiology of the disease.

1.5 Pathogenesis of AD

AD is characterized by the presence of senile plaques, containing the Aβ and neurofibrillary tangles (NFTs), consisting of hyperphosphorylated forms of τ protein; in AD a selective death of the cholinergic system, atrophy and synaptic and neuronal loss occurs; all these features contribute to the progressive cognitive decline that characterizes AD (Selkoe D. J., 2002; Ling Y. et al., 2003).

1.5.1 Amyloid plaques

Amyloid plaques (Fig. 3) are extracellular microscopic deposits, which are found in close proximity to degenerating axons and dendrites. In the mid-1960s Michael Kidd and Robert Terry used the electron microscope to describe the ultrastructural properties of AD brain lesions, demonstrating that plaques are composed of a filamentous material with a structure identical to amyloid, and revealing that this fibrillar substance was different from that of neurofibrillary tangles (Kidd M. 1964; Terry R. D. et al., 1964).

The amyloid plaque core protein is known today as Aβ, a short peptide fragment of ~37-43 amino acids, with a high propensity to aggregate (Jarrett J. T. et al, 1993).

Amyloid plaques exhibit different sizes (10-120 μm) and degrees of compactness, such that two main types can be distinguished: 1) diffuse and 2) neuritic plaques.

1.5.1.1 Diffuse amyloid plaques

Diffuse amyloid plaques are abundant in cortex and hippocampus but can be also found in other brain regions not typically implicated in AD symptomatology (i.e. thalamus, cerebellum, striatum). They are composed of non-fibrillar aggregates of Aβ, mostly ending at amino acid 42, a slightly longer, more hydrophobic form, prone to aggregation. Diffuse plaques are characterized by the absence of a compact, dense core, which is associated with little or no neuritric dystrophy. It has been hypothesized that these structures may be the precursors of neuritic plaques. However diffuse plaques can be found in the brains of non-demented subjects, in regions vulnerable to AD pathology (Bennett D. A. et al., 2006).

1.5.1.2 Senile plaques

Senile plaques are extracellular deposits characterized by Aβ peptide (about 4 kDa) that aggregates (Kang J. et al., 1987). They are composed by extracellular compact deposits of Aβ40 and Aβ42 peptides, soluble oligomers and insoluble fibrils. Electron microscopy studies revealed that the ultrastructure of dense-core plaques is comprised of a central mass of extracelullar filaments that radially extend toward the periphery, where they are intermingled with neuronal, astrocytic, and microglial processes. These neuronal processes, known as dystrophic neurites, often contain packets of paired helical filaments, as well as abundant abnormal mitochondria and dense bodies of probable mitochondrial and lysosomal origin (Fiala J. C. et al., 2007). Plaque-associated neuritic dystrophies represent the most notorious evidence of Aβ-induced neurotoxicity

and feature many of the pathophysiological processes downstream Aβ (Su J. H. et al., 1993).

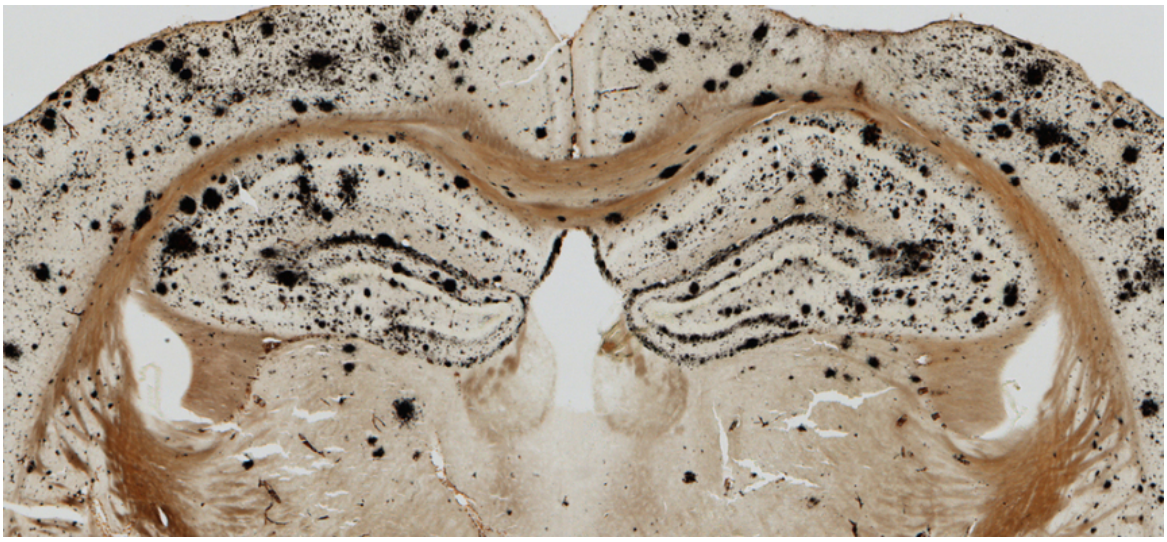

Fig. 3 *Amyloid deposits in brain tissue from a mouse model of AD (Zhao et al., 2014).*

1.5.2 Neurofibrillary tangles

NFTs (Fig. 4) are insoluble intracellular protein inclusions found inside neurons, typically occupying the entire cytoplasm. The core of NFTs is composed of bundles of paired helical filaments (PHFs), containing all brain isoforms of the τ protein in a hyperphosphorylated state (Goedert M. et al, 1988). τ is a microtubule- associated protein, which is essential for axonal microtubule assembly and stability. As a result of hyperphosphorylation, τ loses affinity for microtubules, interfering with the neuron's metabolic transport system (Bramblett G. T. et al, 1993). This chemical modification renders it insoluble, highly prone to aggregation and favours filament formation (Haase C. et al, 2004). Increased τ phosphorylation may be a consequence of an altered balance between the actions of specific kinases (i.e. GSK-3b, cdk5, MAPK) and phosphatases (PP2A, PP-1), which may exhibit enhanced and reduced activities, respectively (Wang J. Z. et al., 2013).

Notably, the extent of NFT pathology positively correlates with the severity of dementia (Arriagada P. V. et al., 1992), suggesting that τ tangles play a prominent role in neurodegeneration.

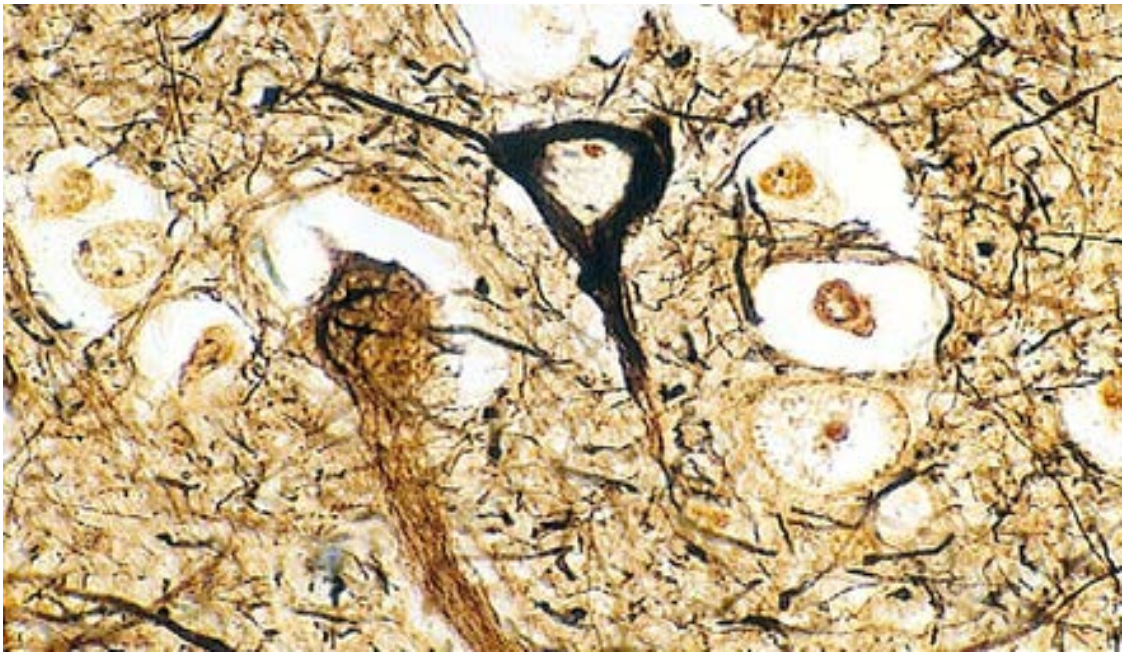

Figure 4: *NFTs in a brain section from an AD patient.*

1.5.3 The APP

The amyloid precursor protein (APP) is a highly conserved transmembrane, type-1, integral glycoprotein that is ubiquitously expressed and around 110-135 kDa in size (Selkoe D. J. et al., 1988; Tharp W. G. and Sarkar I. N., 2013). It has two mammalian homologs, APP-like protein 1 (APLP1) and 2 (APLP2) (Coulson T. et al., 2000) with several common conserved motifs. Howeveer, Aβ domain in not conserved and unique to the APP protein (Bayer T. A. et al., 1999).

The APP gene, in human, is located on chromosome 21.

APP695, APP751 and APP770 (among 8) are the three major isoforms containing 695, 751, and 770 amino acids, respectively (Sandbrink R. et al., 1996) generated by alternative splicing. The isoform mainly expressed in the central nervous system (CNS), is APP695, in particular in neurons in the cerebellum, cortex and hippocampus, while APP751 and APP751 areexpressed in other tissues (O'Brien R. G. and Wong P. C., 2011).

The physiological role of APP remains unclear. It may be able to trafficking neuronal protein along the axon, neurite outgrowth and synaptogenesis, transmembrane signal transduction, cell adhesion and calcium metabolism (Zheng H. and Koo E. H., 2006). APP is sequentially cleaved to generate Aβ protein, the main component of plaques (Korenberg J. R. et al., 1989). Two cleavage pathways for APP are possible: the amyloidogenic and non-amyloidogenic (Fig. 5) (Montoliu-Gaya L. and Villegas S.,

2015).

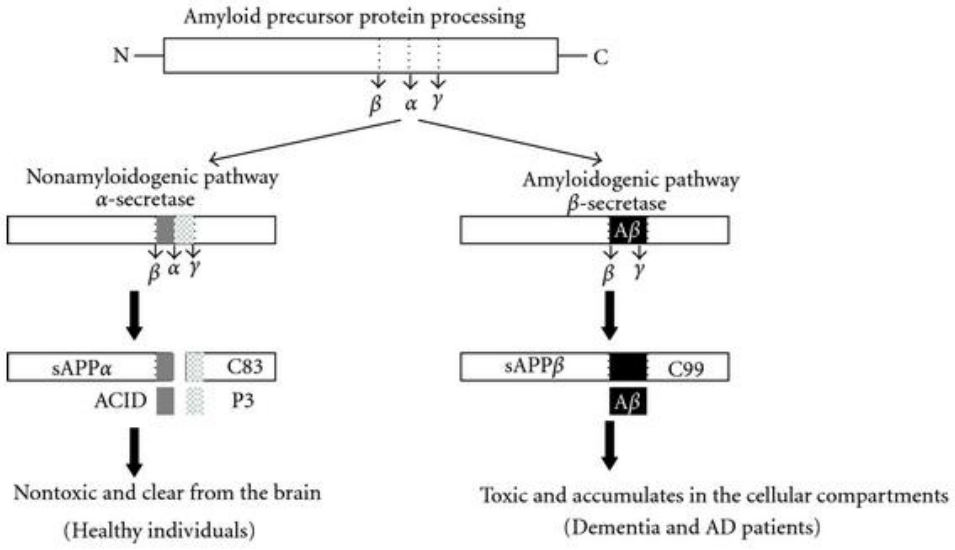

Fig 5: *The APP processing.*

1.5.3.1 The non- amyloidogenic pathway

In this pathway, APP is cleaved by a α-secretase releasing a large soluble N-terminal fragment called sAPPα and a smaller C-terminal fragment (C83) (Esch F. S. et al., 1990).

While sAPPα is released into the extracellular space, the 83-residue C-terminal fragment (C83) is membrane-bound. C83 is further cleaved by γ-secretase into the soluble, 3 kDa C-terminal fragment (p3) and the APP intracellular domain (AICD). The p3 peptide is rapidly degenerated (Zheng H. and Koo E. H., 2011). While AICD is subjected to act as a transcriptional regulator, the function of p3 is unknown (Chow V. W. et al., 2010). The cleavage of APP by α-secretase occurs within the Aβ domain, preventing the generation of amyloid peptides (Sisodia S. S., 1992). In contrast to Aβ, several studies suggest that sAPPα is neuroprotective. sAPPα plays a role in neuronal plasticity, synaptogenesis, cell adhesion as well as early CNS development (Mattson M.P., 1997).

Recent studies shown, that ADAM-10 acts as the main α-secretase in neurons (Kuhn P. H. et al., 2010).

1.5.3.2 The amyloidogenic pathway

The amyloidogenic pathway leads to neurotoxic Aβ generation. β-secretase (BACE1) mediates the first proteolysis step, which releases a soluble large N-terminal fragment (sAPPβ) into the extracellular medium. A 99-amino acid C terminal fragment (C99) remains in the membrane (Hussain I. et al., 1999), cleaving Aβ at two known positions: the aspartate at position 1 and the glutamate at position 11 (Vassar R. et al., 1999).

The newly exposed C99 N-terminus corresponds to the first amino acid of Aβ. Successive cleavage of this fragment by γ-secretase (between residues 38 and 43) releases the Aβ peptide of various lengths and AICD. γ-secretase is a complex of enzymes consisting of presenilin 1 or 2 (PS1 and PS2), nicastrin, anterior pharynx defective (APH-1) and presenilin enhancer 2 (PEN2) (Francis R. et al., 2002) and they regulate each other coordinately. Together, these proteins have a molecular weight of approximately 170 kDa, whereas glycosylation of the nicastrin extracellular domain (ECD) provides an additional mass of 30-70 kDa (Schedin-Weiss S. et al., 2014).

Under normal physiological conditions Aβ is mainly 40 aminoacids long and the 42 aminoacid long. Aβ, the main component of amyloid plaques, represent only a minor portion of the total Aβ (Zhang W. et al., 2011).

BACE1 is a membrane-bound aspartyl protease, highly expressed in the brain, especially in regions affected by AD including the cortex and the hippocampus (Johnston et al., 2005), was identified by several groups as the primary β-secretase (Yan R. et al., 1999). BACE1 has been shown to be concentrated in the pre-synaptic terminals of neurons (Cai H. et al., 2001), suggesting a role in synaptic functions. Although under healthy conditions BACE1 is present almost exclusively in neurons, an increase in BACE1 levels has been reported to occur in astrocytes with aging and inflammatory conditions, as well as in the brain of AD patients and AD animal models (Tamagno E. et al., 2012). Next to BACE1, BACE2 (a homolog of BACE1) and cathepsin B, have been discovered additional β-secretases. In several studies the inhibition of the protease cathepsin B reduced Aβ generation *in vivo* and *in vitro*. (Hook V. Y. et al., 2009).

1.6 The amyloid cascade hypothesis

The amyloid cascade hypothesis, proposed by Hardy and Higgins in 1992, Aβ deposition can be the causative event of AD leading to NFTs, neuron loss, vascular damage and cognitive deficits. The hypothesis claims that increased Aβ production or decreased Aβ clearance leads to accumulation of hydrophobic Aβ40 and Aβ42 and the

formation of insoluble extracellular plaques resulting in synapse loss, neuron loss, brain atrophy and dementia (Pimplikar S. W., 2009).

1.7 The cholinergic hypothesis

A cholinergic theory of AD was developed in 1970. The degeneration of the cholinergic system in AD mainly affect the Nucleus Basalis Magnocellularis (NBM) (Whitehouse P. J. et al., 1981), a nucleus implicated in memory, behaviour and attentional functions. There is another cholinergic nucleus that degenerate in AD, that is the medial septum (MS), vertical (vDB) limb of the diagonal band of Broca (Lehéricy S. et al., 1993) projecting to the hippocampus, the main structure in memory formation, where also the total enzyme choline acetyltransferase (ChAT) amount is reduced in AD (Perry E. K. et al., 1977). Cognitive deficits in AD patients can be related to that degeneration of these cholinergic neurons. The basal forebrain cholinergic system is not the only neurotransmitter system that undergoes degeneration in AD, also other systems, such as the serotonergic and noradrenergic systems are affected (Palmer A. M. et al., 1987). It is important to note that Aβ plaques and NFTs are associated with neurons independently from the transmitter. The cholinergic therapy for AD subjects is mainly symptomatic, although if there is evidence suggesting that acetylcholine may be related to APP processing (Aztiria E. et al., 2008) and then that cholinergic therapy could reduce amyloid accumulation (Schliebs R., 2005). Nevertheless, considering that in the last stages of the pathology cell loss is very high, the beneficial symptomatic effects of cholinergic therapy are likely to be achieved only at the early stages of AD.

1.8 Amyloid aggregation

Amyloid fibrils share common structural features despite the considerable diversity in the primary sequence of the constituent proteins. Amyloid deposits extracted from tissues are typically composed of unbranched fibrils (7 to 10 nm in diameter) assembled from two to three 3 nm filaments (protofilaments) twisted around each other. They are rich in β-sheet structures and the ordered regions adopt a cross-β structure in which individual strands in the β-sheets run perpendicular to the long axis of the fibril with the inter β-sheet hydrogen bonds oriented parallel to the fibril axis (Sunde M. et al., 1997). Amyloid fibril formation in bulk solution generally occurs through a nucleation-dependent polymerization process consisting of two phases (Fig.6):

- nucleation (lag phase)

- extension (growth phase).
- possible secondary nucleation

The lag phase is relative to the time required for "nuclei" to form. The initial step of nucleus formation consists in the slow and reversible association of monomers. This process is thermodynamically unfavorable and it is the rate limiting step of the fibrillation process. Once a nucleus has formed, the further addition of monomers to the nucleus becomes thermodynamically favored and this results in a rapid extension of amyloid fibrils (Iannuzzi C. et al., 2015). In some cases, oligomers coming from fiberil fragmentation can induce secondary nucleation.

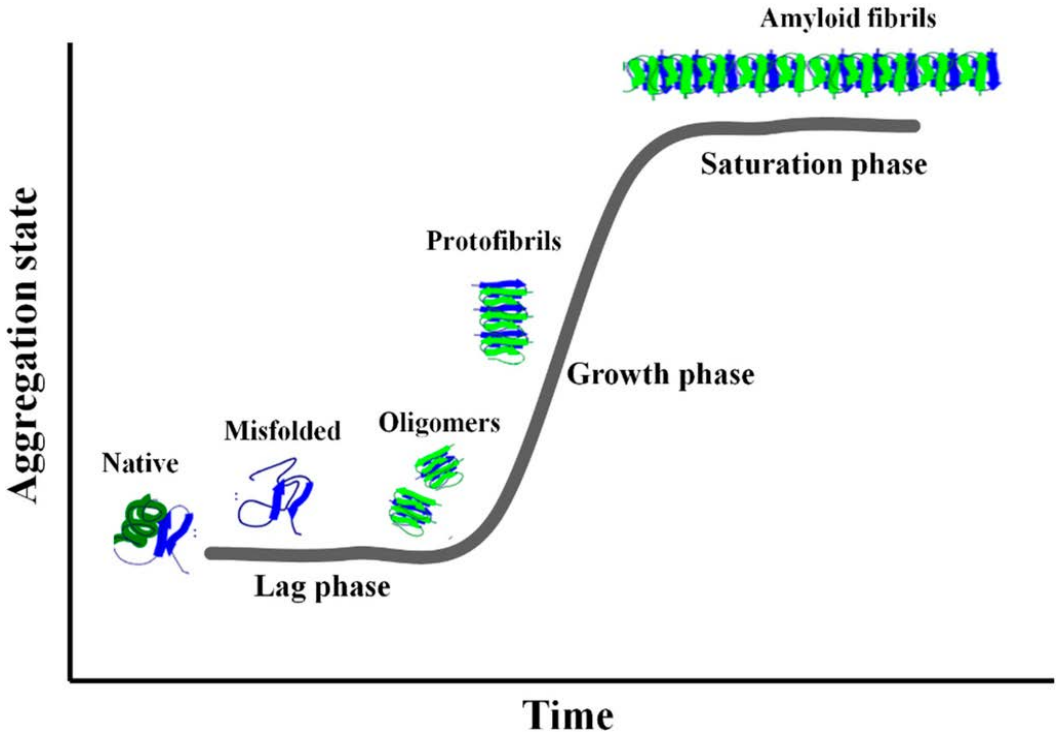

Fig. 6. *Fibril formation process.*

Increased formation and aggregation of neurotoxic Aβ oligomers have been reported to be crucially involved in the initiation and progression of AD (Li S. et al., 2009). Further, it has been demonstrated that soluble oligomeric Aβ42 rather than plaques correlates best with the synaptic loss and cognitive decline in AD patients (Bayer T. A. and Wirths O., 2010). Oligomers are able to disrupt synaptic plasticity and inhibit hippocampal LTP. Due to its amphiphilic nature, Aβ aggregates and forms higher oligomers *in vitro* (Pimplikar S. W., 2009).

1.9 Generation of pyroglutamate-modified amyloid-ß

Various post-translational modifications of Aβ have been described (Kummer M. P. and Heneka M. T., 2014).

Post-translational N-terminal truncation of the first two amino acids of the Aβ peptide and subsequent pyroglutamate formation at the N-terminal glutamate resulting in pE3-Aβ (Saido T. C. et al., 1995) as well as phosphorylation of serine 8 of Aβ (Kumar S. et al., 2011) have been described. Both pE3-Aβ and phosphorylated Aβ occur in AD plaques (Saido T. C. et al., 1995). pE3-Aβ increases the aggregation propensity of Aβ by changing the biophysical properties of Aβ fibrils (Schlenzig D. et al., 2009). Phosphorylated Aβ, on the other hand, promotes the formation of Aβ oligomers that serve as nucleation sites for fibril formation (Kumar S. et al., 2011). These modifications of Aβ play a role in AD pathogenesis and the development of dementia.

pE3-Aβ formation is a multi-step process requiring as a substrate amino-terminally truncated Aβ beginning at glutamate 3 or 11, followed by the cyclisation of exposed glutamate to pyroglutamate (Gunn A. P. et al., 2010). The formation of pE3-Aβ is catalysed by the metal-dependent enzyme glutaminyl cyclase (QC) (Fig.7) (Schilling S. et al., 2004). QC is highly expressed in the human brain and has been shown to be upregulated in AD (Schilling S. et al., 2008), thereby causing an increase in pE3-Aβ formation. The process causes the loss of 3 charges for pE3-Aβ, and 6 charges for pE11-Aβ.

The N-terminal formation of pE3-Aβ renders the Aβ peptide more hydrophobic (Schlenzig D. et al., 2009, 2012) and triggers rapid oligomerization, which negatively interferes with synaptic and neuronal physiology (Schlenzig D. et al., 2012). These data suggest that Aβ oligomers formed from pE3-Aβ structurally differ from those of Aβ42, and it is assumed that these structural modifications constitute the basis for the increased toxicity (Nussbaum J. M. et al., 2012). Recent studies also suggest that the abundance of pE3-Aβ correlates with the appearance of τ PHFs (Mandler M. et al., 2014) and that the concentration of pE3-Aβ in cortical tissue of postmortem human AD brain samples inversely correlates with the cognitive status of the patients (Morawski M. et al., 2014). In contrast to the content of unmodified Aβ in plaques, the level of pE3-Aβ increases correlates with disease stages.

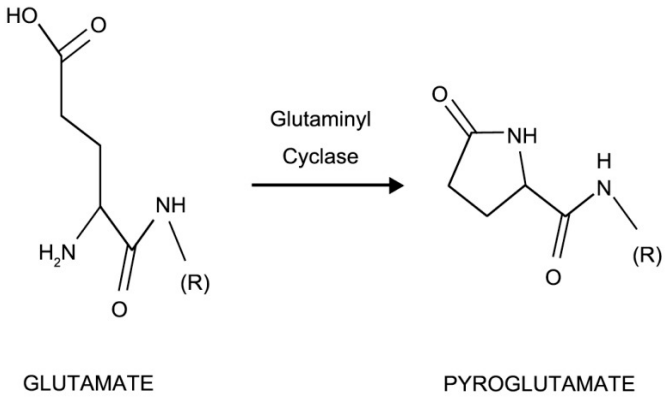

Fig.7 *Activity of Glutaminyl cyclase (Garmendia R. P. et al., 2013).*

1.10 Treatments for AD

There is currently no cure for AD, however there are multiple drugs that have been proven to slow disease progression and treat symptoms. Symptoms can be divided into "cognitive" and "behavioral and psychiatric" categories (Khachaturian Z. S. et al. 1996). Cognitive symptoms affect memory, language, judgment, and thought processes. Behavioral symptoms alter a patient's actions and emotions.

1.10.1 Treatments for Cognitive Symptoms

The U.S. Food and Drug Administration (FDA) has approved two types of medication to treat cognitive symptoms of AD: (1) cholinesterase inhibitors (ChEIs) and (2) memantine.

AD has a devastating impact on the brain's cholinergic system. This cell network is critical for learning and memory. Acetylcholine is an important neurotransmitter involved in learning and memory. Normal aging causes a slight decrease in acetylcholine concentration, causing periodic forgetfulness. However, in AD, the concentration can be decreased by as much as ninety-percent resulting in significant memory and behavioral decline (Zimmerberg B., 1998).

Cholinesterase inhibitors increase the availability of acetylcholine. Specifically, they block the activity of acetylcholinesterase (AChE), the enzyme that breaks acetylcholine down.

Increased acetylcholine levels temporarily improve function in the cholinergic system. Clinical research has found that cholinesterase inhibitors postpone the worsening of Alzheimer's symptoms for an average of six months to a year.

Four ChEIs are commonly used to treat cognitive symptoms in mild to moderate AD: Exelon®(rivastigmine tartrate; Novartis AG, Basel, Switzerland); Reminyl® (galantamine hydrobromide; Johnson and Johnson Inc, New Brunswick, NJ); Aricept® (donepezil hydrochloride; Pfizer Inc, New York, NY; Eisai Inc, Tokyo, Japan); and the rarely prescribed Cognex® (tacrine hydrochloride; First Horizon Pharmaceutical Corp., Roswell, GA).

1.10.1.1 Tacrine

Tacrine is a reversible ChEI, it was the first such drug to be introduced for the treatment of AD in a range of countries such as South America, Asia, Europe, Australia, and the United States in 1995 and 1996. For its liver toxicity and attendant requirement for monitoring liver function, tacrine prescriptions dropped after other acetylcholinesterase inhibitors were introduced, and its use has been largely discontinued (Alzforum.it).

1.10.1.2 Donepezil

Donepezil is approved in more than 90 countries around the world to treat mild to moderate AD, and is approved for the treatment of severe AD in the United States, Japan, Canada, and several other countries. Donepezil reversibly inhibits AChE and has a long duration of action. Donepezil and other AChE inhibitors increase the availability of the acetylcholine in cholinergic synapses, enhancing cholinergic transmission. Donepezil delays the progressive worsening of cognitive symptoms of AD.

Donepezil's most common side effects are gastrointestinal, diarrhea, nausea, and vomiting; dizziness, sleeplessness/fatigue, and urinary incontinence also have been reported. These are known class effects of cholinergic therapies, and often occur primarily at the beginning of treatment.

The formulations available for donepezil are immediate release (5 or 10 mg), sustained release (23 mg), and orally disintegrating (5 or 10 mg) tablets, all of which are intended for oral-route administration. Since the oral donepezil therapy is associated with above described adverse events in the gastrointestinal system and in plasma fluctuations, an alternative route of administration, such as the transdermal one, has been recently attempted.

Donepezil has the longest plasma half-life at about 70 hours compared with 6 hours for galantamine, 3 hours for tacrine, and 1.5 hours for rivastigmine (this has the practical

advantage that it is excreted quickly from the body and so relief from side effects is much more speedy than with the longer-acting compounds). The half-life also has implications for the daily dosing regimen: the advantage of donepezil is that it only needs to be given once a day (Alistairs B., 2003).
Donepezil is the only cholinesterase inhibitor approved to treat severe AD (alzforum.it).

1.10.1.3 Galantamine

Galantamine is an alkaloid isolated from *Galanthus nivalis* in 1950's. Galantamine crosses the blood brain barrier (BBB) and has been used in humans for decades in anaesthesia and the treatment of neuropathic pain. It act as ChEI and as an allosteric potentiator of both nicotinic and muscarinic acetylcholine receptors. In 2000 it was registered for the treatment of AD and it was approved in the European Union, the United States, Canada, Japan, and many other countries around the world.
It is available as a tablet, as solution, and, since 2005, as a once-daily extended-release capsule. Also in 2005, the name Reminyl was replaced with Razadyne to avoid confusion with the diabetes drug Amaryl (glimepiride).
The most common side effects of galantamine are gastrointestinal, including nausea, vomiting, and diarrhea. Dizziness, insomnia or nightmares, agitation, mild arryhthmia, and other effects have also been reported (Wilcock G. et al., 2003) (Alzforum.it).

1.10.1.4 Rivastigmine

Rivastigmine is a reversible inhibitor of both the AChE and butyrylcholinesterase enzymes. It is used for AD tratment across its mild, moderate, and severe stages, as well as for the treatment of dementia associated with Parkinson's disease. It was approved in 1997 in Switzerland, and from 2000 it became available in several countries including the United States, Canada, and Europe. It is available in oral capsule (twice-daily) and as a skin patch, sold in three doses that provides continuous delivery of the drug over 24 hours, that bypassing first-pass metabolism of intestine and liver, it enables patients to receive a higher therapeutic dose (Cummings J. et al., 2007).
The most common side effects are nausea, vomiting, diarrhea, and loss of appetite, and less frequently agitation, depression, dizziness, fatigue/sleeplessness, and rare skin sensitivity reactions for the patch (Alistairs B., 2003).

1.10.2 Noncholinergic approaches

1.10.2.1 Glutamatergic antagonist

Glutamate is an excitatory neurotransmitter involved in learning and memory in the brain and is probably present in 70% of neurones. In excess, glutamate is excitotoxic and activates *N*-methyl-D-aspartate (NMDA) receptor leads to a disruption of cell-to-cell communication and cell death in the brain and to deposition of β-amyloid. The most side effects for drugs with high affinity to NMDA are schizophreniform psychoses.

Memantine, an NMDA receptor antagonist, works by regulating the activity of glutamate. Memantine may protect cells against excess glutamate by partially blocking NMDA receptors. It is currently approved for tratment of moderate-to-severe AD, but not for mild AD. Clinical studies have found the drug may offer a modest benefit in patient memory, daily activity and overall function.

Significant improvements in global ratings of dementia, and cognitive function have been demonstrated for dosages of 10 or 20 mg/day (escalating from 5 mg/day over 1 week) (Alistairs B., 2003).

Adverse side effects of memantine include headache, constipation, confusion and dizziness.

1.10.2.2 Chelating Agents

Amyloid beta aggregation is dependent on Cu^{2+} and Zn^{2+}. Furthermore, both metals are markedly accumulated in plaques (Bush 2008). Furthermore, compounds like the antibiotic clioquinol, a Cu^{2+}/Zn^{2+} chelator, promote solubility of Aβ42 and prevent plaque formation. Aluminum has been found in high concentrations in both senile plaques and NFTs in the brains of subjects with AD, which suggests that this metal may be involved in the etiopathology of AD (Christen Y. Et al., 2000).

1.10.2.3 Nicotinic Acetylcholine Receptor Agonists and Muscarinic Receptor Modulators

AD is characterized by a progressive phenotypic downregulation of markers within cholinergic BF neurons, cholinergic BF cell loss and reduced cortical choline acetyltransferase activity associated with cognitive decline. Delaying cholinergic BF neurodegeneration or minimizing its consequences is the mechanism of action for most currently available drug treatments for cognitive dysfunction in AD (Mufson E. J. et al., 2008). This cholinergic deficit is also associated with the loss of acetylcholine receptors

(nAChRs) (Engidawork E. et al. 2001). The stimulation of these receptors by nAChR agonists may improve cognitive deficits observed in AD patients. Furthermore, nAChRs play an important role in neuroprotection against β amyloid-induced cytotoxicity (Mudo G. et al. 2007), and thus, nAChR agonists may even counter the loss of synapses and neurons.

1.10.2.4 5-hydroxytryptamine (5-HT, Serotonin) Receptor Modulators

The loss of serotonergic neurons leads not only to cognitive decline but also to behavioural symptoms like anxiety, insomnia and depression (Schmitt F. A. et al., 2006). Serotonins probably unique among the monoamines in that its effects are subserved by as many as 13 distinct heptahelical, G-protein-coupled receptors (GPCRs) and one (presumably a family of) ligand-gated ion channel(s). The action of 5-HT (serotonin) are mediated through seven major receptor classes (5-HT_{1-7}), largely classified on the basis of their structural and operational characteristics. (Baez M. et al., 1995). Which receptor subtype needs to be modulated in which way (inhibition or stimulation) to positively influence the cognitive and/or behavioural symptoms observed in AD patients, is not fully understood.

Researchers have shown the positive influence of the 5-HT_4 and 5-HT_6 receptor in AD treatment.

An impressive article highlighting the role of 5-HT4 receptor in AD was written with specific emphasis on Aβ peptide secretion (Robert S. J. and Lezoualc'h. F., 2008).

Researchers also shown that 5-HT_6, predominating in brain regions, is associated with cognition and behaviour. It seems to be involved in the regulation of putatively cholinergic-mediated behaviours, anxiety and memory performance (reviewed by Upton N. et al. 2008).

1.10.2.5 Ion Channel Modulators

The dysregulation of calcium homeostasis may be a key factor accelerating some pathological processes in AD (Bojarski L. et al., 2008). Presenilins, mutated in some patients with familial AD, were shown to form low conductance calcium channels in the endoplasmatic reticulum (Tu H. et al., 2006) where, the elevated cytosolic calcium concentration caused by the mutation facilitates Aβ generation (reviewed by Bojarski L. et al., 2008). Recent studies shown that nilvadipine, a dihydropyridine calcium antagonist, reduced Aβ load in a mouse model of AD.

1.10.2.6 Phosphodiesterase (PDE) 4 Inhibitors

Phosphodiesterase-4 (PDE4) belongs to an important family of proteins that regulates the intracellular level of cyclic adenosine monophosphate (cAMP). Cyclic adenosine monophosphate (cAMP) is a second messenger that plays an important role in biochemical processes regulating cognition and memory consolidation (Isiegas C. et al., 2008). PDE4 inhibitors can counteract deficits in long-term memory caused by pharmacological agents, aging or overexpression of mutant forms of human amyloid precursor proteins. (Ghavami A. et al., 2006). PDE-4 with selective inhibitors can offer a novel therapeutic approach for slowing the progression of AD.

1.10.2.7 Diet

Diet may play an important role in the prevention of AD. (Solfrizzi V. et al., 2003). Caloric restriction in animals extends their life span and increases the resistance of neurons to degeneration (Mattson M. P., 2000). Higher intake of vitamin C, (Zandi P. P. et al., 2004) vitamin E, (Zandi P. P. et al., 2004) flavonoids, (Cammenges D. et al., 2000) unsaturated fatty acids, (Kalmijn S. et al., 1997) fish (Morris M. C. et al., 2003) higher levels of vitamin B12 and folate (La Rue A. et al., 1997) modest to moderate ethanol (Ruitenberg A. et al.,2002) and lower total fats (Kalmijin S. et al., 1997) have been related to a lower risk for AD or slower cognitive decline.

Dietary pattern analysis in relation to many other diseases (ie, cirrhosis or various cancers) has recently received growing attention because individuals do not consume foods or nutrients in isolation, but rather as components of their daily diet. Defining diet by dietary patterns has the ability to capture its multidimensionality whereas reducing its apparent complexity because patterns can integrate complex or subtle interactive effects of many dietary constituents and bypass problems generated by multiple testing and the high correlations that may exist among these constituents (Jacques P. F. et al., 2001) Dietary patterns can be developed a priori from previous knowledge concerning a favorable or adverse health effect of various dietary constituents.

One such dietary pattern is the Mediterranean diet (MD), which has received increased attention in recent years because evidence relating it to lower risk for cardiovascular disease, several forms of cancer, and overall mortality. The MD is characterized by high intake of vegetables, legumes, fruits, and cereals; high intake of unsaturated fatty acids (mostly in the form of olive oil), but low intake of saturated fatty acids; a moderately

high intake of fish; a low-to-moderate intake of dairy products (mostly cheese or yogurt); a low intake of meat and poultry; and a regular but moderate amount of ethanol, in the form of red wine and generally during meals (Trichopolou A. et al., 2003). Therefore, the MD appears to include many of the components reported as potentially beneficial for AD and cognitive performance (Scarmeas N., 2006).

1.10.3 Treatment for Behavioral and Psychiatric Symptoms.

In addition to cognitive and functional decline, AD can cause severe behavioral and psychiatric symptoms. These symptoms include anxiety, sleeplessness, agitation, hallucinations, and delusions. Possible treatment methods involve non-drug interventions and medications to treat the symptoms being presented. Altering the environment to eliminate obstacles and increase security or investigating any potential interactions between the patient's medications that could cause adverse effects to behavior or psychiatric health are effective non-drug approach (Alzheimer association, 2010).

If these interventions do not improve the symptoms, medication may be required. For example, antipsychotics and anxiolytics may be taken to reduce hallucinations and anxiety, respectively (Alzheimer association, 2010).

2. The blood brain barrier

Strong barrier surrounds the human brain, rigorously and reliably shielding off most substances and pathogens to protect the fragile CNS homeostasis. However, this barrier also often prevents successful drug treatment in case of brain-associated diseases.
Substances may often show promising *in vitro* results in preclinical testing, but they often fail to benefit *in vivo*. The limiting factor is the delivery to the brain.

2.1 The structure of the blood-brain barrier

Three barrier layers contribute to the separation of the blood and neural tissues: (Ehrlich P. et al., 1885) a highly specialized endothelial cells (EC) layer comprising the BBB and partitioning the blood and brain interstitial fluid. The blood-CSF barrier (BCSFB) with the choroid plexus epithelium which secretes the specialized cerebral spinal fluid (CSF) into the cerebral ventricles, and the arachnoid epithelium separating the blood from the subarachnoid CSF (Kandanearatchi A. et al., 2003).
The BBB components include the EC layer and its basement membrane, adjoined by tight cell-to-cell junction proteins (TJ) with specific transport mechanisms and pinocytic vesicles. The endothelium is surrounded by cellular elements including pericytes and astroglial foot processes, forming an additional continuous stratum that separates blood vessels from brain tissue. Around penetrating vessels and venules there is some distance between EC and brain tissue forming the Virchow-Robin space in which perivascular macrophages, executing some of the immune functions of the CNS, are found. The intimate contact between neurons, astrocytes, microglia, pericytes and blood vessels, and the functional interactions and signaling between them form a dynamic functional unit, known as the ***neurovascular unit*** (Serlin Y. et al., 2015).
TJ proteins play a key role in maintaining barrier function. Without accurate TJ protein expression, the cellular barrier lacks appropriate resistance and is permeable to various substances. The first identified TJ protein was zonula occludens (Daneman R. et al., 2010). Later, occludin and claudin group were shown (Hamilton N. B. et al., 2010). Claudin derives from the Latin word “claudere”, meaning “to shut, to block”.
Transporters on both sides of the endothelial layer import valuable nutrients and export noxious metabolites. For example glucose and aminoacids that have their own transport system to maintain brain homeostasis, while other valuable molecules are transported by receptor-mediated transcytosis, such as insulin, transferrin or apolipoproteins bind to their specific receptor protein at the BBB and are imported by clathrin-mediated

endocytosis. This process required the formation of endosomes that are later acetated by proton pumps (and then are called lysosomes) before degradation. Another import process is adsorptive transcytosis, that involves the fusion of plasma protein with the plasma membrane of the endothelial cells and then transported across the barrier and released at the brain site.

Different enzymes in the brain parenchyma contribute to the metabolic barrier function: peptidases and nucleotidases outside of the cell can degrade peptides and adenosine triphosphate (ATP); monoamine oxidase (MAO) and members of the cytochrome P450 (CYP) family inside the cell inactivate many neuroactive and toxic compounds (Armulik A. et al., 2010).

2.2 *In vitro* models of BBB

Numerous *in vitro* models of the BBB have been emerged to simplify and to make possible in vivo and in human studies (Wilhelm I. et al., 2011). *In vitro* approaches have numerous advanges respect to animal models:

- Less expensive;
- High throughput for drug permeability experiments;
- Simplified working environment;
- Less variability;
- Higher reproducibility;
- Higher versatility (manipulating possibilities).

In vitro models of BBB need to have certain criteria (Gumbleton M. et al., 2001):

- It must represent the permeability data for low (e.g., inulin or sucrose) and high (e.g., diazepam or propranolol) brain-penetrating substances;
- It must reflect the limited paracellular pathway, which forces substances to take the transcellular route across the BBB. In this case are recommended permeability studies with marker solutes.
- It should possess a cell architecture that resembles the *in vivo* conditions, including for nanoparticle the espression of receptors of BBB.

Immortalized cell lines have their advantages. They are ready to be purchased from reliable commercial sources at relatively low costs in comparison with all the work and effort that have to be put into primary cell culture. Also, no significant difference was

observed between samples made from different passages of the cells, thus the consistency of the in vitro samples was made possible.

2.3 Nanoparticles

Nanoparticles are defined as solid, biodegradable colloids, with diameters ranging from 1 to 1.000 nm, and bearing drugs or other biologically active substances (Kreuter J., 2001). They are characherizesized by at least two components: a basis polymer to form the particles and one pharmaceutically active substance that can be incorporated, adsorbed or chemically bound (Kreuter J., 2001). Natural macromolecules as basis material include human serum albumin (HSA), sodium alginate, chitosan or gelatin (Lai P. et al., 2014). Common examples for synthetic, biocompatible polymers for nanoparticle preparation are poly(lactic acid) (PLA) and poly(glycolic acid) (PGA), or a copolymers from PLA and PGA, resulting in poly(lactic-co-glycolic acid) (PLGA), which are approved by the FDA (Rieger J. et al., 2009) and frequently serve as basis material for nanoparticle preparation (Hines D. J. and Kaplan D. L. et al., 2013). The human body metabolizes these polyesters into glycolic acid and lactic acid. These acids then are decomposed within the citric acid cycle to form water (H_2O) and carbon dioxide (CO_2), which explains their excellent biocompatibility (Kumari A. et al., 2010).
Different types of nano-sized carriers, such as polymeric nanoparticles, ***solid lipid nanoparticles***, ceramic nanoparticles, magnetic nanoparticles, polymeric micelles, polymer-drug conjugates, nanotubes, nanowires, nanocages and dendrimers, etc., are being developed for various drug-delivery applications (Sahoo S. K. et al., 2007). Polymeric nanoparticles can be fabricated from polysaccharides (Liu Z. et al., 2008; Fernandez-Urrusino R. et al., 1999), proteins (Farrugia C. A. et al., 1999) and synthetic polymers (Fattal. E. et al., 1998). Nanoparticles made from natural hydrophilic polymers have several advantages such as better drug-loading capacity and biocompatibility (Liu Z. et al., 2008). Systems based on proteins including gelatin, collagen, casein, ***albumin*** and whey protein (Kuijpers A. J. et al., 2000).
Albumin is a macromolecular carrier that has been shown to be biodegradable, nontoxic, metabolized in vivo to produce innocuous degradation products, non-immunogenic, easy to purify and soluble in water allowing ease of delivery by injection and thus an ideal candidate for nanoparticle preparation (Kratz F. et al., 1997).

2.3.1 Albumin nanoparticles

Albumin-based nanoparticles (NPs) (Fig. 8) carrier systems represent an attractive strategy where significant amount of drug can be incorporated into the particle matrix because of the different drug binding sites present in the albumin molecule (Patil G. V., 2003). Albumin is the most abundant plasma protein (35–50 g/L human serum) with a molecular weight of 66.5 kDa (Carter D . C. et al., 1989). It is synthesized in the liver where it is produced at a rate of approximately 0.7 mg/h for every gram of liver (i.e. 10–15 g daily); human serum albumin (HSA) has an average half-life of 19 days. Albumin is an acidic, very soluble protein that is extremely robust: it is stable in the pH range of 4–9, soluble in 40% ethanol, and can be heated at 60 °C for up to 10 h without deleterious effects (Naveen R. et al., 2016).

For its primary structure and to the high content of charged amino acids (e.g. lysine), albumin-based nanoparticles could allow the electrostatic adsorption of positively (e.g. ganciclovir) or negatively charged (e.g. oligonucleotide) molecules without the requirement of other compounds (Irache J. M. et al., 2005). In addition, albumin nanoparticles can be easily prepared under soft conditions by coacervation, controlled desolvation or emulsion formation. They show a smaller size (50 to 300 nm) compared to other microparticles and, in general, better controlled release properties than liposomes which may improve patient acceptance and compliance. Commercially, albumins are obtained with significant quantities from egg white (ovalbumin), bovine serum (bovine serum albumin, BSA), and HSA and also available from soybeans, milk, and grains (Arshady R., 1989).

There are several advantages of using albumin as drug carrier: 1) as an endogenous protein, HSA is native to the body. It is biodegradable in nature, nontoxic and non-immunogenic; 2) Albumin is a robust protein. It is stable over a wide pH range 4-9, could be heated at 60°C for up to 10 h without deleterious effect, is unaltered by denaturing agents and solvents at moderate concentrations (Neumnann E. et al., 2010). Therefore, albumin could remain stable under typical processing conditions; 3) as the most abundant protein in plasma, albumin is readily available. It has been used in clinical setting for more than 30 years; 4) the half life of albumin is 19 days in blood circulation (Naveen R. et al, 2016).

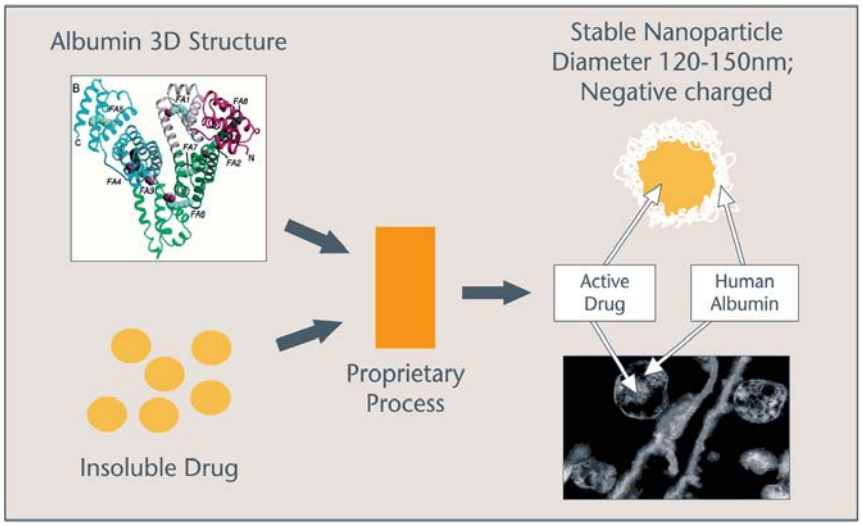

Fig. 8 *Albumin as a drug carrier.*

2.3.2 Solid Lipid Nanoparticles

Solid lipid nanoparticles (SLNs) (Fig.9) represent an alternative carrier system to traditional colloidal carriers. Nanoparticles made from solid lipids are attracting major attention as novel colloidal drug carrier for intravenous applications as they have been proposed as an alternative particulate carrier system. The system consists of spherical solid lipid particles in the nanometer ranges, which are dispersed in water or in aqueous surfactant solution. Generally, they are made of solid hydrophobic core having a monolayer of phospholipids coating. The solid core contains the drug dissolved or dispersed in the solid high melting fat matrix. The hydrophobic chains of phospholipids are embedded in the fat matrix. They have potential to carry lipophilic or hydrophilic drugs or diagnostics (Yadav N. et al., 2013).

They have many advantages such as good biocompatibility, non toxic, stable against coalescence, drug leakage, hydrolysis, biodegradable, physically stable and good carrier for lipophillic drugs (Mukherjee S. et al., 2008; Surrender V. and Deepika M., 2016).

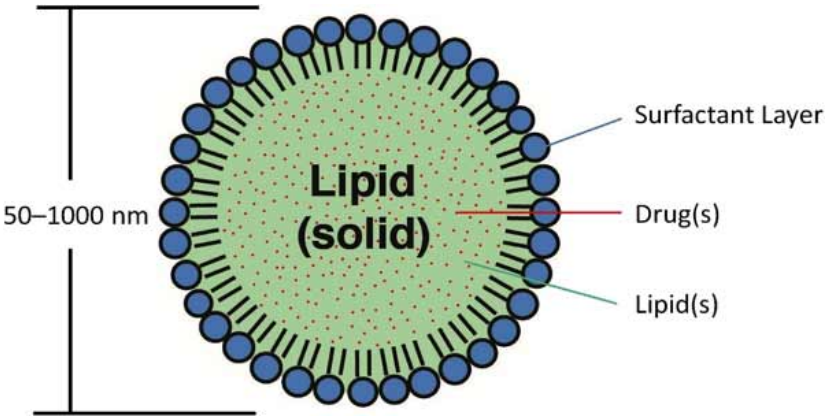

Fig.9 *SLNs.*

2.3.2.1 Advantages of SLNs

1. SLNs have better stability and ease of upgradability to production scale as compared to liposome;
2. In SLNs the lipid matrix is made from physiological lipid which decreases the danger of acute and chronic toxicity;
3. Very high long-term stability;
4. It is easy to manufacture than bipolymeric nanoparticles;
5. Better control over release kinetics of encapsulated compound;
6. SLNs can be enhancing the bioavailability of entrapped bioactive;
7. Chemical protection of labile incorporated compound.;
8. Raw material which are to be required are same as that of emulsion;
9. Large scale production is possible;
10. High concentration of functional compound can be achieved;
11. Lyophilization possible.

2.3.2.2 Disadvantages of SLNs

1. Poor drug loading capacity;
2. Drug expulsion after polymeric transition during storage;
3. Relatively high water content of the dispersions (70-99.9%);
4. The low capacity to load hydrophilic drugs due to partitioning effects during the production process (Ekambaram P. et al., 2012).

2.4 Nanoparticles for AD

Nanoparticles are an elegant way to overcome the challenging BBB with minimal invasive damage. By masking the original physic-chemical properties of a drug, nanoparticles allow barrier.

It have been shown (Kreuter J., 2004) the influence of BBB transit capability by dozen different surfactant. Incubate nanoparticles with polysorbate 80 (Tween80) or poloxamer 188 (Pluronic F-68) causes the anchorage of lipoproteins from blood plasma or serum of the culture medium (Wohlfart S. et al., 2012). Apolipoproteins E and/or A-I, for example, adsorb to the nanoparticle' s surface and can interact with receptors at the BBB, resulting in cellular uptake of the drug-loaded nanoparticles *in vitro* and *in vivo* (Wagner S. et al., 2010).

Loperamide, an opioid drug that cannot enter the brain and therefore has no analgesic effect, when is loaded to nanoparticles and injected into mice, these animals become less sensitive in nociceptive experiments (Kreuter J. et al., 2002).

Nanoparticles can act in a variety of ways by breaking down Aβ plaque formation, degrading Aβ toxic peptide and modulating some enzymes as secretases.

3. Autophagy

Autophagy is regarded as a “self-digestion”process, which degrades a cell's own cytoplasmic content through lysosomes, for the maintenance of cellular homeostasis (Li L. et al., 2010).

There are three forms of autophagy (Zare-Shahabadi A. et al., 2015):

- ***Chaperone-mediated autophagy (CMA)*** that involves cytoplasmic proteins being selectively delivered into the lysosome through recognition of specific motifs that are biochemically related to the pentapeptide KFERQ. First, CMA substrates are recognized in the cytoplasm by the heat shock cognate protein of 70 KDa (hsc70). The chaperone–KFERQ-containing protein complex then binds LAMP (lysosome-associated membrane protein)-2A receptors on the lysosomal membrane, and translocates the target proteins into the lysosomes for degradation (Kaushik S. et al., 2011)
- ***Microautophagy*** in which the cytoplasmic components are directly engulfed by the lysosome (Li W. et al., 2012);
- ***Macroautophagy*** in which ordered activity of autophagy-related (Atg) proteins leads to the cytoplasmic components being engulfed by autophagy vacuoles (AV) and degraded by proteases after fusion with lysosomes (Zare-Shahabadi A. et al., 2015).

3.1 The process of macroautophagy

Autophagy is a primarily degradative pathway that takes place in all eukaryotic cells. It is used for recycling cytoplasm to generate macromolecular building blocks and energy under stress conditions, to remove superfluous and damaged organelles to adapt to changing nutrient conditions and to maintain cellular homeostasis. The most prevalent form of autophagy is macroautophagy, and during this process, the cell forms a double-membrane sequestering compartment termed the phagophore, which matures into an autophagosome. Following delivery to the vacuole or lysosome, the cargo is degraded and the resulting macromolecules are released back into the cytosol for reuse (Feng Y. et al, 2014).

It can be devided in the following steps:

- ***Initiation of autophagosome formation***

  The initiation of macroautophagy involves the assembly of ULK protein complex comprising ULK1, Atg13, FIP200 and Atg101 at the isolation

membranes, where it works with other Atg proteins to initiate autophagosome formation (Mizushima N., 2010). The assembly of ULK complex is facilitated by a Ras-like small G protein RalB and the exocyst complex (Bodemann B. O. et al., 2011), and its activation requires dissociation from the negative regulator mTOR complex 1 (mTORC1) (Hosokawa N. et al., 2009) during cellular stress such as starvation. Macroautophagy can also be induced via mTOR-independent mechanisms, altering the transcription of macroautophagy genes or reducing the cellular level of inositol 1,4,5-trisphosphate (IP3) (Mehrpour M. et al., 2010). Recent studies shown that macroautophagy is induced when IP3 production is reduced by treatment with IP3 receptor antagonists (Vicencio J. M. et al. 2009) that determine decreases intracytosolic calcium ion concentration and calpain activity to lower the cAMP level and conseguent activation of macroautophagy (Mehrpour M. et al., 2010). Also Atg5 and Atg12 can regulate macroautophagy (Mizushima N. et al., 2001). Interaction of Atg5 with Atg16L1 at the isolation membranes facilitates autophagosome formation (Fujita N. et al., 2008).

- ***Autophagosome nucleation***

  Autophagosome formation appears to start with nucleation and elongation of the isolation membranes to generate a phagophore (Simonsen A. and Tooze S. A., 2009). The formation of phagophore requires the activity of Class III phosphatidylinositol-3-kinase (PI3K) that form a large protein complex with Beclin-1 and is coordinated by the interactions of other proteins including Beclin-1, UV irradiation resistance-associated tumor suppressor gene (UVRAG), Atg14, B-cell leukemia/lymphoma-2 (Bcl-2), p150, ambra1, endophilin B1, and Vacuolar protein sorting 34 (Vps34), which activates PI3K to produce phosphatidylinositol-3-phosphate (Mehrpour M. et al., 2010). When Beclin-1 is bound to Bcl-2 the activity of this complex is inhibited (Pattingre S. et al., 2005), but is stimulated upon UVRAG recruitment (Liang C. et al., 2006). Ambra1 also directly binds Beclin-1 to regulate Beclin-1/PI3K complex formation (Fimia G. M. et al., 2007). Then, endophilin B1, also known as Bax-interacting factor 1 (Bif-1) (Cuddeback S. M. et al., 2001) or SH3GLB1 (Pierrat B. et al., 2001), is the only N-terminal Bin-Amphiphysin-Rvs (N-BAR) domain-containing protein identified in this Beclin-1/PI3K complex, and is believed to generate membrane curvature during vesicle nucleation (Takahashi Y. et al., 2009). The function of endophilin B1 requires both its N-BAR domain and Src-homology 3 domain,

with the latter shown to mediate its interaction with UVRAG to recruit Beclin-1 and facilitate PI3K activation (Takahashi Y. et al., 2007). The activity of Endophilin B1 is required to coordinate in Atg9 activity (Takahashi Y et al., 2010). PTEN-induced putative kinase 1 (PINK1) (Minchiorri S. et al., 2010), death-associated protein kinase (DAPK) (Zalckvar E. et al., 2009), IP3 receptor (Vicencio J. M. et al., 2009) and high mobility group box 1 (Tang D. et al., 2010) can act also as regulators of the complex.

- ***Autophagosome elongation***

  For autophagosome elongation other Atg proteins are recruited (Simonsen A. and Tooze S. A., 2009).

  Atg5 and Atg12 are involved in the first of two ubiquitylation-like reactions that control autophagy. Atg12 is conjugated to Atg5 in a reaction that requires Atg7 (ubiquitin-activating-enzyme (E1)-like) and Atg10 (ubiquitin-conjugating-enzyme (E2)-like).

  Also, Atg5-Atg12 interacts non-covalently with Atg16L (Atg16-like) to form a complex of approximately 800 kDa (Ohsumi Y. and Mizushima N., 2004).

  Together with Atg3, Atg4 and Atg7, the Atg5–Atg12–Atg16L1 complex mediates the conjugation of phosphatidylethanolamine (PE) to the microtubule associated protein 1 light chain 3 (LC3)-I to form LC3-II, leading to the translocation of LC3 from cytoplasm to the membrane of the preautophagosomes (Mizushima N. et al., 2001). Once autophagosome formation is completed, the Atg proteins are released back to the cytoplasm by a yet uncharacterized mechanism.

- ***Autophagosome maturation and degradation***

  Subsequent to the elongation step, autophagosomes are fused with lysosomes for degradation of their contents, a process known as autophagosome maturation (Simonsen A. et al., 2009; Eskelinen E. L., 2005). Autophagosome maturation and degradation require the action of late endosome marker protein Rab7 and lysosomal membrane protein LAMP-2 (Jager S. et al., 2004). Have been shown the activity of UVRAG (Liang C. et al., 2008), Rubicon (Zhong Y. et al., 2009), presenilin-1 (Lee J. H. et al., 2010), valosin containing protein (VCP) (Tresse E. et al., 2010), and syntaxin-5 SNARE complex proteins (Renna M. et al., 2011) as regulators of autophagosome maturation and degradation. UVRAG interacts with the class C vacuolar protein sorting complex, and requires Rab7 activity for

promoting autophagosome fusion with late endosomes and lysosomes (Liang C. et al., 2008), regulating negatively the autophagosome maturation (Matsunaga K. et al., 2009). On the other hand, inhibition of presenilin-1 (Lee J. H. et al., 2010) VCP (Tresse E. et al., 2010) or syntaxin-5 SNARE complex (Renna M. et al., 2011) impairs lysosomal degradation of autophagosomes. (Alan S. L., 2011).

3.2 The process of CMA

Selectivity of CMA is conferred by the presence of a pentapeptide motif in the amino acid sequence of the substrate proteins, KFERQ, that when recognized by a cytosolic chaperone targeting substrates to lysosomes. In KFERQ motif, Q should be the flanking amino acid but could be located at the beginning or at the end of the sequence; there could be up to two of the allowed hydrophobic residues (I, F, L or V) or two of the allowed positive residues (R or K), but only one negative charge provided either by E or D. In certain proteins, Q can be replaced with N, but this exchange does not work in all proteins, suggesting that the surrounding amino acid context might be important in this case. The “pure” KFERQ motif is only present in ribonuclease A, the first protein identified as a CMA substrate.

3.2.1 Hsc70

Cytosolic heat shock cognate protein of 70 kDa (cyt-hsc70), the constitutively expressed member of the 70-kD family of chaperones, interacts with substrate proteins via their KFERQ motif. In addition, Hsp90, Hsp40, Bcl-2 associate athanogene 1 (Bag-1), Hsc70-Hsp90 organizing protein (Hop), and Hsc70-interacting protein (Hip) have been shown to interact with the CMA substrate–chaperone complex at the lysosomal membrane. Researchers have shown that Hsc70 can bind to protein aggregates and target them to lysosomes via chaperone-assisted selective autophagy. Also lysosome associates Hsc70 (lys-Hsc70) play a key role in substrate translocation. Only those lysosomes containing Hsc70 in their lumen are competent for uptake of CMA substrates. It remains unclear how this chaperone acts. Researchers suggesting that it may be act by facilitating substrate internalization in an energy-dependent manner, or passively, by binding the portion of substrate already translocated and preventing its retrograde movement to the cytoplasm.

3.2.2 LAMP2A

This recognition at the lysosomal membrane is mediated by the lysosome-associated membrane protein type 2A (LAMP2A), a single-span membrane protein with a very heavily glycosylated luminal region and a short (12–amino acid) C-terminus tail exposed on the sur- face of the lysosomes, where substrate proteins bind. LAMP2A is one of the three splice variants of the lamp2 gene, all of which contain identical luminal regions, but different transmembrane and cytosolic tails (Ferreira J. V. et al., 2015).

CMA substrate protein binds to the c-terminal cytosolic tail of LAMP2A at the lysosomal membrane and the interaction determine its multimerization to form a 700-kDa complex at the lysosomal membrane. Hsp90 is shown essential to preserve the stability of LAMP2A. However, once that the substrate crosses the membrane, LAMP2A rapidly disassembles in a process mediated by the Hsc70 present on the cytosolic side of the lysosomal membrane.

3.3 Macroautophagy and AD

Dysregulation of autophagy occurs in both AD animal models and AD patients and autophagy regulation has been proposed as a possible therapeutic target for several diseases including neurodegenerative ones (Rubinsztein D. C. et al., 2005).

Rubinsztein et al. have reported increased levels of expression of lysosomal proteases in the early phase of AD patients (Rubinsztein D. C. et al., 2005). Atg1, atg8a, and atg18 genes were down-regulated in *Drosophila melanogaster* with aging, with conseguent reduction of autophagy activity and the accumulation of Aβ load, both considered to correlate with late-onset neuronal dysfunction and AD phenotype (Omata Y. et al. 2014). These results indicate that the age-induced reduction of Atg gene expression is associated with late onset of AD.

Studies from ApoE4 transgenic mice showed that overexpression of ApoE4 elevated Aβ42 load in lysosome and finally led to hippocampus neurons death (Belinson H. et al. 2008).

Proper formation and degradation of autophagosome is critical for normal autophagic flux. In healthy neurons, low-basal autophagic activity was detected because of the quick subsequent degradation of autophagosome by lysosome. In hippocampus neurons of AD mice, abnormal accumulation of immature AVs in axon was observed before synaptic and neuronal loss (Sanchez-Varo R. et al., 2012). However, as either autophagosome axonal trafficking deficiency or insufficient lysosome acidification

could cause AVs accumulation in axon.

3.4 CMA and AD

Similar to macroautophagy, CMA is also considered to connect with neurodegeneration diseases such as AD and PD. CMA plays an important role in both τ tangles and Aβ plaques generation and its activity is impaired with aging process (Koga H. and Cuervo A. M., 2011). In certain τ mutations, the first cleavage will happen in wrong amino acid position. After this cleavage, although remaining c-truncated τ could still be recognized by hsc70 and recruited onto lysosome membrane, it cannot translocated into lysosome luminal efficiently, thus resulting in accumulation of truncated τ on lysosome membrane. Some of these products organize as oligomeric structures at the lysosomal membrane and with time, these oligomers can promote disruption of the lysosomal membrane and the subsequent leakage of lysosomal enzymes (Koga H. and Cuervo A. M., 2011).

Another recent study found APP also bears KFERQ motif which could be recognized by hsc70. Deletion of this sequence will keep APP away from lysosome and increase its secretase cleavage products, without influence its ability to bind to hsc70 (Qian Li et al., 2017).

4. Epigenetic and AD

The term “epigenetics” was introduced by the biologist Conrad Hal Waddington (1905–1975) in the early 1940s. He defined epigenetics the branch of biology that studies the causal interactions between genes and their products, which bring the phenotype into being (Waddington C. H., 1942, 2012). Then it was defined as the heritable traits resulting from changes in a chromosome without altering the DNA sequence (Berger S. L. et al., 2009). Nowadays, epigenetics is considered as the study of structural changes of the chromatin that modify the phenotype without altering the genotype (Jaenisch R. and Bird A., 2003), independently of whether the cells divide or not. (Sanchez-Mut J. V. and Gräff J. et al., 2015).

So epigenetic mechanisms modify heritable and non-heritable traits without necessarily altering the underlying DNA sequence. These effects are typically accomplished by inhibition of transcriptional access to various genes, leading to their repression or silencing. Conversely, release from normal epigenetic repression can enhance gene expression. These are modifications that can occur at specific gene loci in specific cells to generate specific cellular phenotypes (Wilson V. L. and Jones P. A., 1983).

Epigenetic modifications can also cause genetic mutations.

About half of the genes that cause familial or inherited forms of cancer are turned off by methylation. Most of these genes normally suppress tumor formation and help repair DNA, including O^6-methylguanine-DNA methyltransferase (*MGMT*), MLH1 cyclin-dependent kinase inhibitor 2B (*CDKN2B*), and *RASSF1A*. For example, hypermethylation of the promoter of *MGMT* causes the number of G-to-A mutations to increase (Mastroeni D. et al., 2011).

It is also known that epigenetic mechanisms participate in the processes of learning and memory formation (Woldemichael B. T. et al., 2014), and that – on the other end of the spectrum – lifestyle (Fraga M. F. et al., 2005), aging (Heyn H. et al., 2012), nutrition (Cooney C. A. et al., 2002), and environmental toxins (Anway M. D. et al., 2005) associated with AD can modify the epigenetic makeup and might thereby contribute to the pathophysiology of AD (Bennett D. A. et al., 2015).

At the molecular level, it is generally accepted that epigenetics encompasse two main mechanisms: the direct methylation of the DNA, and the modification of the proteins that package the DNA, the histones (Sanchez-Mut J. V. and Gräff J. et al., 2015).

4.1 DNA Methylation

DNA methylation is an heritable epigenetic mark involving the addition of a methyl group to the aromatic ring of a single DNA base.

This modification mainly occurs in cytosines that precede guanines and concerne a covalent transfer of a methyl group to the C-5 position of the cytosine (CpG dinucleotides) that tend to accumulate in CpG-dense regions (so-called CpG islands, or CGI). CpG islands are usually unmethylated in normal cells, whereas when they are methylated (mCpG) they are involved in gene silencing. The enzymes that carry out the active DNA methylation, so called DNA methyltransferases (DNMTs), are all associated with the nucleosomes (Jeong S. et al., 2009), suggesting that DNA methylation and nucleosome positioning are intimately related.

DNA methylation is regulated by a family of DNMTs: DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L.

DNMT1 is proposed for the maintenance of DNA methylation patterns during cell division, and DNMT3A and DNMT3B, have preference for unmethylated CpG dinucleotides and perform *de novo* methylation during development, while DNMT3L has no catalytic activity itself. The methyl group that is transferred to cytosine by the DNMTs ultimately derives from methyltetrahydrofolate through its interactions with S-adenosylmethionine (SAM) in the homocysteine-methionine cycle.

Global hypomethylation appears to be the most important feature of aging cells and tissues. DNA hypomethylation occours in several age-related diseases such as AD (Wierda R. J. et al., 2010). DNA hypermethylation is also important in aging, whereby specific regions such as tumor suppressor genes are highly methylated and may play an important role in cancer development (Esteller M., 2000).

Interestingly, DNMTs also shown high levels of expression in post-mitotic neurons (Guo J. U. et al., 2014), suggesting that their importance in the adult brain. A deficit of DNMTs can leading to DNA demethylation (Rhee I. et al., 2002), but DNA can also be actively demethylated by the action of several enzymatic reactions. These include the 10–11 translocation proteins (TET), which mediate the oxidation of 5-methylcytosines (5mC) to 5-hydroxymethylcytosine (5hmC), and later on to 5-formilcytosine (5fC) and 5-carboxycytosine (5caC); and the thymine-DNA glycosylases (TDG), which causes the final excision and conversion to cytosines (Kohli R. M. and Zhang Y., 2013).

70% of annotated genes contain CpG regions in their promoters (Deaton A. M. and Bird A., 2011) and most of them are non-methylated. Methylation of CpG sequences may

alter gene (Bird A. P. and Wolffe A. P., 1999; Zhang H. et al., 2002). Highly methylated genes can be repressed, while hypomethylation of a gene can leading to enhanced expression or overexpression compared to the normally repressed, methylated state. It has been also shown that in nearly half the genes hypomethylation resulted in silencing rather than upregulation (Yakovlev A. et al., 2009).

It is also shown another linked mechanism, methyl-CpG-binding proteins (MeCPs) such as MeCP2, by which DNA methylation that may modify gene expression. MeCP2 can bind to methylated DNA, and recruit HDACs, which, inducing a more condensed chromatin state and decreased or silenced gene transcription (Wade P. A. et al., 1998).

MeCP1, instead, can bind only a single methyl-CpG-binding domain protein but not methylated DNA (Mastroeni D. et al., 2011).

4.2 Histone Modifications

The nucleosome is the fundamental repeating unit of chromatin, consisting of an octamer protein complex containing two molecules of each of the core histones (H2A, H2B, H3, and H4) in conjunction with 147 base pairs of genomic DNA wrapped around them. Histone proteins have some amino-terminal tails that make them accessible to modification of their amino acid residues, of which acetylation, methylation, phosphorylation, and ubiquitination are the most thoroughly studied, and they have been associated with activation or repression of transcription (Kouzarides T., 2007). Acetylation is generally associated with an active gene transcription profile, while the consequence of methylation can be either positive or negative with respect to transcriptional expression, depending on the position of the residue within the histone (Kouzarides T., 2007). Histone modification seems to contribute to the progression of aging and age-related diseases. (Longo V. D., 2009).

4.2.1 Histone acetylation

Histone acetylation can be associated with increased gene activity (Kouzarides T., 2007), reducing the basic charge of histones and the electrostatic interaction with the negatively charged DNA chains, facilitating the access of the transcriptional machinery to the DNA (Li G. and Reinberg D., 2011). Histone acetylation is a process in which acetyl coenzyme A transfers acetyl to the lysine residues of core histone N terminal domains under the catalysis of histone acetyltransferases (HATs).

Histone acetylation include lysine acetylation modifications such as the acetylation of

lysine 9 on histone 3 (H3K9ac) and (H3K27ac) in gene promoters and H3K4ac and H4K12ac in gene bodies, among others (Wang H. C. et al., 2008).

Three families of HATs – GNAT, MYST, and CBP/p300 – are responsible of the acetylation of these amino acids (Bannister A. J. and Kouzarides T., 2011). Zinc-dependent class I, II and IV HDACs as well as the NAD- dependent class III HDACs, the sirtuins, antagonize the activity of HATs. Similarly to the HATs, HDAC proteins show a low level of specificity that is mainly regulated by the interaction with other non-catalytic proteins and complexes (Yang X. J. and Seto E., 2007).

4.2.2 Histone Methylation

Histone methylation is a process by which methyl groups are transferred to amino acids of histone proteins.

The effect of histone methylation depends on both the type of modification and the residue on which it occurs. For example, the mono-methylation of lysine 27 of histone 3 (H3K27me1) is enriched in promoters of active genes, whereas the tri-methylation of the same amino acid (H3K27me3) is mainly found in repressed genes (Wang H. C. et al., 2008). Therefore, the enzymes that regulate histone methylation have an high degree of specificity. Have been shown two classes of lysine HMTs: SET domain and non-SET domain-containing HMTs. Also, the activity of the HMTs is antagonized by two classes of HDMTs – LSD1- and Jumonji- related HDMTs – that also show a high degree of specificity. For example, LSD1 demethylates the monomethylation and dimethylation of H3K4 and H3K9, but not their trimethylation (Bannister A. J. and Kouzarides T., 2011).

4.3 miRNAs

In the last decade, few areas of biology have been transformed as thoroughly as RNA molecular biology. This transformation has occurred along many fronts but one of the most significant advances has been the discovery of small (20–30 nucleotide [nt]) noncoding RNAs that regulate genes and genomes called microRNAs (miRNAs).

miRNAs are transcribed by RNA polymerase II as large RNA precursors called pri-miRNAs and comprise of a 5' cap and poly-A tail (leey et al., 2004). The pri-miRNAs are processed by an RNase III enzyme Drosha (Han J. et al., 2004), and the double-stranded-RNA-binding protein, Pasha/DGCR8 (Denli A. M. et al., 2004). The resulting pre-miRNAs (70-nucleotides) are folded into imperfect stem-loop structures. The pre-

miRNAs are then exported into the cytoplasm by the karyopherin exportin 5 (Exp5) and Ran-GTP complex (Yi R. et al., 2003) Ran (ras-related nuclear protein) is a small GTP binding protein belonging to the RAS superfamily that is essential for the translocation of RNA and proteins through the nuclear pore complex (Moore M. S. et al., 1993). The Ran GTPase binds Exp5 and forms a nuclear heterotrimer with pre-miRNAs (Yi R. et al., 2003; Lund E. et al., 2001). Once in the cytoplasm, the pre-miRNAs undergo an additional processing step by the RNAse III enzyme Dicer (Bernstein E. et al., 2001) generating the miRNA, a double-stranded RNA aproximately 22 nucleotides in length. Dicer also initiates the formation of the RNA-induced silencing complex (RISC) (Hammond S. M., 2000). RISC is responsible for the gene silencing observed due to miRNA expression and RNA interference (Hammond S. M. et al., 2000) (Sigma.it).

The effects of small RNAs on gene expression and control are generally inhibitory, and the cor- responding regulatory mechanisms are therefore collectively subsumed under the heading of RNA silencing. (Carthew R. W. and Sontheimer E. J., 2009)

Like histone modifications, miRNA has been implicated in both broad and specific CNS processes and disorders. For example, miRNA-206 appears to promote neuromuscular synapse regeneration (Williams A. H. et al., 2009), and miR-329, miRNA-134 and miRNA-381, which are induced by neuronal activity, have been suggested to be essential for activity-dependent dendritic outgrowth of hippocampal neurons (Khudayberdievet S. al., 2009). In turn, these and other miRNA-mediated mechanisms have been investigated in various CNS disorders, including HIV-dementia (Witwer K. W. et al., 2010), amyotrophic lateral sclerosis (Williams A. H. et al., 2009), Tourette's syndrome (Abelson J. F. et al., 2005), AD, and other neurologic conditions (Mastroeni D. et al., 2011).

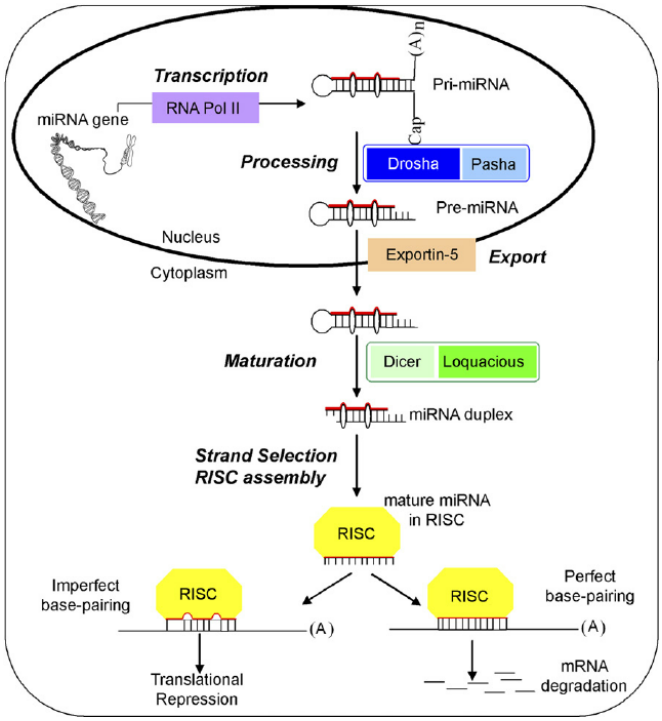

***Fig.10** mRNA processing.*

4.4 Epigenetic regulation of aging

Aging is considered one of the most risk factors for AD (Gao S. et al., 1998; Kukull W. A. et al., 2002). Seems to be deleterious changes in mitochondria/oxidative stress (Crouch P. J. et al., 2007), gonadotropins (Fuller S. J. et al., 2007), calcium (Thibault O. et al., 2007), glucocorticoids (Landfield P. W. et al., 2007), inflammation (Duenas-Gonzalez A. et al., 2008), trace metals (Brewer G. J., 2007), insulin (Craft S., 2005), cerebrovascular supply (Bailey T. L. et al., 2004), the cell cycle (Macaluso M. et al., 2005), Aβ (Selkoe D. J., 2003), tau (Maeda S. et al., 2006), and hundreds to thousands of genes (Parachikova A. et al., 2007) occur both in aging and AD (Mastroeni D. et al., 2011).

DNA methylation and histone modifications are implicated in the phenotypic alterations and then in cellular senescence of various organisms (Wilson V. L. and Jones P. A., 1983).

DNA methylation undergoes remodeling during ageing in various tissues in mice and humans.

Although changes in DNA methylation in heterogeneous tissues may partly reflect alterations in cell composition (Zou J. et al., 2014), it is interesting to note that age-dependent changes in DNA methylation are detected in more homogeneous cell populations, such as hematopoietic stem cells (HSCs) (Beerman I. et al., 2013)

In HSCs, regions with altered DNA methylation overlap binding sites of specific sets of transcription factors. This observation raises the possibility that these methylation changes could influence the targeting of transcription factors, and thereby contribute to misregulation of gene expression during ageing (Sun D. et al., 2014).

It is also shown that the folding of cellular genomes into higher-order chromatin structure affects nearly all cellular processes that are linked to ageing, including transcription, DNA repair and DNA replication (Cavalli G. et al., 2013). The expression of core histones is reduced during replicative ageing in yeast, as well as in other species and cell types (Feser J. and Tyler J., 2011).

Histone acetylation directly impacts the physical association of histones and DNA. Histone acetylation is a key conserved player in longevity, and evidence suggests that its pattern changes during normal ageing. Changes in histone acetylation thus may be a consequence and a cause of the failure of older cells to transduce external stimuli to downstream transcriptional responses, a process that is particularly detrimental for rapid cell-to-cell signaling in the brain (Benayoun A. B. et al., 2015).

In addition to aging, epigenetics plays a major role in development (reviewed in Reik W. and Dean W., 2001). These mechanisms could be relevant to AD since overt clinical symptoms of the disorder virtually never appear until after the developmental stages of infancy, childhood, and early adolescence have been completed, and this is true not simply in LOAD patients but in patients carrying APP, PS1, or PS2 mutations. Thus, epigenetic changes earlier in life might be a necessary but not sufficient step toward AD in susceptible individuals, a key concept in the “LEARn” (latent early-life associated regulation) model of age-related neurologic disorders (Wu X. and Hua X., 2008). Support for this hypothesis comes from APP transgenic mouse research wherein earlier epigenetic manipulations appear to accelerate or delay the expression of Aβ pathology (Fuso A. et al., 2008).

5. Poly ADP-ribosylation

The pathological hallmarks of AD include the accumulation of Aβ. Recently, it has been discovered that Aβ activates the protein poly(ADP-ribosyl) polymerase-1 (PARP-1) specifically in astrocytes, leading indirectly to neuronal cell death. PARP-1 is a DNA repair enzyme, of 113 kDa, normally activated by single strand breaks associated with oxidative stress. PARPs include 17 putative isoforms based on protein sequence homology to a PARP catalytic domain. PARP-1 is the founding member of the PARP family and accounts for more than 90% cellular PARP activity (Smith S., 2001). In response to DNA damage, PARP-1 uses NAD+ as a substrate and attaches polymers of ADP ribose on different acceptor proteins (heteromodification) or on PARP-1 itself (automodification) (D'Amours D. et al., 1999). Because Poly(ADP-ribose) (PAR) is negatively charged and noncovalently couples with nuclear proteins, (PARylation), PAR can act as a scaffolding for chromatin remodeling and DNA repair processes.

The most prominent target protein (acceptor) of the poly(ADP-ribosyl)ation reaction is PARP-1 itself, but many other acceptor proteins have been described, including p53, NF-kB, histones, DNA-topoisomerases and the catalytic subunit of the DNA-dependent protein kinase (DNA-PK_{CS}) (De Murcia G. et al., 1995).

Recently, poly(ADP-ribose) polymerase-1 (PARP-1) has been shown to play a key role in nuclear factor kappa B (NF-kB)-driven expression of inflammatory mediators by glia during the neuroimmune response (Chiarugi A. and Moskowitz A., 2003). Pharmacological inhibition of PARP-1 reduces infact, both NF-kB activity and the synthesis of inflammatory mediators during glial activation. Importantly, these effects correlate with reduced neurotoxicity of activated glial cells *in vitro* and *in vivo*. These results underscore the importance of PARP-1 enzymatic activity and poly(ADP-ribosyl)ation during glial cell transcriptional activation and identify PARP-1 activity-dependent regulation of NF-kB as a possible treatment target for neuroinflammatory and neurodegenerative disorders (Chiarugi A. and Moskowitz A., 2003).

The turnover of PAR polymers is managed by PAR glycohydrolase (PARG) (Davidovic L. et al., 2001).

In various disease beyond cancers, enhanced cellular stress generates unregulated PARP-1 activation and the consequent depletion of NAD+ and subsequent inflammation, cell death, and tissue damage. In this respect, PARP inhibitors have been investigated as treatment channels to protect cell death, tissue damage, and aging or oxidative damage-related pathologies.

Recent studies using PARP inhibitors suggest that poly(ADP-ribosyl)ation may affect signaling pathways which are linked to cell survival (Capone R. et al., 2012). To better understand the implication of PARP-1 activation in cell death, have been analyzed the effect of PAR synthesis on the MEK/ERK signaling pathway in HeLa cells exposed to the alkylating agent N-methyl-N^0 –methyl-nitro-N-nitrosoguanidine (MNNG). Results shown that massive PAR synthesis in HeLa following MNNG exposure affect the MEK/ERK signaling pathway leading to cell death and that sustained activation of ERK1/2 by inhibition of massive PAR synthesis with the PARP inhibitor PJ34, leads to cytoprotection (Abeti R. and Duchen M. R., 2012).

Studies show that oxidized and reduced Nicotinamide Adenine Dinucleotide (NAD+ and NADH respectively) are crucial metabolites in metabolic reactions. Continuous PARP activation can reduce intracellular levels of NAD+ by 80% and raises nicotinamide (NAM) (Bai P. and Cantò C., 2012). It has been known that the decrease of NAD+ and the increase of NAM ensured by enhanced PARP activity correlates with a downregulation of SIRT1 activity, a class III histone deacetylase (Bai P. and Cantò C., 2011). Similarly, the activation of SIRT1 reduced PARP activity (Kolthur- seetharam U. et al., 2006). These observations raised a possible crosstalk between SIRT1 and PARP-1 (Chung H. T. and Joe Y., 2014).

5.1 Competition for NAD+

PARPs and SIRT1 may compete for the limiting NAD+ substrate because they are NAD+ dependent enzymes (Fig.11) (Houtkopper R. H. et al., 2010). The SIRT1 activity induced the fluctuations in NAD+ levels as the Km of SIRT1 falls in the bounds of physiological cellular NAD+ change (Houtkopper R. H. et al., 2010). It is well known that NAD+ levels upon excessive DNA damage is decreased due to PARP activation. In the excessive DNA damage, NAD+ levels may decrease to 20–30% of the control that is likely to be rate limiting of SIRT enzymes (Houtkopper R. H. et al., 2010). SIRT1 activity is dramatically declined under these conditions which might consequently lead to decreased SIRT1 expression.

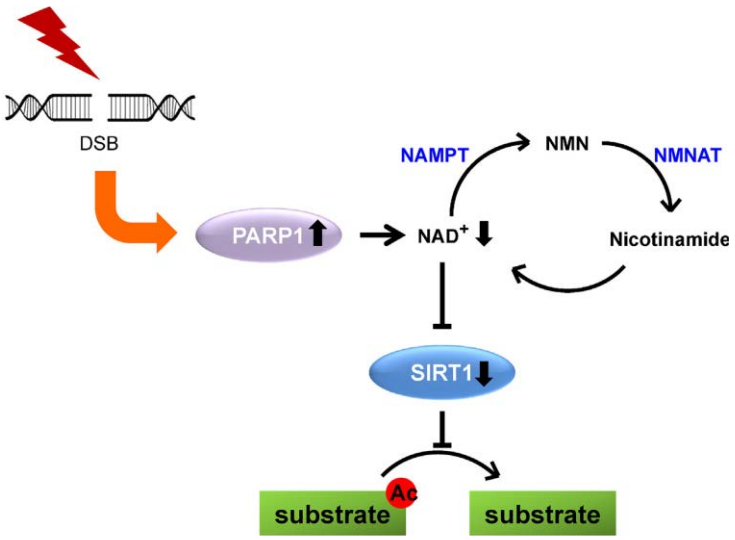

Fig. 10 *Competition for NAD+ between PARP1 and SIRT1.*

5.2 Sirtuins

Sirtuins are a highly conserved family of proteins. The silent information regulator 2 (SIR2) gene was first described. In mammals seven sirtuin family members exist, including three members, Sirt3, Sirt4, and Sirt5, that localize exclusively within the mitochondria. Sirt1 appears to be the closest mammalian homolog to yeast Sir2, the first member of the sirtuins linked to aging. Each of the mammalian sirtuins has a distinct subcellular localization. Sirt1, Sirt6, and Sirt7 are nuclear proteins, although a fraction of Sirt1 can be found in the cytosol. Sirt2, on the other hand, is predominantly cytosolic, although again it can be found in the nucleus in certain situations (North B. J. and Verdin E., 2007). The ability of sirtuins to influence metabolism and potentially life span is believed to revolve around the ability of sirtuin family members to function as protein deacetylases. In addition to this enzymatic function, Sirt4 can further act to ADP ribosylate target proteins. Unlike other protein deacetylases, sirtuins require NAD as a cofactor in the deacetylation reaction (Imai S. et al., 2000). The link among NAD, NADH, and sirtuin activity has led many to believe that this family of proteins acts as a sensor of energetic status. This may particularly be true in the mitochondria, where levels of NAD and NADH are high and where a disproportionate fraction of proteins appear to be acetylated (Kim M. Y. et al., 2006).

In addition to their differential cellular location, the sirtuin family members can also be distinguished by their different enzymatic activities. SIRT1 and SIRT5 act as deacetylases (Vaziri H. et al., 2001), while SIRT4 seems to be mono-ADP-ribosyl transferase (Haigis M. C. et al., 2006). SIRT2, SIRT3 and SIRT6 can display both

activities (Liszt G. et al., 2005; Michishita E. et al., 2008; North B. J. et al., 2003; Shi T. et al., 2005). The activity of SIRT7 has not been clearly established, even though it has been hypothesized to act as a deacetylase (Vakhrusheva O. et al., 2008). Of note, SIRT5 was recently described to demalonylate and desuccinylate proteins (Peng L. et al., 2011).

5.2.1 SIRT1

Among all sirtuins, SIRT1 is the best characterized. Human SIRT1 contains the conserved catalytic core of sirtuins and both N- and C-terminal extensions that all span ~240 aminoacids. These extensions serve as platforms for interaction with regulatory proteins and substrates. In total the human SIRT1 spans 747 amino acids. SIRT1 contains 2 nuclear localization signals (NLS) as well as 2 nuclear exportation signals (NES) (Tanno M. et al., 2007). SIRT1 activity is generally increased in situations of energy/nutrient stress. The fact that SIRT1 activity is regulated by NAD^+ raised the hypothesis that NAD^+ could act as a metabolic sensor in situations of energy stress, where NAD^+ levels are generally affected. Changes in intracellular NAD^+ rarely fluctuate more than 2-fold (Chen D. et al., 2008), which is a likely range to impact on sirtuin activity. In general, NAD^+ levels increase in mammalian tissues in response to energy/nutrient stresses like exercise, fasting (Cantò C. et al., 2010) or calorie restriction (Chen D. et al., 2008). SIRT1 activity is also controlled by other NAD^+-derived metabolites. It was proposed that NADH would compete with NAD^+ binding to SIRT1 and inhibit SIRT1 activity (Lin S. J. et al., 2004). The balanced functionality of these signals determines the presence of SIRT1 in either the nuclear or the cytoplasmic compartment, and explains that SIRT1 location may differ depending on the cell type or tissue evaluated.

In agreement with its dual cellular localization, SIRT1 targets can be found in both the nuclear and cytosolic compartments. SIRT1 activity in the nucleus articulates dynamic and varied transcriptional responses through the deacetylation of a large spectrum of transcriptional regulators. Therefore, the deacetylation by SIRT1 can lead to the direct activation or inhibition of transcriptional regulators and modify their interaction profiles, depending on the cellular context.

Recent sudies reported that SIRT1 has marked anti-inflammatory effects in diverse tissues and cell models (Pfluger P. T. et al., 2008), probably through the negative

regulation of the NF-kB pathway (Yeung F. et al., 2004). Also, SIRT1 activity has a strong influence on cell proliferation, apoptosis and cancer.

Another field where SIRT1 may be of interest is in the CNS. It was recently proven that SIRT1 has key roles modulating cognitive function and synaptic plasticity (Gao J. et al., 2010). Additionally, there is evidence that enhanced SIRT1 activity could be protective in diseases as neurodegeneration, AD and amyotrophic lateral sclerosis (Donmez G. and Guarente L., 2010).

Also it has been identified p53 as a SIRT1 substrate. Two different laboratories have reported how SIRT1 interacts with and deacetylates p53 (Luo J. et al., 2001; Vaziri et al., 2001). While p53 can be acetylated in up to 6 residues, SIRT1 seems to preferentially deacetylate Lys^{379} (human Lys^{382}). The deacetylation of p53 by SIRT1 attenuated its activity on the p21 promoter and inhibited p53-dependent apoptosis (Cantò C. et al., 2012).

5.3 PARP-SIRT interaction in age-related diseases

Muiras et al. (Muiras et al., 1998) found increased PARP activities in cells from centenarians, suggesting that larger capacity of PARP activation is related with successful aging. The larger PARylation ability is postulated to be reliable DNA repair that prevents the DNA damage-related diseases (Nicolas L. et al., 2010). However, age-related pathologies such as inflammatory diseases is increasingly occurred by PARP-1 overexpression (Magerich A. et al., 2010) Aging is strongly related with enhanced oxidative stress. This oxidative stress and increased PARP activation (Massudi H. et al., 2012) in old age place a considerable strain on NAD+ homeostasis. As a result, NAD+ levels and accompanied SIRT1 activities are decreasing with aging. The decrease in SIRT1 activity induces mitochondrial dysfunction, which represents a characteristic of aging and is probably a leading cause of various age-related chronic inflammatory and metabolic diseases. (Chung H. T., Joe Y., 2014).

6. The Mediterranean diet

6.1 History of the Mediterranean diet

MD was described by Ancel Keys in the 1950s. For Ancel Keys it included 'home made mine-strone...pasta in endless variety...served with tomato sauce and a sprinkle of cheese, only occasionally enriched with some bits of meat or served with a little local sea-food...a hearty dish of beans and short lengths of macaroni...lots of bread never more than a few hours from the oven and never served with any kind of spread; great quantities of fresh vegetables, a modest portion of meat or fish perhaps twice a week, wine of the type we used to call 'Dagored'...always fresh fruit for dessert'(Nestle M., 1995).

Later Keys cites 'The heart of what we now consider the MD is mainly vegetarian: pasta in many forms, leaves sprinkled with olive oil, all kinds of vegetables in season and often cheese, all finished off with fruit and frequently washed down with wine...it is much lower in meat and dairy products and there are some differences in dessert. What we call pie is almost unknown, as are cornstarch and steamed puddings. Cakes are mostly special types for Christmas and Easter and fresh fruit is the standard dessert (Davis C. et al., 2015).

For Trichopoulou et al., (Trichopoulou A. et al., 1995) studying older people in Greek villages, the eight characteristics of the traditional Greek diet are:

1. A high ratio of monounsaturated to saturated fat.
2. Moderate alcohol consumption.
3. High consumption of legumes.
4. High consumption of cereals (including bread).
5. High consumption of fruits.
6. High consumption of vegetables.
7. Low consumption of meat and meat products.
8. Low consumption of milk and dairy products.''

So during last years studies of the MD has advanced, and the definition originally introduced by Keys has evolved and varied.

Not a specific pattern but a collection of eating habits traditionally followed by the populations of the Mediterranean basin first defined the MD called "traditional". This dietary pattern is characterized by abundant plant foods consumption in the form of fruits, vegetables, breads, other forms of cereals, potatoes, beans, nuts and seeds; fresh

fruit as the typical dessert; olive oil as the main source of monounsaturated fat; dairy products as principally cheese and yogurt; a low to moderate consumption of fish depending on the proximity of the sea; a low to moderate consumption of poultry; fewer than four eggs consumed per week; low amount of red meat and wine consumed in low to moderate amounts, normally during meals (Willet W. C. et al., 1995; Courtney D. et al., 2015).

The definitions from Willett et al. Panagiotakos et al. and Dilis et al. add some description of traditional definitions; olive oil was added to vegetables and legumes to make them palatable, fruits were eaten as desserts or snacks, cheeses accompanied salads and stews, and red meat was eaten only on special occasions.

Most commonly, recommendations of numbers of servings for these food groups are represented as a diet pyramid. Diet pyramids are considered a useful way to display the general principles of a diet including approximate recommendations for quantities of food groups (f.e., those consumed in greatest quantities appear in the largest section of the pyramid). Over the years, some MD pyramids were elaborated by the USA (Menotti A. et al., 1999; Kromhout D. et al., 1995) and by Mediterranean countries, such as Greece (Kafatos A. et al., 2000), Spain (Bach-Faig A. et al., 2011) and Italy (Willet W. C. et al., 1995), to give their populations dietary advice based on both cultural heritage and scientific evidence in order to prevent chronic diseases and promote longevity.

In the new MD pyramid (Fig. 11), adapted to the specific realities of different countries, the pattern includes all food groups along with adequate frequencies and quantities in the daily diet that make it healthy or unhealthy.

Plant-origin foods are situated at the base of the pyramid. They provide key nutrients, fibre and protective substances that contribute to general wellbeing, satiety and the maintenance of a balanced diet, and thus should be consumed in high proportions and frequency.

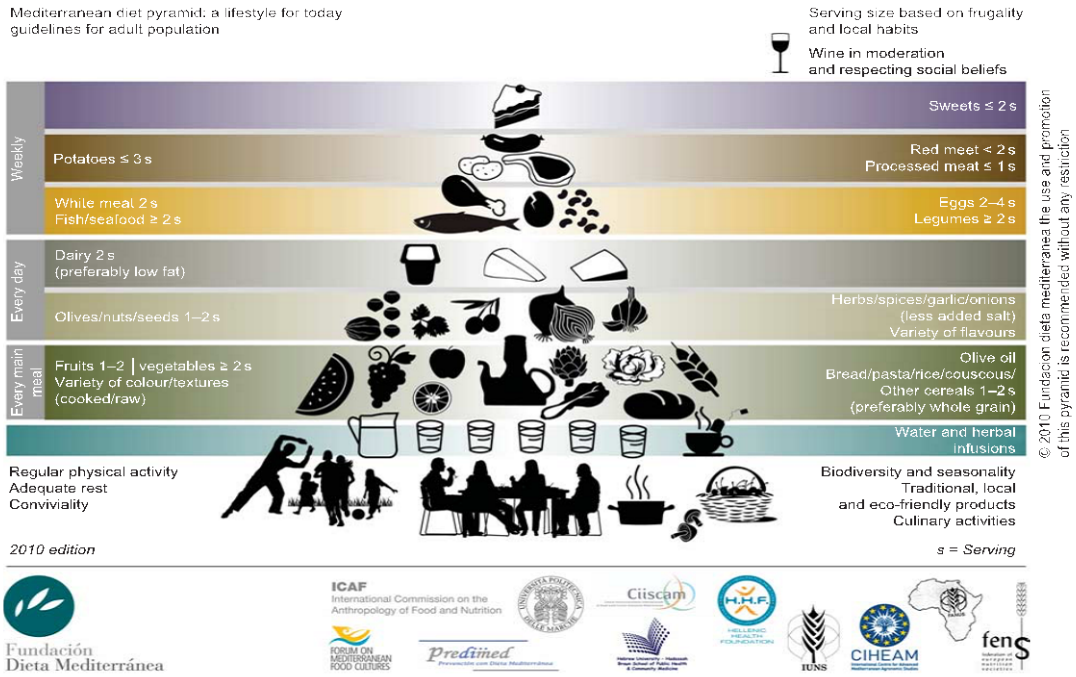

Fig. 11 *The MD pyramid.*

At the base of the pyramid, food items that should sustain the diet and provide the highest energy intake, and at the upper levels, foods to be eaten in moderate amounts such as those of animal origin and/or rich in sugars and fats that should be eaten in moderation and some of them left for special occasions. The pyramid establishes dietary daily, weekly and occasional guidelines in order to follow a healthy and balanced diet.

Every day

Main meals should contain three basic elements, which can also be found throughout the day:

Cereals: one or two servings per meal in the form of bread, pasta, rice, couscous and others. Preferably whole grain, since processing normally removes fibre and some valuable nutrients (Mg, Fe, vitamins, etc.) (Braida-Bruno L. et al., 2013).

Vegetables: two or more servings per meal. In order to ensure vitamin and mineral daily intakes, at least one of the servings should be consumed raw (one meal/d) (La joie R. et al., 2013).

Fruit: one or two servings per meal, variety in colours and textures', as the most frequently chosen dessert, in order to ensure a wide variety of antioxidants and protective compounds (Becker J. A. et al., 2011).

People who consume fruit and vegetables regularly have been compared with others who don't, and benefit and a decrease in the risk of AD have been found in consumers;

this may be due to the fact that fruit and vegetables are a source of antioxidants and bioactive compounds, as well as to their low contents of saturated fat (Hu N. et al, 2013).

Strong protection against AD has been demonstrated for the consumption of vegetables, especially those with green leaves, which contain vitamin E. The consumption of certain grains related to the MD has also been proven useful (Hjorth E. et al., 2013; Hunberto H. L. et al., 2015).

Dairy products should be present in moderate amounts (two servings per day), with a preference for low-fat dairy, traditionally in the form of yoghurt, cheese and other fermented dairy products. Although their richness in Ca is important for bone and heart health, dairy products can be a major source of saturated fat (Zhang X. et al., 2013).

Olive oil is located at the centre of the pyramid; it represents the principal source of dietary lipids because of its high nutritional quality (especially extra virgin olive oil, EVOO). Its unique composition gives it a high resistance to elevated temperatures, and it is recommended for both cooking (Tyagi E. et al., 2013) and dressings. Several studies have suggested that EVOO has several health promoting effects that could protect from and decrease the risk of AD. Feeding mice with EVOO-enriched diet for 6 months, beginning at an age before Aβ accumulation starts, has significantly reduced total Aβ and tau brain levels with a significant improvement in mouse cognitive behavior. On the other hand, although feeding mice with EVOO-enriched diet for 3 months, beginning at an age after Aβ accumulation starts, showed improved clearance across the BBB and significant reduction in Aβ levels, it did not affect tau levels or improve cognitive functions of TgSwDI mouse (Qosa H. et al., 2015). The double transgenic TgCRND8 mice (overexpressing the Swedish and Indiana mutations in the human APP), aged 1.5 and 4, and age-matched wild type control mice were used to examine in vivo the effects of 8 weeks dietary supplementation of oleuropein aglycone (50 mg/kg of diet), the main polyphenol found in extra virgin olive oil. We have reported in our laboratory that dietary supplementation of oleuropein aglycone strongly improves the cognitive performance of young/ middle-aged TgCRND8 mice, a model of amyloid-ß deposition, respect to age-matched littermates with un-supplemented diet (Grossi C. et al., 2013). We also studied the effects of dietary intake of a mix of polyphenols present in olive mill waste water administered at the same dose of OLE (50 mg kg^{-1} of diet) previously investigated. We also showed that the mix of polyphenols improved significantly cognitive functions of Tg mice and significantly reduced Aß42

and pE3-Aß plaque area and number in the cortex and in the hippocampus (Pantano D. et al., 2017).

Traditionally, vegetables and other plant foods are cooked with olive oil, thus amplifying their nutritional value.

Olives, nuts and seeds are good sources of healthy lipids, proteins, vitamins, minerals and fibre (Ouitschke W. W. et al., 2013). A reasonable consumption of olives, nuts and seeds (such as a handful) make for a healthy snack choice.

Spices, herbs, garlic and onions are a good way to introduce a variety of flavours and palatability to dishes and allow for a reduction in salt use, as salt is one of the main contributing factors to the development of hypertension among predisposed individuals (Villaflores O. B. et al., 2012). Herbs and spices are good sources of micronutrients and antioxidant compounds and also contribute to the regional identities of Mediterranean dishes.

Moderate consumption of ***wine*** and other fermented beverages during meals (one glass per day for women and two glasses per day for men, as a generic reference) (Davinelli S. et al., 2012).

Weekly

Consumption of a variety of plant- and animal-origin proteins is recommended.

Fish and shellfish (two or more servings), ***white meat*** (two servings) and ***eggs*** (two to four servings) are good sources of animal protein. Fish, white meat (poultry, turkey, rabbit, etc.) and eggs provide high-quality protein. Fish and shellfish are a good source of healthy protein and lipids. Varied consumption (of oily fish, lean fish and shellfish) is recommended.

The essential fatty acids, which include the omega-6 (n-6) and omega-3 (n-3) fatty acids, are crucial components of the MD. Docosahexanoic acid (DHA) is an n-3 polyunsaturated fatty acid (PUFA) found predominantly in marine fish and algae. Although it has a low conversion rate, it can also be biosynthesized in vivo in mammals from dietary n-3 sources, notably linolenic acid (Salem et al., 1996b). It is essential for prenatal brain development and normal maintenance of brain function and vision in adults (Mitchell et al., 1998). Deficiencies in the level of DHA, such as low serum levels as well as high dietary intake ratios of n-6/n-3 fatty acids, have been linked to cognitive impairment (Connor W. E. et al., 1990) and AD.

DHA or its enzymatically generated metabolites may be neuroprotective by reducing Aß toxicity (Mukherjee P. K. et al., 2004).

White meat is also a good source of lean protein without the high levels of saturated fat found in some red meat cuts. Egg consumption, including those used in cooking as well as baking, should be between two and four times per week.

Consumption of red meat (less than two servings, preferably lean cuts) and processed meats (less than one serving) should be small in both quantity and frequency as the intake of such meats has been consistently associated with some chronic diseases (Ashare R. L. et al., 2013; Hu N. et al., 2013).

The combination of ***legumes*** (more than two servings) and cereals is a healthy plant protein and lipid source that should be considered as a meat alternative (Dhungana H. et al., 2013; Dodge H. H. et al., 2012).

Potatoes are also included on the weekly level (three or fewer servings per week, preferably fresh), as they are a part of many traditional recipes with meat and fish. They should be consumed in moderation (Pallauf K. et al., 2013) as they have a high glycaemic index (Shinto L. et al., 2014) and are most commonly prepared fried.

Occasionally in the top of the pyramid appear foods rich in sugars and unhealthy fats (sweets). Sugar, candies, pastries and beverages such as sweetened fruit juices and soft drinks should be consumed in small amounts and set aside for special occasions. These foods are energy dense and are likely to contribute to weight gain (Pallauf K. et al., 2013). Simple sugars, which are abundant in sweets, pastries, fruit juices and soft drinks, have been associated with an increased occurrence of tooth decay (Hu N. et al., 2013).

Physical activity

Regular practice of moderate physical activity (at least 30 min throughout the day) serves as a basic complement to the diet by balancing energy intake, maintaining healthy body weight and providing many other health benefits (Hu N. et al., 2013). Physical activity not only involves sports such as football, dancing, jogging, cycling, etc. but also walking, taking the stairs v. the lift, housework, gardening, etc. Practising leisure activities outdoors (Lim H. J. et al, 2013), and preferably with others, makes them more enjoyable and strengthens the sense of community (Anna B. F. et al., 2011).

6.2 The MD indexes

Recently, it has been suggested that there is no specific food or component that is as benficial as the whole diet pattern and, possibly, the broader traditional cuisine and

lifestyle habits which may be significant elements. However, some studies have attempted to identify the relative importance of individual foods within predictive index tools, as an holistic approach to study items that are highly interrelated and influence their impact on the risk of non-communicable chronic diseases (Hu F. B., 2002).

There are two distinct approaches: *a priori* and *a posteriori*.

The *a priori* approach expected the construction of an index on the relationship between foods and diseases (Kant A. K., 2004).

The *a posteriori* approach is based on multivariate statistical techniques that identify sets of dietary exposures strongly correlated together and not previously known.

Mediterranean Diet Score, Dietary Score, Mediterranean Adequacy Index, and PREvención con DIeta MEDiterránea (PREDIMED) score are such of *a priori* dietary indexes used to evaluate dietary exposure in such studies (D'Alessandro A. and De Pergola G., 2015).

6.3 MD and AD

The health benefits of a Mediterranean-style diet are well documented. The Mediterranean-style diet has been first associated with a significant decrease in overall mortality among 182 men and women aged 70 years and more in three Greek villages (Trichopoulou A. et al., 1995). Advantages of a type of diet that adheres to the principles of the traditional Mediterranean one on health were mentioned in a recent meta-analysis in which greater adherence to a Mediterranean-style diet was associated with longer survival, reduced risk for cardiovascular mortality and cancer incidence and mortality (Sofi F. et al., 2008). A growing body of evidence has then emerged and the concept of MD as a healthy eating model has been widely recognized (Roman B. et al., 2008).

Previous observational studies already indicated that specific food or nutrient that take part of the traditional MD (i.e. fish, unsaturated fatty acids, antioxidant as vitamin E, vitamin B12, folates, carotenes, flavonoids, moderate alcohol) may have potential protective effect against dementia or cognitive decline (Kroger E. et al., 2009).

However, these results about isolated nutrient or food seem so far conflicting (Devore E. E. at al., 2009). Compared with traditional single-food or nutrient methods, the dietary pattern approach is appealing for several reasons. Indeed, the analyses of single nutrients ignore important interactions (additive, synergistic or antagonist effects)

between components of diet and more importantly people did not eat isolated nutrients (Kant A. K. et al., 2004).

Different biological mechanisms could be evoked to explain the association between foods or nutrients of the MD and potential better brain health. Oxidative damages have been implicated in the pathogenesis of AD, and the MD is also a dietary pattern with antioxidant properties (Dai J. et al., 2008).

Natural products (fruits and vegetables) are rich in diverse anti-oxidative components, including vitamins and essential minerals. Such balanced dietary formulas may play a beneficial role to prevent AD. Although the mode of action and targets of the dietary anti-oxidants remains unclear, multiple anti-oxidative species found in fruits and vegetables have been shown to activate a multitude of cell protective pharmacological pathways and offer beneficial roles in mitigating age-related pathologies.

Flavonoids and polyphenols are major anti-oxidative molecules found in fruits and vegetables, and a daily healthy diet is highly attractive as a potential preventive therapeutic approach against degenerative diseases. Curcumin is a powerful anti-oxidative polyphenol known as a beneficial agent in dozens of aging related degenerative diseases, including AD (Ringman J. M. et al., 2005).

Increasing evidence shows that hundreds of different flavonoids and polyphenols potently scavenge reactive oxygen species (ROS) and reactive nitrogen species (RNS) and play beneficial roles in aging related degenerative diseases (Dierckx N. et al., 2003). In vitro studies show the protective effects of multiple flavonoid (e.g., bi-flavonoids) and polyphenol (e.g., tannins) containing compounds in AD (Thapa A. et al., 2011). However, larger flavonoids or polyphenols may have poor blood brain permeability. It is also possible that in vivo degradation of products of these large molecules may function as individual flavonoid or polyphenol moiety (Mori T. et al., 2012). Phenolic groups found in curcumin as individual, ferulic acid, and styryl benzene are also strong anti-oxidants (Picone P. et al., 2013). These suggest multiple polyphenol and flavonoid groups containing natural anti-oxidants may be better and more efficient anti-oxidant molecules (Thapa A. and Carroll N. J., 2017).

Moreover, inflammation is another mechanism involved in the pathogenesis of AD which is in general reduced with higher adherence to the MD (Panagiotakos D. B. et al., 2009).

Polyphenols have been shown to induce hypoacetylation of RelA/p65 inhibiting the activity of HAT leading to the down-regulation of NF-kB function associated to the

inflammatory response. Moreover they could induce protein deacetylase activity such as HDAC2/SIRT1 wich inhibits transription of pro-inflammatory genes (Chung S. et al., 2010).

In an another recent study it has been shown that resveratrol inhibits inflammatory cytochines expression in response to lipopolysaccharide challenge in rat lungs (Sangwoon C. et al., 2010).

Furthermore, the expression of various genes encoding other pro-inflammatory factors, such as COX-2, MMPs, adhesion molecules, and inducible nitric oxide synthase (iNOS) can be significantly inhibited by resveratrol, quercetin, curcumin and catechins via down-regulating NF-kB and AP-1 transcription factors (Biesalski H. K. et al., 2007).

6.4 AD-diabetes relation and MD

Recent studies suggest that AD may be linked to brain insulin resistance, a key alteration in prediabetes and diabetes mellitus (reviewed in Biessels G. J. and Reagan L. P., 2015), and patients with diabetes are at increased risk of developing AD as compared to healthy individuals. Indeed, insulin resistance, increased inflammation, and impaired metabolism are key pathological features of both AD and diabetes (reviewed in Bitra V. R. et al., 2015; Bedse G. et al., 2015), and the AD–diabetes relation has led to propose some AD symptoms as a type 3 brain diabetes (reviewed in De La Monte S. M., 2014). Recently it has been reported antidiabetic effects of many polyphenols, including those found in the EVOO (Rigacci S. and Stefani M., 2016), that can provide possible explanations of their efficiency against aging-related neurodegeneration (Casamenti F. and Stefani M., 2017). Diabetes, notably type 2 diabetes (T2DM), is a condition closely associated with obesity and cardio vascular disease and these states concur with the so-called metabolic syndrome; the latter also includes non-alcoholic fatty liver disease, a condition whose severity spans from simple triglyceride accumulation in the liver parenchyma (steatosis) to non-alcoholic steatohepatitis. T2DM and other pathological states associated with deregulation of carbohydrate and lipid metabolism can be positively targeted by olive oil polyphenols.

The positive outcome of the administration of oleuropein aglycone (OLE) and other olive polyphenols against derangement of carbohydrate metabolism is supported by many studies.

The reported anti-diabetic effects comprise: (i) the inhibition of the amylin tendency to aggregate into amyloid, whose toxic deposits in the pancreatic β-cells are considered to

affect cell viability in T2DM (Rigacci S. et al., 2010); (ii) the reduction of serum glucose and cholesterol levels with restoration of the antioxidant perturbations in rat (Sangi S. M. A. et al., 2015) and rabbit (Al-Azzawie H. F. and Alhamdani M. S., 2006), models of diabetes; (iii) the modification of the expression of genes implicated, among others, in lipogenesis, thermogenesis and insulin resistance in high-fat-diet mice (Shen Y. et al., 2014); (iv) the reduction of the digestion and intestinal absorption of dietary carbohydrates both in the mucosal and in serosal sides of the intestine of diabetic rats (Sangi S. M. A. et al., 2015), together with the improvement of glucose homeostasis with a reduction of glycated hemoglobin and fasting plasma insulin levels in humans (De Bock M. et al., 2013); (v) a significant rise in insulin sensitivity and pancreatic β-cell secretory capacity in middle-aged overweight men (De Bock M. et al., 2013) with a measurable and rapid change of the expression of genes mechanistically related to insulin sensitivity and to the metabolic syndrome (Konstantinidou V. et al., 2009); (vi) the reduction of the metabolic activity, cardioprotection and prevention of inflammation and of cytokine-induced oxidative damage of pancreatic β-cells (Wang S. et al., 2014); (vii) the improvement of the antioxidant status in healthy elderly people (Oliveras-López M. J. et al., 2013) and the protection of insulin-secreting β-cells against H_2O_2 toxicity by modulating redox homeostasis with protection of cell physiology against oxidative stress (Cumaoğlu A. et al., 2011) ; (viii) the prevention of the inflammatory response and of cytokine-mediated oxidative cell damage with downregulation of the genes involved in adipocyte differentiation (Parzonko A. et al., 2013); (ix) the downregulation of Wnt10b inhibitory genes and upregulation of β-catenin protein expression as well as of key adipogenic and thermogenic genes in high-fat-diet mice (Drira R. et al., 2011) as well as of genes involved in galanin-mediated signaling; (x) the upregulation of genes involved in Wnt10b-signaling in C57BL/6N mice (Kuem N. et al., 2014) and (xi) the increase of signal molecules active in fasting conditions (IL-6, Insulin-like growth factor-binding protein 1 and 2, IGFBP-1 and IGFBP-2 (Al-Azzawie H. F. and Alhamdani M. S., 2006). These results provide a molecular basis for some of the benefits potentially coming from EVOO consumption and pave the way to further studies on the possible pharmacological use of OLE to prevent or to slow down the progression of type II diabetes.

6.5 Chemical composition of olive oil

Olive oil is composed for 98-99% from a saponifiable fraction consisting of triglycerides, diglycerides (2-3%) and monoglycerides (0.1-0.2%).

While this fraction is qualitatively the same for all the olive oils, it can change quantitatively. The remaining part (1-2%) is constituted by the unsaponifiable fraction that, even if present in small quantities, plays a very important role in the oil quality. This fraction consists of hydrocarbons such as squalene and waxes, tocopherols and tocotrienols, higher aliphatic alcohols, sterols, triterpenic and biterpenic alcohols, pigments such as carotenoids and chlorophylls, and phenols. Conversely, the un-saponifiable fraction is both qualitatively and quantitatively able to differentiate the olive oils both in organoleptic and nutritional properties.

6.5.1 Phenols

The phenolic compounds described by Cantarelli in 1961, are charachterized by a common feature a benzene ring that can then be attached to one or more hydroxyl groups and other functional groups such as glycosides, esters etc. Since then researchers have been highlighted the antioxidant properties of phenols in their studies. The concentration of phenolic compounds, in olive drupe, ranges between 1-3% of fresh pulp weight (Garrido F. A. et al., 1997).

Phenols can be divided in: phenolic acids, phenolic alcohols, flavonoids (flavones glycosides and anthocyanins), lignans and secoiridoids, which are present exclusively in the Oleaceae family (Servili M. et al., 2004). They are hydrophilic, but are present in the EVOO around water droplets thanks to their amphiphilic characteristics (Lozano-Sanchez J. et al., 2010). During the crushing and malaxation steps, esterases and glucosidase act on the phenol substrate, modifying the phenols profile (Romero-Segura C. et al., 2009).

In EVOO there are both (i) benzoic acids, such as vanillic acid, gallic acid, syringic acid, etc., and (ii) cinnamic acids, such as coumaric acid, ferulic acid, caffeic acid, etc.

Flavonoids consists of two benzene rings joined by a linear three-carbon chain. More than 6000 different flavonoids have been identified, and surely this number will increase (Ferrer et al., 2008). The different flavonoids have diverse biological functions, including protection against ultraviolet (UV) radiation and phytopathogens, signaling during nodulation, male fertility, auxin transport, as well as the coloration of flowers as a visual signal that attracts pollinators (Bradshaw H. D. and Schemske D. W., 2003).

Flavonoids are also responsible for the display of fall color in many plants, which may protect leaf cells from photooxidative damage, enhancing the efficiency of nutrient retrieval during senescence (Field T. S. et al., 2001). Flavonols are probably the most important flavonoids participating in stress responses; they are the most ancient and widespread flavonoids, having a wide range of potent physiological activities (Pollastri S. and Tattini M., 2011).These secondary metabolites, widely distributed in plants, are classified in six major subgroups: chalcones, flavones, flavonols, flavandiols, anthocyanins, and proanthocyanidins or condensed tannins and a seventh group is found in some species, the aurones that differ in the level of oxidation (Middleton E. Jr., 1998).

Secoiridoids, produced from the secondary metabolism of terpenes, are characterized by presence of elenoic acid (EA) or its derivatives, such as dialdehydic form of elenolic acid in their molecular structure, esterified with hydroxytyrosol or tyrosol, a phenil ethyl alcohol (Carrasco-Pancorbo A. et al., 2005). EA esterification with hydroxtyrosol (3,4 DHPEA) determine the formation of oleuropein (3,4-DHPEA-EA), while EA esterification with tyrosol (p-HPEA) determine the formation of ligstroside (p-HPEA-EA). Oleuropein is a major constituent of secoiridoids group, wich is an ester of hydroxytyrosol and the elenolic acid glucoside (Soler-Rivas C. et al., 2000). Oleuropein and ligstroside are mainly present in their glycosidic form in fruits while in the aglycon forms in EVOO, due to the enzymatic modifications occurring during crashing and malaxation. The aglycon forms can exist in a number of ketoenolic tautomeric equilibria involving the opening of the heterocyclic ring, yielding to compounds of different structures (Angerosa et al., 1996). The most abundant secoiridoids found in EVOO are the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA) or to tyrosol (p-HPEA-EDA), and an isomer of the oleuropein aglycon (aldehydic form of oleuropein or ligstroside aglycons) (Servili M. et al., 2004) such as 3,4DHPEA-EDA from oleuropein and demethyloleuropein and p-HPEA-EDA from ligstroside, respectively (Rovellini P. and Cortesi N., 2002).

The last group of phenols, lignans, are formed by combination of two phenylpropane units (Pereira D. M. et al., 2009). Two major lignans recognized in olive oil are (+)-pinoresinol and (+)-1-acetoxypinoresinol (Bendini A. et al., 2007).

6.5.1.1 Bioavailability and Metabolism of Olive Oil Phenolic Compounds

The majority of research regarding the bioavailability of olive oil phenolic compounds

has focused on three major phenolics: **hydroxytyrosol,** tyrosol, and **oleuropein**, and in general, **phenolics from EVOO** have been demonstrated to be readily bioavailable.

Hydroxytyrosol and tyrosol have been shown that are absorbed after ingestion in a dose-dependent manner (Visioli F. et al., 2000; Caruso D. et al., 2001) and that the bioavailability of hydroxytyrosol and tyrosol increased when administered as an olive oil solution compared to an aqueous solution (Tuck K. L. et al., 2001). The high antioxidant content of EVOO compared to water may protected the breakdown of phenolics in the gastrointestinal tract prior to absorption (Tuck K. L. et al., 2001) that for ligstroside-aglycone, hydroxytyrosol, tyrosol, and oleuropein-aglycone was as high as 55–66% in humans (Vissers M. N. et al., 2002). Tyrosol and hydroxytyrosol are polar compounds and their absorption seems to be via passive diffusion (Manna C. et al., 2000), while the polar but larger phenolic, oleuropein-glycoside may be absorbed via diffusion through the lipid bilayer of the epithelial cell membrane and be absorbed via a glucose transporter or by two other mechanisms such as the paracellular route or transcellular passive diffusion (Edgecombe S. et al., 2000).

A low quantity of phenolic components in urine after ingestion indicates that these phenolics are readily absorbed. In recent studies phenolics (mainly in the form of hydroxytyrosol and tyrosol) were determined to be between 30–60% and 20–22% of the total ingested (Visioli F. et al., 2000). It demonstrates that humans absorb a significant portion (~40–95%, using hydroxytyrosol and tyrosol as proxy) of phenolic compounds ingested (Vissers M. N. et al., 2002).

The metabolism of olive oil phenolic compounds is important in determining their availability. If phenolics are broken down and converted to other phenolics this may have a notable effect on their bioavailability. Phenolic compounds, oleuropein-glycoside and oleuropein and ligstroside-aglycones are converted to hydroxytyrosol or tyrosol and excreted in urine (Vissers M.N et al., 2002). Hydroxytyrosol and tyrosol themselves are sometimes conjugated to glucuronic acid and excreted in urine as glucuronides (Cicerale S. et al., 2010).

6.5.2 Oleuropein aglycone

Phenolic compounds are found in all parts of the olive plant, but their nature and concentration varies greatly between the various tissues. In Olea europaea, oleuropein, demethyl-oleuropein, ligstroside, and oleoside represent the predominant phenolic oleosides (Soler-Rivas C. et al., 2000), whereas verbascoside (Ryan D. et al., 1999) is

the main hydroxycinnamic derivative of the olive fruit (Servili M. et al., 1999). Oleuropein, the most phenolic compound of EVOO, can reach concentrations of up to 140 mg g−1 on a dry matter basis in young olives (Amiot M. J. et al., 1986) and 60–90 mg g−1 of dry matter in the leaves (Omar S. H., 2010).

Oleuropein is an ester of 2-(3,4-dihydroxyphenyl)ethanol (hydroxytyrosol, HT) and has the oleosidic skeleton that is common to the secoiridoid glucosides of Oleaceae (Soler-Rivas C. et al., 2000), mainly in its aglycone form, which makes the sugar moiety insoluble in oil (Fig.12).

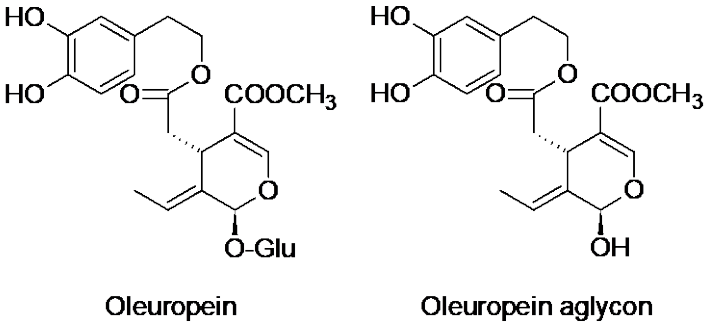

Fig. 12 *Chemical structures of Oleuropein and OLE.*

6.5.2.1 Neuroprotective activity of Oleuropein

It has been reported that oleuropein prevents Aβ aggregation in AD patients. In our laboratory we report that dietary supplementation of OLE strongly improves the cognitive performance of young/ middle-aged TgCRND8 mice, a model of amyloid-ß deposition (Grossi C. et al., 2013).

Recent data show that OLE given orally for eight weeks to TgCRND8 mice, a model of Aβ deposition, downregulates HDAC2 (Luccarini I. et al., 2015), an enzyme known to be upregulated in AD (Graff J. et al., 2012). In these mice, the downregulation of HDAC2 resulted in a significant increase in the level of histone acetylation, in particular of H3 at K9 and of H4 at K5. Histone acetylation has been reported to improve cognitive deficits in animal models of AD and its indication is considered a promising novel therapeutic strategy against AD (Adwan L. et al., 2013).

It is also shown that OLE could provide a protective and therapeutic effect against a number of pathologies, including AD as well as obesity, type 2 diabetes, non-alcoholic hepatitis, and other natural or experimentally-induced pathological conditions. Such a protection could result, at least in part, in a remarkable improvement of the pathological

signs arising from stress conditions including oxidative stress, an excessive inflammatory response, and the presence of cytotoxic aggregated material. In particular, recent studies highlihted OLE neuroprotection suggesting that it could also play a therapeutic role against AD (Casamenti F. et al., 2015).

6.5.3 HT

HT (Fig. 13) is one of the main phenolic components of olive oil. It is present in the fruit and leaf of the olive (*Olea europaea L.*) mainly as a product of oleuropein hydrolysis, the other product being elenolic acid. HT was early identified as the strongest in vitro anti-oxidant potential among all the olive oil polyphenols (Visioli F. et al., 1998). The content of this compound is largely dependent on the oil quality. Amounts range from 2 mg/Kg to 14 mg/kg in EVOO. (Romero C. et al., 2004).
HT in oil is found free, in acetate form or as a part of more complex compounds like oleacein, oleuropein, and verbascoside (Boskou D. et al., 2008).

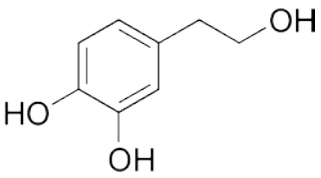

Fig. 13 *Chemical structure of Hydroxytyrosol.*

Oleuropein is responsible for the bitter taste of olives and decreases as the fruit ripens turning into unglycosylated form, OLE by enzymatic hydrolysis, and finally converted into HT, being this one an indicator of maturation of the olives (Charoenprasert S., et al., 2012) (Fig. 14)

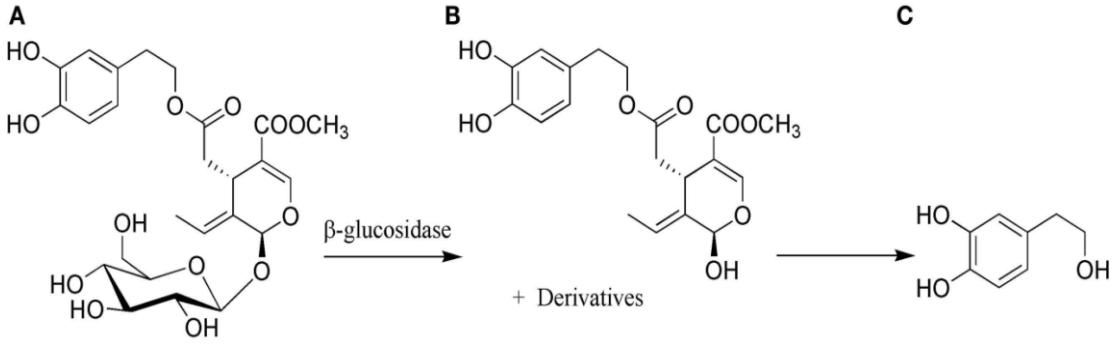

Fig. 14 *Oleuropein (A), OLE (B), and HT (C).*

HT, has a molecular weight of 154.16 is a white powder with melting point around 55°C and is fairly soluble in water and polar organic solvents such as low-molecular

weight alcohols. Like other polyphenols, it is easily oxidized in aqueous solutions unless adequate precautions, acid pH, and absence of oxidants, are taken.

Another natural source of HT is red wine (Fernandez-Mar M. I. et al., 2012), although the concentrations found are lower than those found in olive oil.

6.5.3.1 Absorption and Bioavailability of HT

The term bioavailability is commonly used to indicate the proportion of a substance that reaches the systemic circulation after oral administration considering both its absorption and its local metabolic transformation.

There are several assays dealing with the absorption of olive oil phenolic compounds both in animals (Bai C. et al., 1998; Tuck K.L. et al., 2000) and human beings. Visioli et al. (Visioli F. et al., 2000) found a high correlation for tyrosol and HT in human urine respect the ingested of different phenolic amounts in olive oil. The urine hydrolyzed using glucuronidase enzyme revealed the high grade of conjugation of HT and an increasing conjugation degree in higher phenolic content oils was found. Researchers has also demonstrated that the absorption of HT was 121.1% when was administered as a maintenance dose of 25 ml/day of olive oil for 1 week (Tuck K. L. et al., 2000).

Related to phenolic compounds absorption, the influence of the delivery vehicle seems to be crucial. In human beings, HT absorption was higher when subjects ingested EVOO than in refined oil (phenol-free) enriched with phenolic compounds, or when HT was incorporated into a yogurt as functional food, with urinary recoveries of 44, 23, and 5.8%, respectively (Visioli F. et al., 2003). In this direction, clinical trials have demonstrated that the amount of HT in free form was undetectable being 98% in conjugated form, mainly glucuronides, in plasma, and urine (Miro-Casas E. et al., 2003). These data provided an idea of the bioavailability of hydroxytyrosol, to highlight that olive oil phenolics are well absorbed at the intestinal level, suggesting that the intestinal/hepatic metabolism of the ingested phenolics is extensive. Since the amount of free HT in plasma and urine is almost undetectable, some authors suggest to attribute the biological activity to metabolites of HT (Vilaplana-Pérez C. et al., 2014).

6.5.4 Andrographolide

Andrographis paniculata is an herbaceous plant of the Acanthaceae family, often cultivated in India, China, and other countries for medicinal purposes. Due to its

extremely bitter taste, it is often referred to as the “the king of bitters,” and is used as a bitter tonic in Ayurvedic and other traditionally known health care systems of India and many other Asian countries. Andrographolide, structurally a labdane diterpenoid, is quantitatively the major secondary metabolite of the plant, and it is now often considered to be the major bioactive constituent of the plant involved in its observed therapeutically interesting bioactivities (Johansson B. E. and Brett I. C., 2007). Although a very broad spectrum of therapeutically interesting pharmacological properties of *A. paniculata* extracts and pure andrographolide have now been revealed, many questions concerning their pharmacological targets and sites of actions still remain unanswered (Sidwell R. W. et al., 2005).

Apart from andrographolide, > 20 other structurally analogous diterpenoids as well as > 10 flavonoids have been isolated and pharmacologically characterized from diverse types of *A. paniculata* extracts currently commercialized for medicinal purposes (Chotpitayasunondh T. et al., 2005).

Andrographis paniculata contains diterpenes, lactones, and flavonoids. Flavonoids mainly exist in the root, but also have been isolated from the leaves. The aerial parts contains alkanes, ketones, and aledehydes. The major constituents in andrographis are diterpene lactones known as andrographolides. Four lactones Chuaxinlian A (deoxyandrographolide), B (andrographolide), C (neoandrographolide) and D (14-deoxy-11, 12-didehydroandrographolide) were isolated from the aerial parts. The leaf and stem extracts were assayed for the presence of glycosides, flavonoids, gums, steroids, terpenoids, tannins, saponins and phenolic compounds. The ethanol, acetone, methanol, petroleum ether and chloroform extracts of Kalmegh were screened for the presence of secondary metabolites.

It has been shown that *Andrographis paniculata* recovers spatial memory and cognitive decline in *Octodon degus* (degu, the only wild-type South American rodent that develops Alzheimer's-like pathology with age). In addition, andrographolide treatment recovers the synaptic transmission and reduces phosphorylated tau protein and amyloid aggregate maturation in aged degus (Rivera D. S. et al., 2016).

AIM OF THE STUDY

Mouse

- To study the effects of oleuropein aglycone (OLE), the main polyphenol present in extra virgin olive oil (EVOO) on the activation state of PARP1 and its crosstalk with SIRT1, both *in vitro* in N2a cells and *in vivo* in 6-month-old TgCRND8 mice fed, for 8 weeks, with a low fat diet supplemented with OLE (50 mg/kg of diet).
- To define whether the neuroprotective effects of EVOO consumption are due to the content of specific phenols, such as OLE, or they could also be provided by a mix of olive polyphenols or their metabolites. Accordingly, we investigated in the same transgenic mice the effects on cognitive functions and Aß burden of dietary intake for 8 weeks of the mix of polyphenols present in olive mill waste water or of pure HT administered at a total dose as high as the highest dose of OLE (50 mg/kg of diet) previously found to be effective.
- To check the effects of andrographolide coniugated with fluorescent albumin nanoparticles (NPs) *in vitro* on N2a cells viability and *in vivo* on behavioral performance of TgCRND8 mice

Rat

- To check in adult rats injected with quisqualic acid (QA) into the right nucleus basalis magnocellularis (NBM) the ability of intravenously administered cholinergic neurons from human fetal NBM (hfNBMs) to migrate to the injected site and to ameliorate cognitive functions.
- To check the ability of intravenously administered solid lipid nanoparticles (SLNs) to cross the blood brain barrier (BBB) and to penetrate the central nervous system of adult rats.

MATERIALS AND METHODS

1. Mouse experiments

1.1 TgCRND8 mice

The TgCRND8 (Tg) mice express two mutated human APP genes implicated in AD (Swedish, KM670/672NL and Indiana, V717F) under the regulation of Syrian hamster prion promoter gene (Chishti et al., 2001). The mice were maintained on a hybrid (C57)/(C57/CH3) background by crossing transgenic heterozygous TgCRND8 males with wild-type (wt) female mice.

The main feature of this model is a very rapid development of amyloid pathology in the brain: amyloid β deposits start quite early since the two mutations involve both the β and γ secretase APP cleavage sites; all mice display amyloid plaques in the cortex and hippocampus by three months of age (Chishti et al., 2001). These neuropathologic manifestations are accompanied by impaired acquisition and learning deficits.

The transgenic mice were generated and supplied by the group of Dr. P St George Hyslop (Center for research in neurodegenerative Diseases, Toronto, Canada) and then the colony was bred in our animal house, Ce.S.A.L. (Centro Stabulazione Animali da Laboratorio), University of Florence.

1.2 Genotyping

Transgenic CRND8 mice were identified by DNA extraction from tail samples and PCR analysis using specific primers (5'-aggactgaccactcgacca-3' and 5'-tggattttcgtagccgttc-3') for the human mutated (KM670/672NL+V717F) cDNA insert (Bellucci et al., 2006).

The primers directed against the insert used the following program: lid heating to 105°C, five minutes at 99°C in order to degrade any DNAses, then five minutes at 95°C followed by 35 cycles: each cycle involved 1 min at 95°C, 1 min at 52°C and 1 min at 72°C. After cycles were completed, samples were held at 72°C for an additional 5 minutes and then were stored at 8°C.

In order to check the presence of the transgene, the PCR product was run in a gel electrophoresis using 0.5X Tris/borate/EDTA buffer and 2% agarose gel with etidium bromide (Sigma). DNA was visualized on a UV lightbox imaged with a camera. Only in transgenic samples a single band between 700 and 800 bp is detected.

1.3 Oleuropein deglycosylation

Oleuropein (Extrasynthase, Genay Cedex, France) deglycosylation was performed according to Konno et al. (Konno et al., 1999) with minor modifications. Briefly, a 10 mM solution of glycated oleuropein in 0.1 M sodium phosphate buffer, pH 7.0, was incubated with 7.55 I.U./mL of ß-glycosidase overnight at room temperature in the dark. The reaction mixture was centrifuged at 10,000 × g for 15 min to precipitate the aglycone. The complete oleuropein deglycosylation was confirmed by assaying the glucose released in the supernatant with the Glucose (HK) Assay Kit (Sigma-Aldrich, Milan, Italy). GC-MS analysis showed the absence of any oleuropein in the precipitate and the substantially total recover of OLE in the same precipitate. A 50 or 100 mM OLE stock solution in DMSO was stored protected from light; the solution was diluted immediately before use in seed oil for mice feeding.

1.3.1 Oleuropein aglycone treatment

Since several beneficial effects of OLE have been reported (Rigacci et al., 2010; Diomede et al., 2013), we decided to test its effect on a transgenic mouse model of AD by oral administration of a diet containing OLE.

After deglycosylation, the 100 mM OLE stock solution was protected from light, stored at -20°C and diluted in oil immediately before diet supplementation.

4-month-old TgCRND8 and wt mice were used (n=6/group/genotype, equally divided for sex). The mice were treated for 8 weeks with a modified low-fat (5.0%) AIN-76A diet (10g/day per mouse) composed of 50.0% sucrose, 5.0% fat, 20.0% casein, 15.0% corn starch, 5.0% powdered cellulose, 3.5% AIN-76 mineral mix, 1.0% AIN-76A vitamin mix, 0.3% DL-methionine, and 0.2% choline bitartrate (Piccioni, Milan, Italy), either as such (untreated mice) or supplemented with OLE (50mg/kg of diet) (OLE-fed mice) (Grossi C. et al., 2013; Luccarini I. et al., 2015). In addition, a group of 1.5-month-old OLE-treated TgCRND8 mice and a group of untreated TgCRND8 or wt mice (n=6/group/genotype, equally divided for sex) was used for immunohistochemical and western blotting analysis of PAR polymers only. Paraffin embedded brain slices of 6-month-old OLE-fed wt mice (Grossi C. et al., 2013) were employed for the analysis of PAR immunoreactivity reported in Fig. 19.

1.4 Polyphenols extract

Polyphenols extract was obtained from a concentrate of olive mill waste water. The concentrated solution was obtained by an innovative technology introduced by Alfa Laval and named Blue Vap. Blue Vap is an evaporator-condenser system used to process olive mill waste water in order to produce about 80% of the influent flow as condensate and the remaining 20% as concentrate. Obtained condensate is highly biodegradable and could therefore be treated by biological secondary treatment reactors, such as SBR or CWs, in order to meet the quality target for discharge (Masi et al., 2015). The concentrate is normally mixed with the olive mill pomace before further conventional collection to centralised treatment plants (usually operating further oil extraction and combustion to produce energy).

The polyphenols content was evaluated by HPLC with an Agilent Technologies model 1100 system according to Servili M. et al., 2011 and identified as hydroxytyrosol (3,4 DHPEA, HT) (8.4 ± 0.6 g/kg), thyrosol(p-HPEA) (4.7± 0.04 g/kg), verbascoside (58.7±1.6 g/kg) and OLE di-aldehyde (3,4-DHPEA-EDA) (12.6± 0.5 g/kg).

1.4.1 Polyphenols treatment

4-month-old Tg mice (n = 6/group/genotype, equally divided for sex) were used for diet treatment. As we have reported for OLE treatment, the animals were treated for 8 weeks with a modified low-fat (5.0%) AIN-76A diet (10 g/day per mouse) supplemented with a mix of polyphenols contained in an extract from olive mill concentrated waste water, at 50 mg/kg of diet. The animal were divided into two groups: i) Tg mice fed with low-fat diet as such (untreated mice); ii) Tg mice fed with the same diet supplemented with polyphenols in the olive mill waste water extract at 50 mg/kg of diet (kindly provided by Alfa Laval, Italy). Polyphenols treatment was well tolerated and no treated animals died.

1.5 HT extract

As described above for polyphenols, HT extract was obtained from a concentrate of olive mill waste water by the innovative technology introduced by Alfa Laval.

HT was evaluated by HPLC with an Agilent Technologies model 1100 system according to Servili M. et al., 2011 and identified as HT (712,3±14,3mg/g) (3,4 DHPEA, HT) and at a lower concentration as thyrosol(p-HPEA) (1,6±0,4 mg/g).

1.5.1 HT treatment

As we reported for polyphenol treatment were used 4-month-old Tg mice (n = 6/group/genotype, equally divided for sex) and treated for 8 weeks with a modified low-fat (5.0%) AIN-76A diet (10 g/day per mouse) supplemented with HT, at 50 mg/kg of diet. The animal were divided into two groups: i) Tg mice fed with low-fat diet as such (untreated mice); ii) Tg mice fed with the same diet supplemented with HT at 50 mg/kg of diet (kindly provided by Alfa Laval, Italy). Also in this way, HT treatment was well tolerated and no treated animals died.

1.6 Andrographolide coniugate with fluorescent albumin NPs (NPs-ANDRO) treatment

4-month-old Tg mice (n = 6/group/genotype, equally divided for sex) were treated intraperitoneally (IP), 3 times a week, with 4.0 mg/kg NPs-ANDRO dissolved in saline. According to body weight, the dose to be administered intraperitoneally was recalculated. The treatment was well tolerated and no treated animals died.

1.7 Body weight

The animals were weighted once a week during the treatment; body weight was slightly, not significantly, increased in all mice. The treatment was not toxic and animals were healthy.

1.8 N2a cell treatment and western blotting analysis

N2a murine neuroblastoma cells (European Collection of Authenticated Cell Cultures, ECACC) were plated in Eagle's minimum essential medium (MEM) supplemented with non-essential amino acids, 10% FCS (Sigma-Aldrich, Steinheim, Germany), antibiotics, and glutamine. The cells were maintained in a 5.0% CO_2 humidified atmosphere at 37 °C. To induce protein poly-ADP-ribosylation, the cells were incubated for 15 min with 100 μM of the PARP1 activator methylnitronitrosoguanidine (MNNG). After treatment for 24 h with OLE (100 μM) or with the PARP inhibitors 6(5H)-phenanthridinone (PHE, 30 μM) or N-(6-oxo-5,6-dihydrophenanthridin-2- yl)-(N,N-dimethylamino) acetamide hydrochloride (PJ-34, 20 μM), the cells were lysed in Laemmli buffer (60mM Tris–HCl, pH 6.8, 2% (w/v) SDS, 10mM EDTA, 10% (w/v) glycerol) and protein concentration was determined

with a BCA detection kit (Pierce, USA). The samples were added with β−mercaptoethanol and bromophenol blue, boiled for 10 min, clarified at 10,000 × g for 10 min, run on 8% SDS-PAGE, and transferred to PVDF membranes (Amersham GE Healthcare, Buckinghamshire, UK). After blocking with 5.0% (w/v) BSA in 0.1% (v/v) PBS- Tween-20, the membrane was incubated overnight at 4°C with specific primary antibodies (Abs) (Table 1). After washing, the membranes were incubated for 1.0 h with 1:10000 specific secondary Abs. The immunoreactive bands were detected with the Immobilon Western Chemiluminescent HRP substrate (Millipore, Bil- lerica, MA, USA) and quantified by densitometric analysis using a ChemiDoc system and the Quantity One software (Bio-Rad Laboratories, Italy).

1.9 N2a cell treatment and nucleotide measurement

N2a cells were plated in MEM, 10% FCS (FCS, Sigma-Aldrich), antibiotics, and glutamine. The cells were treated for 24h with 100 μM OLE or with 100 μM OLE plus 100 μM MNNG (90 min). The NAD+ levels were measured by means of an enzymatic cycling procedure according to Buonvicino D. et al., 2013. Briefly, the cells grown in a 48 well plate were killed with 50 μl of 1N $HClO_4$ and the mixture was neutralized by adding an equal volume of 1 N KOH. Then, 100 μl of cells extract was added with 50 mM bicine (50 μl) and mixed with an equal volume of bicine buffer containing 23.0 μl/ml ethanol, 0.17 mg/ml MTT, 0.57 mg/ml phenazine ethosulfate, and 20 μg alcohol dehydrogenase. The mixture was left standing at room temperature for 10 min before absorbance measurement at 550 nm.

1.10 MTT Assay

N2a cells were seeded into 96-well plates at a density of 5,000 cells/well in fresh complete medium and grown for 24 h. The cells were also treated with ANDRO at three different times: 48h, 24h and 4h a three different concentrations: 1 μM, 2μM and 3 μM. After 48 h, the culture medium was removed and the cells were incubated for 2.0 h in a 0.5 mg/mL MTT solution. Finally, cell lysis solution (20% (w/v) SDS, 50% (v/v) N,Ndimethylformamide) was added, and the multi-well plate was kept overnight at 37 °C in a humidified incubator. The blue formazan absorbance was measured at 570 nm using an automatic plate reader (BioRad) as reported in Rigacci et al., 2011.

1.11 Behavioral experiments

At the end of the diet treatment memory performance was investigated by three widely used behavioural tests.

TgCRND8 mice, as previously demonstrated in our laboratories by performing rotarod, hole platform and grip strength test, don't show any motor deficits or exploration impairment (Bellucci et al., 2006).

1.11.1 Morris Water Maze test

The Morris Water Maze (MWM) (Fig. 16) was first established by neuroscientist Richard G. Morris in 1981 in order to test hippocampal-dependent learning, including acquisition of spatial memory and long-term spatial memory (Brits-Bromley K. et al., 2011).

The water maze apparatus consisted of a circular pool (1.2 m in diameter and 0.47 m high) made of white plastic, as described by Chishti et al. 2001; and Janus et al. 2004. The pool was filled to a depth of 20 cm with water (24°C–25°C) that was made opaque by the addition of non-toxic white paint. During a conventional reference memory MWM training, an escape platform (10 cm in diameter), made of white plastic with a grooved surface for a better grip, was submerged 0.5 cm under the water level.

All mice underwent a reference memory training with a hidden platform placed in the centre of one quadrant of the pool (northwest) for four days, with four trials per day, with the four starting locations varied between trials. If the platform was not located within the maximum time of 60 s, the mouse was guided to the location. The mouse was allowed 20 s on the platform. Extra-maze visual cues around the room remained in fixed positions throughout the experiment. For each trial, latency to find the platform (maximum 60 s) was recorded by a video-tracking/computer- digitizing system (HVS Image, Hampton, UK). On day 4, five hours after the last trial, the platform was removed from the pool and each mouse received one 30 s swim "probe trial". The starting point was set in the south-east quadrant. Swim speed (cm/s), percentage of time spent in the target quadrant, and the number of platform area crossings, determined as one entrance and one exit from the area, were recorded (Grossi et al., 2009).

Fig. 16: *Morris Water Maze test.*

1.11.2 Step down inhibitory passive avoidance task

In this test, the animals learn to associate exploration of a compartment with a foot shock delivered through the floor grid. On subsequent exposure to the same environment, the mice will avoid stepping down or will increase the latency before ‘‘stepping down’’ onto the floor grid.

The term “passive avoidance” (Netto and Izquierdo, 1985) is used to indicate in vivo experiments in which the animal learns to avoid a specific event, usually concerned an innate behavior, that is dangerous due to a linked punishment.

A mouse in an open field naturally spends most of the time in dark places, as near walls and corners; when the mouse is placed on an elevated-platform in the center of the field, it will try to get off the platform,to explore the environment and go near the walls. Based on this natural behavior, several different variants of the step down inhibitory avoidance task have been used (Abdel-Hafez et al., 1998; Chorover and Schiller, 1965; Kubanis and Zornetzer, 1981; Zoernetzer et al., 1982)

In this study, the following procedure was used:

- the apparatus was an open field plexiglas box (40 × 40 cm) with a steel rod floor and a plexiglas platform (4 × 4 × 4 cm) set in the centre of the grid floor to which intermittent electric shocks (20 mA, 50 Hz) were delivered (Fig. 17).
- on day 1 (training test, TT), each mouse was placed on the platform and received an electric shock (1 Hz, 0.5 s, 40 V DC) for 3 s when it stepped down placing all paws on the grid floor (Hiramatsu et al., 1995) considering 180 s as the upper cut-off, then the mouse was put back into the cage.

Responsiveness to the punishment in the TT was assessed by animal vocalization; only those mice that vocalized touching the grid (about 70% of mice) were used for retention test (RT).

- 24 h after TT, each mouse was placed on the platform again (RT). The latencies were measured, considering 180 s as the upper cut-off. The test ended when the mouse stepped down or when the cut-off time was over. During retention test no electric shock was released.
- latencies recorded during TT and RT were compared: a latency longer in the RT than in TT meant the mouse learnt and could remember the punishment received the day before, otherwise the mouse showed memory impairment.

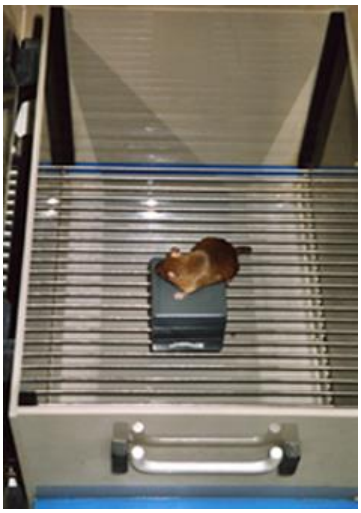

Fig. 17: *Step down inhibitory avoidance task.*

1.11.3 Object recognition test

The object recognition paradigm utilized the innate tendency of mice to explore a novel object over a familiar one. The test measures spontaneous behavior, and thus requires no length training or preparation (Ennaceur and Delacour, 1988).

Pre-training: To avoid neophobic interference a habituation phase preceded the testing. Habituation consisted in placing the mice for 5 min into a square open-field arena (60 x 50x 25 cm). During this phase the mice could explore freely the empty arena with one object inside (Fig. 18).

Testing: The object recognition task was conducted 1 day after the habituation phase and comprised two trials (T1 and T2). During the T1 the animals could explore two identical objects while in the T2 one object was replaced by a novel one. Each trial lasted 5 min, with an intertrial interval of about 60 min. All objects were made of a biologically neutral material such as plastic or metal, and animals could not move them around in the arena. Objects were not known to have any ethological significance for the mice and they never had been associated with a reinforcer (Ennaceur et al., 2005).

Fecal boli were removed after each tested animal.

Frequency and duration of object exploration was recorded by a video-tracking/computer-digitizing system (HVS Image, UK).

Exploration of an object was defined as directing the nose towards an object at a distance of less than a half head length and/or touching the object with the paws. Sitting on an object was not considered as exploratory behaviour. Mice without any exploration behaviour towards the objects were excluded from the analysis of learning behaviour.

To analyze the object recognition test, a discrimination score was calculated: discrimination score=exploration time of the novel object (s)/total exploration time of both objects (s). A discrimination score above 0.5 indicates the ability of mice to discriminate between the familiar and novel objects while a score below or equal to 0.5, reflecting a novel object exploration time less or equal to the half of the total time spent between the two objects, indicates memory impairment in this task. Mice with a total exploration time of less than 5 s in either the T1 or the T2 were excluded from the analysis of learning behaviour on this test day.

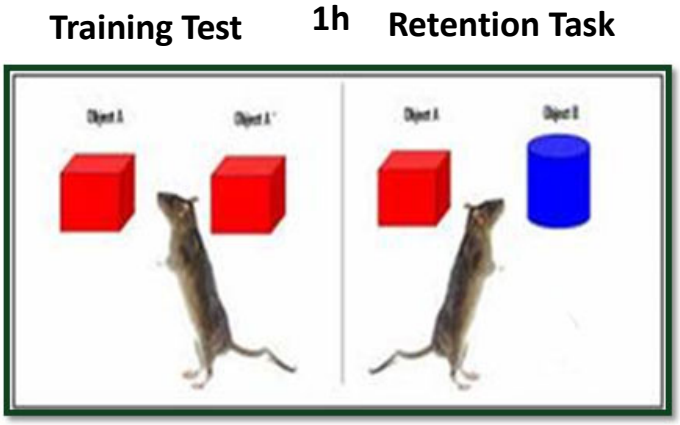

Fig. 18: *Object Recognition test (ORT).*

1.12 Animal tissue processing

After completing the behavioral tests, mice were sacrificed by cervical dislocation and the brains were rapidly removed and divided sagittally. For protein analysis, cortical and hippocampal samples from one hemibrain were immediately sectioned, snap-frozen in dry ice or liquid nitrogen and stored at -80 °C. The other hemibrain was postfixed in phosphate-buffered 4.0% paraformaldehyde, pH 7.4, at 4 °C for 48 h, rinsed in PBS and paraffin embedded for immunohistochemistry.

1.13 Immunohistochemistry

Immunohistochemical analyses were performed on 5.0 µm coronal paraffin-embedded sections obtained by microtome sectioning (Leica Microsystem, UK).

The sections were incubated overnight at 4 °C with the specific primary antibodies (Abs) (Table 1) diluted in 0.1 M PBS, pH 7.4, containing Triton X-100 (0.3%) and BSA (5.0 mg/mL). For pE3-Aß and Aß plaque identification, tissue sections were incubated for 1.0 h with the biotinylated secondary Ab (Vector Laboratories, Burlingame, CA, USA) diluted 1:1000 in 0.1 M PBS/BSA (1.0 mg/ml); immunostaining was visualized using the avidin-biotin system (Vectastain, Vector Laboratories, Burlingame, CA, USA) and 3,3'- diaminobenzidine plus Nickel (DAB Kit) (Vector Laboratories, Burlingame, CA, USA) as the chromogen.

Fluorescent immunohistochemistry experiments were carried out as previously reported (Bellucci et al., 2007). The sections were incubated for 1.0 h with blocking solution (BS) containing 0.25% Triton X-100, 5.0 g/L BSA and 5.0% normal horse serum in 0.1 M PBS, pH 7.4, and then overnight at 4 °C with the primary Ab (Table1). On day 2, the sections were incubated for 1.0 h in the dark with the appropriate fluorescent secondary Ab diluted 1:400 in BSA. For double immunostaining, the sections were incubated overnight with the second primary Ab

(Table 1) in the dark at 4 °C and, on day 3, for 1.0 h at room temperature with the second fluorescent Ab diluted 1:400 in BSA. The analysis of negative controls (not treated with the primary Ab) was simultaneously performed to exclude the presence of non-specific immunofluorescence staining, cross-immunostaining, or fluorescence bleed-through (Fiorentini et al., 2010).The sections were cover slipped using ProLong (Lyfe Technologies, New York, USA) mounting medium with or without DAPI for nuclear staining. Representative images were acquired by an Olympus BX600 microscope coupled to CellSens Dimension Imaging Software (Olympus, Milan, Italy).

1.14 Determination of Aβ plaque-load

To quantify Aβ plaque burden, cortices and hippocampi of the sections stained with an anti-N3pE (Ab against pE3-Aß) and an anti-Aβ42 antibody were digitized (Olympus BX600, Olympus, Germany) under constant light and filter settings. Six to seven coronal brain sections, each separated by 60 µm interval, from each mouse (4-5 animals per group) were analyzed.

Morphometry was conducted by using CellSens Dimension software (Olympus, Germany). Colour images were converted to greyscale by extracting blue to grey values to obtain best contrast between positive immunoreactivity and background. A constant threshold was chosen for all images to detect immunoreactive staining. Plaque number, size (maximum area, minimum area) and total area were determined automatically in the cortices and hippocampus. Brain regions were based on the Paxinos and Franklin mouse brain atlas (2008). Data from the six-seven sections were summed to derive representative values for each animal for the total plaque area. Data were expressed as mean ± S.E.M.

1.15 Western blot

The western blot is an analytical technique used to detect specific proteins in the given sample of tissue homogenate. A gel electrophoresis is used to separate denaturated proteins by the length of the polypeptide; proteins are then transferred to a membrane, where they are stained with antibodies specific to the target protein. Proteins were then visualized using specific antibodies and a chemiluminescence detection system.

For western blotting analysis, TgCRND8 and wt tissue brain samples were homogenized in ice-cold RIPA lysis buffer composed by:

-2X lysis buffer (50 mMTris-HCl pH 7.5; 50 mMNaCl; 10 mM EGTA; 5 mM EDTA);

- Phosphatase inhibitors (2 mMNaPP; 4 mM PNFF; 1 mM Na_3VO_4);

- Proteases inhibitor (1 mM PMSF, 20 µg/ml leupeptin; 30 µg/ml aprotinin).

The samples were homogenized in 100 µl of RIPA buffer, then centrifuged for 15 min at 12,000 rpm and supernatant was recovered and used for the next steps.

In order to detect the amount of proteins in each sample, a protein assay was performed. Dilutions ranging from 1µg/ml to 0.075 µg/ml of BSA were prepared to outline the standard curve of reference. Into a 96-multi well plate 4µl of each sample (blank, BSA dilutions and supernatant of the homogenized tissues) and 200 µl of Bradford reagent (Bio Rad, Hercules, CA) were put in each well: the Bradford colouring depending on the amount of proteins, became from deep to light blue. After 10 min incubation at RT, samples were assayed with a spectrophotometer associated a computer (E1800, Universal Microplate Reader, Bio-Tex Instruments, Inc). Samples absorbance was detected and the standard samples were useful to correlate absorbance measurement detected to known proteins amount; comparing the absorbance values obtained with the standard curve, the amount of protein in unknown samples could be extracted.

Before loading on a gel, loading buffer (300 mMTris-HCl pH 6.8; 12% SDS; 60% glycerol; 0.6% bromophenol blue; 600 mM DTT; H_2O) was added to unfold and charge negatively the proteins: the samples were boiled for 10 min at 100°C in order to denature the proteins, then they were placed on ice, briefly centrifuged to spin down the samples to the bottom of the tubes, and finally loaded on a gel.

Then 40 µg of proteins were applied to 4-12% Criterion XT Bis-Tris Gel (Novex NuPAGE, Lyfe Technologies, New York, USA) for electrophoresis with XT MES running buffer (Lyfe Technologies, New York, USA). The separated proteins were transferred onto 0.2 µm nitrocellulose membrane by the iBlot dry blotting system (Invitrogen, New York, USA) and incubated overnight at 4 °C with the specific primary Ab (Table 1). The day after, the membrane was washed three times for 15 minutes with TBST and incubated for 1.0 h with the HRP-conjugated secondary Ab (Thermo Scientific, Waltham, MA, USA) diluted 1:4000 in BSA. The immunocomplexes were visualized using enhanced chemiluminescence (Immobilon

Western, Millipore, Billerica, MA**,** USA) and acquired using the ImageQuant 350 system (GE Healthcare, Buckinghamshire, UK). Densitometric band analysis was performed using the Image Quant TL software (GE Healthcare, Buckinghamshire, UK). All primary Ab concentrations were titrated to provide optimal staining.

The membrane could then be reused to perform further experiments: it could be put in 10 ml of stripping buffer (Bio rad, Hercules, CA) shacking thoroughly for 15 min and after a 10 min wash with H_2O it's ready to be used again to determine house-keeping proteins levels, like β-actin or GAPDH, necessary to normalize the samples levels detected and to check the amount of proteins loaded.

The normalized data were collected and the statistical analysis was performed using Graph Pad (Graph Pad Prism Software version 4.0, San Diego, CA, USA).

1.16 Real-time PCR for PARP1

Total RNA was isolated from wild type or transgenic mouse treated or not with OLE, as described above, by the RNeasy mini kit (Qiagen, Germany), subjected to RNase-free DNase digestion (Ambion, USA) and analyzed spectroscopically. 1.0 μg of RNA was retro-transcribed using iScriptTM (Bio- Rad, USA) and amplified with specific primers, as described below. For mouse PARP1 mRNA: forward 5'-CACTCTGCACACGTCCTGGAGAAAG-3', reverse 5'-GGAGGGCATCTGCTCAAGTTTGTT AC-3'; for 18S rRNA: forward 50-AAAACCAA CCCGGTGAGCTCCCTC-30, reverse 50-CTCAGG CTCCCTCTCCGGAATCG-30. All primers were purchased from IDT (IDT, Germany). PCR amplification was performed by means of SSOADV UNIVER SYBR SMX (Bio-Rad, USA) according to manufacturer instruction. PCR amplification of 18S rRNA was used as the normalizer. Real-time PCR assays were performed using the Rotorgene 6000 cycler system (Qiagen, Germany).

1.17 Reverse transcript and Real-Time PCR for TNF-α

Total RNA from tissue was isolated using Trizol Reagent (Life Technologies) and DNase treated to removal genomic DNA contamination. One microgram of RNA was retro-transcribed using iScript (Bio-Rad) and amplified with the following specific primers for 18S forward 5_-AAAACCAACCCGGTGAGCTCCCTC-3_and reverse 5_-CTCAGGCTCCCTCTCCGGAATCG-3_and for TNF-Alpha forward 5'-GCATGATCCGCGACGTGGAACT-3' and reverse 5'-

CGAATGAGAGGGAGGCCATTTGG-3'. All primers were purchased from IDT (IDT, Germany). PCR amplification was carried out by means of SsoAdvancedTMUniversal SYBR®Green Supermix (Bio-Rad, USA) according to manual instruction, using a RotorGene 3000 (Qiagen) Instrument. The RT-qPCR was performed using the following procedure: 98◦C for 1 min, 40 cycles of 98◦C for 5 s, 60◦Cfor 10 s. The program was set to reveal the melting curve of each amplicon from 60◦C to 95◦C with a read every 0.5 °C.

1.18 Data Analysis

One-way ANOVA, followed by Bonferroni's post-hoc test was used to analyse MWM, ORT and step down data. Unpaired two tailed Student's t-test was used to analyse pE3-Aß and Aß42 plaque burden. Statistical analyses were carried out with GraphPad Prism version 5.0 for Windows (San Diego, CA, USA) and the statistical significance was defined as $P < 0.05$. Data are reported as mean values ± standard error of the mean (S.E.M).

Table 1: Antibodies employed in the study

Antibody	Specific	Dilution		Antigen retrieval for IHC	Host	Source
		WB	IHC			
Aβ42	Aß peptide, aa 1-42	ND	1:200		Rabbit	Millipore, Billerica, MA**,** USA
GFAP	Glial fibrillary acidic protein	ND	1:500		Rabbit	Dako, Glostrup, DK
LC3	microtubule-associated protein light chain 3	ND	1:200	Citrate Buffer pH=6	Rabbit	Novus Biologicals, Littleton, CO, USA
NeuN	Neuronal nuclei	ND	1:100	Citrate Buffer pH=6	Mouse	Millipore, Billerica, MA**,** USA
pE3-Aß (N3pE)	Third N-terminal pyroglutamate residue on Aß	ND	1:200	Citrate Buffer pH=6	Rabbit	IBL International, Hamburg, D
α-tubulin (HRP-conjugated)	α-tubulin	1:5000	ND		Mouse	Proteintech, Chicago, IL, USA
Anti-Poly (ADP-ribose) [PAR]	Poly(ADP-ribose	1:1000	1:100	Citrate Buffer pH=6	Mouse	AdipoGen
Anti-Poly (ADP-ribose) [PAR]	Poly(ADP-ribose	ND	1:200	Citrate Buffer pH=6	Rabbit	BD Pharmingen
PARP	PARP-1	1:1000	ND		Rabbit	Cell Signaling
Phospho-NF-kB p65 (Ser536)	Phospho-NF-kB p65 (Ser536)	1:1000	1:100	Citrate Buffer pH=6	Rabbit	Cell Signaling
NF-kB p65	NF-kB p65	1:1000	ND		Rabbit	Cell Signaling
Phospho-p53 (Ser46)	Phospho-p53 (Ser46)	1:1000	1:50	Citrate Buffer pH=6	Rabbit	Cell Signaling
p53	p53	1:500	ND		Rabbit	Santa Cruz

SIRT1	SIRT1	1:400	ND		Rabbit	Proteintech
ß-actin	ß-actin	1:1000	ND		Mouse	Santa Cruz
LAMP-2A	LAMP-2A	ND	1:200	Citrate Buffer pH=6	Rat	Santa Cruz
HSP70	HSP70	1:1000	ND	Citrate Buffer pH=6	Mouse	Santa Cruz
TNF-α	TNF-α	ND	1:1000	Citrate Buffer pH=6	Rabbit	Agilent Technologies
Beclin1	Beclin1 protein	ND	1:200	Citrate Buffer pH=6	Rabbit	abcam, Cambridge, UK

2. Rat experiments

2.1 Animals

For experiments described in 2. and 3. three- and one-month-old, respectively, male Wistar rats (Harlan, Milan, Italy) (n=5-6/group and 3-4/group, respectively) were used. Animals were housed in macrolon cages with ad libitum food and water and maintained on a 12-h light/dark cycle at 23 °C. All experiments were carried out according to the EC Directive 86/609/EEC for animal experiments and National guidelines for animal care (Permit Number: 567/2015-PR). All efforts were made to minimize the number of animals used and their suffering.

2.2 Quisqualic acid injection into the nucleus basalis magnocellularis and treatment

0.5 μL of 0.12 M quisqualic acid (Sigma, Milan, Italy), dissolved in 50 mM sodium phosphate buffer saline (PBS) (pH 7.4) or 0.9% saline was injected into the right nucleus basalis magnocellularis (NBM) of the basal forebrain at the following stereotactic coordinates: AP= -0.2; L= - 2.8 and H= 6.8 from Bregma (Paxinos and Watson, 2006) (Baglioni et al., 2006) of anesthetized (Zoletil, 65 mg/kg, intraperitoneously (ip), plus Carprofen, 5 mg/kg, subcutaneously) rats. The animals were equally divided into the following five groups:

Group I: injected into the NBM with 0.5 μL of quisqualic acid, treated with Ciclosporine (2 mg/kg/day) (Wennersten et al., 2006), one day prior the intravenous (i.v.) administration by the tail vein of 300 μl of 1.5ML hfCNs and for all the lenght of the experiment. The animals were sacrificed at 1, 7 and 21 days after hfCNs administration. Group II: injected into the NBM with 0.5 μL of quisqualic acid and sacrificed 21 days after injection. Group III: injected into the NBM with 0.5 μL saline, treated with Ciclosporine (2 mg/kg/day), one day prior the i.v. administration by the tail vein of 300 μl of 1.5M hfCNs and for all the lenght of the experiment. The animals were sacrificed 21 days after hfCNs administration. Group IV: injected into the NBM with 0.5 μL saline; Group V: un-injected rats. For behavioral experiments groups IV and V were pooled (Control).

2.3 SLNs injection

To evaluate the ability of solid lipid nanoparticles (SLN) to cross the BBB rats were acutely dosed with spherical and small-sized (200 nm) fluorescent nanoparticles

(SLN 28.57 mg/ml plus FITC 1.43 g/ml) or vehicle (0.9% NaCl) by intravenous injection by the tail vein. The animals were equally divided into the following groups: Group I: injected with 200 μL of nanoparticles and sacrificed after 3h ; Group II: injected with 200 μL of nanoparticles and sacrificed after 24h; Group III: injected with 200 μL of nanoparticles and sacrificed after 72h; Group IV: injected with 200 μL of 0.9% of saline.

2.4 Animal Tissue Processing

Twenty-four hours, seven and twenty-one days after injection, or quisqualic acid injection and three, twenty-four and seventy-two hours after SLNs injection, rats were deeply anesthetized (Zoletil, 40 mg/kg i.p.) and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4) at a flow rate of 10 ml/min for 30 min. After sacrifice, brains were quickly extracted and fixed in phosphate-buffered 4% paraformaldehyde (pH 7.4) for 48 hours (h) at 4°C. Subsequently brains were rinsed with PBS, dehydrated using an automated machine, and paraffin embedded. Coronal sections (5.0μm) were cut using a microtome and mounted on slides.

2.5 Immunohistochemistry

Sections were subjected to antigen retrieval by microwave incubation in 10 mM Na-citrate buffer, pH 6.0, and immunostained as above described for mouse. Polyclonal anti glial fibrillary acid protein (GFAP) (1:1000 dilution, Dako Cytomation), anti-choline acetyltransferase (ChAT) (1:200 dilution, Millipore), monoclonal mouse anti-CD34 (1:50, Dako Cytomation) and anti-IBA1 (1:200, Wako) antibodies (Abs) were used for rat experiments. Co-localization experiments were performed as previously reported (Fiorentini et al, 2010), sections were cover slipped using ProLong (Lyfe Technologies, New York, USA) mounting medium with or without DAPI for nuclear staining. An Olympus BX600 microscope coupled to CellSense Dimension Software (Olympus, MI, Italy) was used to acquire representative.

2.6 ChAT-positive cells count

ChAT-positive cells in the NBM were counted under a 10 × objective lens, using an Olympus BX600 microscope. Five sections per animal, anteroposterior standardized with respect to the injection site and spaced 50–100 μm from one another, were

analyzed. The total number of ChAT-positive cells in the QA injected NBM was averaged, expressed as a percentage of that counted in the saline-injected NBM, assumed as 100% (n=3/group), and analyzed using Prism 5.0 (GraphPad Software).

2.7 Behavioral experiments

2.7.1 Morris Water Maze test

During the third week after hCNs administration rats were tested in the Morris Water Maze (MWM) (n = 6/group). The MWM apparatus consisted of a circular pool (1.6 m in diameter and 0.36 m high) made of green plastic. The pool was filled to a depth of 20 cm with water (24-25 °C) that was made dark by the addition of non-toxic dark paint. Rats were tested in the reference memory version of MWM with the procedure previously described for mice (Grossi et al., 2009). Briefly, all rats underwent a reference memory training with a hidden platform (13 cm in diameter, submerged 0.5 cm under the water level), placed in the centre of the target quadrant of the pool for 4 days, with 4 trials per day. The 4 starting locations were varied between trials. Upon release into the water, the rat was allowed to search the platform for 60 seconds; if the platform was not located within 60 seconds, the rat was guided to the location. The rat was allowed 20 seconds on the platform. Extra-maze visual cues around the room remained in fixed positions throughout the experiment. Searches were recorded by a videotracking/computer-digitizing system (HVS Image, Hampton, UK).

2.7.2 Step-Down inhibitory avoidance task

The day after the end of MWM task the animals were tested in the Step-Down inhibitory avoidance task. The apparatus and procedures used for the “Step-Down” inhibitory avoidance test were above described for mouse with some modifications. The apparatus was an open field Plexiglas box (50 × 25 × 25 cm) with a steel rod floor and a plexiglas platform (5 × 8 × 25 cm) set on the grid floor to which intermittent electric shocks (20 mA, 50 Hz) were delivered. On day 1 (training test, TT), each rat was placed on the platform and received an electric shock for 3 s when it stepped down placing all paws on the grid floor. Responsiveness to the punishment in the TT was assessed by animal vocalization; only those rats that vocalized touching the grid (about 70% of mice) were used for retention test (RT). 24 h after

TT, each rat was placed on the platform again (RT). The latencies were measured, considering 180 s as the upper cut-off, during TT and RT.

RESULTS

1. OLE TREATMENT

In this study we investigated the effects of OLE *in vitro*, in N2a cells, and *in vivo*, in 6-month-old TgCRND8 mice fed, for 8 weeks, with a low fat diet supplemented with OLE (50 mg/kg of diet) on the activation state of PARP1 and its crosstalk with SIRT1. We also investigated whether the inhibition of the transcription of pro-inflammatory and pro-apoptotic proteins by OLE could underlie the beneficial effects of this polyphenol.

1.1 OLE reduces PARP1 activation in TgCRND8 mice

PARP1 activation generates PAR polymers (Heeres T. J. and Hergenrother P. J., 2007). So we checked for the presence of PAR polymers in the frontal cortex of untreated or OLE-fed 3.5- and 6-month-old TgCRND8 or wt mice. In both age groups, PAR immunoreactivity was higher in the cortex of TgCRND8 mice respect to wt mice, as shown in 6-month-old animals, whereas PAR polymers were markedly reduced in the cortex of 6-month-old OLE-fed Tg mice with respect to untreated Tg mice of the same age (Fig. 19 A). These data were confirmed by western blotting analysis, showing a significant increase in PAR polymer levels in the frontal cortex of 3.5- and 6-month-old Tg animals compared to wt mice of the same age (Fig. 19 B; One way ANOVA plus Bonferroni *post hoc* test *p<0.05), whereas in OLE-fed Tg animals of both ages PAR polymer levels were similar to those found in wt mice.

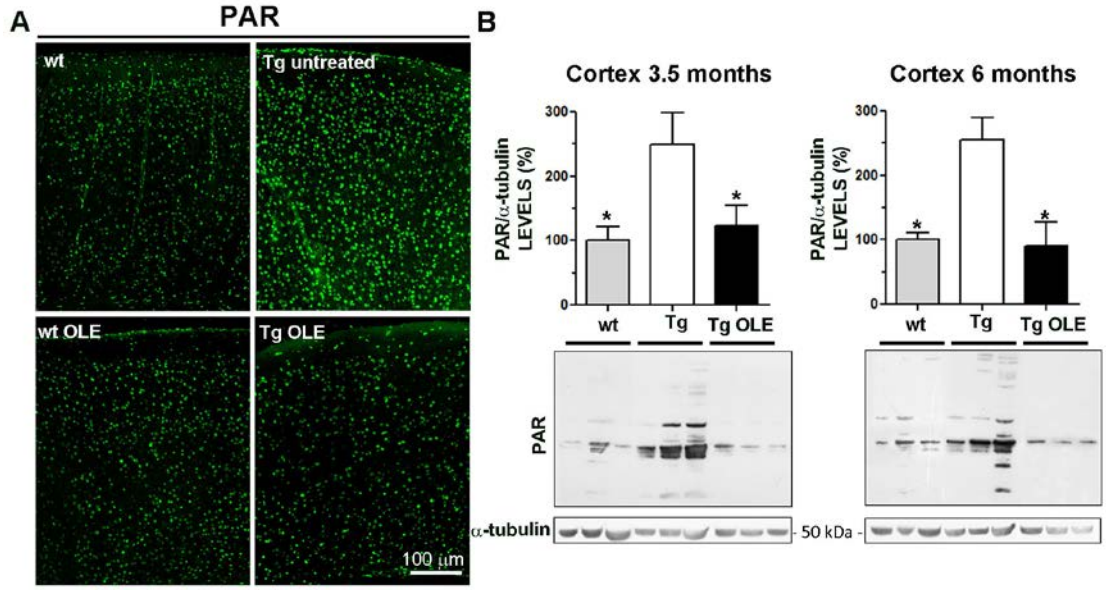

Fig. 19 OLE inhibits PARylation in TgCRND8 mice: *A) PAR immunoreactivity in the cortex of 6-month-old mice. B) Western blotting analysis of PAR protein levels in the cortex of 3.5- and 6-month-old untreated wt and Tg mice and OLE-fed Tg mice. n = 5-6 mice/group. One way ANOVA plus Bonferroni post hoc test*p < 0.05 vs Tg mice.*

Next, we analyzed the type of cells displaying accumulation of PAR polymers in double

immunofluorescence staining using antibody to NeuN (red) and to PAR (green) and antibody to GFAP (red) and to PAR (green) plus DAPI (blue). In Tg and wt animals, PAR immunoreactivity was found mainly within neuronal nuclei, as shown by the positive staining with NeuN antibody plus DAPI (Fig. 19 C), whereas it was also detected within a few astrocytes only in Tg groups, as shown by GFAP antibody staining (arrows in the merged images of Fig. 19 D). Notably, the number of PAR-immunopositive neurons detected in TgCRND8 mice was strongly reduced in OLE- fed Tg mice, confirming a possible inhibition of PARP1 activation by OLE (Fig. 19 C).

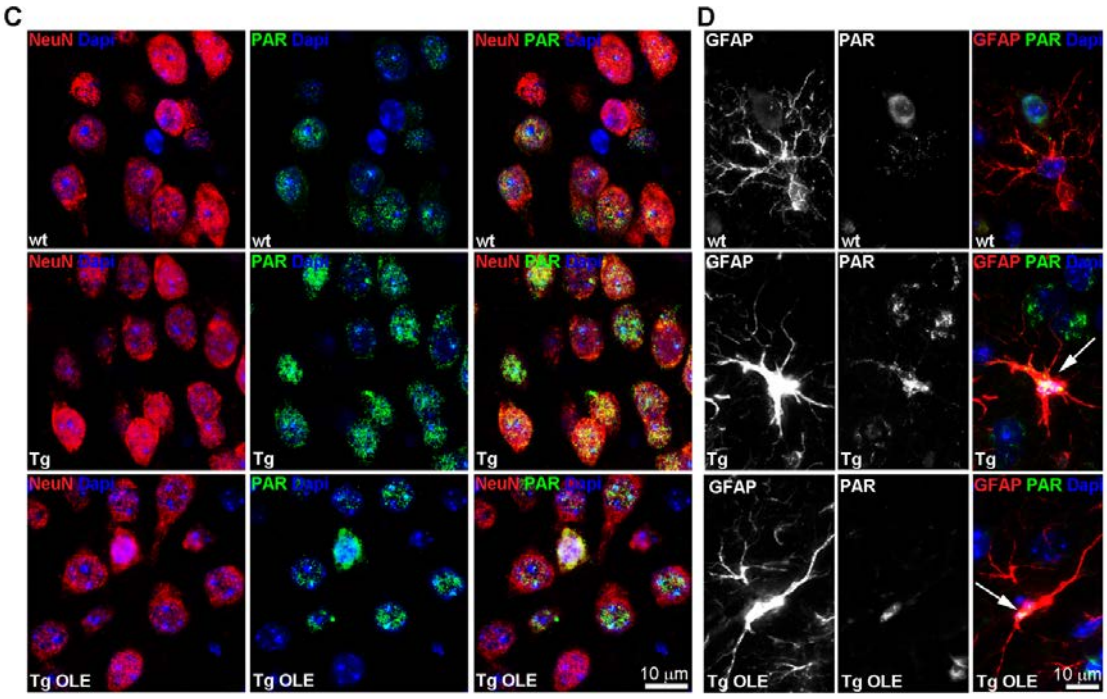

Fig. 19 OLE inhibits PAR immunoreactivity *C) Double immunofluorescence with NeuN (red) and PAR (green) antibody and (D) with GFAP (red) and PAR (green) antibody plus DAPI (blue).*

1.2 OLE inhibits MNNG-induced PARP1 activation and attenuates NAD+ depletion in N2a cells

To confirm inhibition of PARP1 activation by OLE in the mouse brain, we determined the levels of PARylated proteins in cultured N2a neuroblastoma cells. So we exposed N2a cells for 15 min to 100 μM MNNG, a PARP activator, in the absence or in the presence of 100 μM OLE or of two PARP inhibitors (PHE, 30 μM or PJ-34, 20 μM) 24 h prior to treatment with MNNG. As expected, we found that MNNG induced a significant increase of PARylated proteins with respect to untreated control cells (Fig. 20A; One way ANOVA plus Bonferroni post hoc test ** $p < 0.01$ vs controls). However,

the MNNG-induced PAR formation was markedly reduced in cells treated with OLE, PHE, or PJ-34 24 h prior to MNNG exposure, where PAR levels dropped below control values. At the same conditions, cell treatment with OLE, PHE, or PJ-34 alone did not affect PAR formation.

PARP activity needs NAD+ as a substrate and contributes to the modulation of intracellular NAD+ levels. Therefore, we measured NAD+ content in N2a cells exposed to MNNG (100 μM) for 90 min and found a significant reduction (-27%) of the NAD+ content as compared to the levels measured in control cells (One way ANOVA plus Bonferroni post hoc test *p < 0.05vs control cells). The reduction was slightly attenuated by cell treatment for 24 h with 20 μM PJ-34 or with 100 μM OLE (Fig. 20 B). Treatment with OLE did not affect NAD+ levels in control cells (not shown). So these data indicate that OLE administration to cultured neuronal cells displays effects similar to those elicited by well known PARP inhibitors, thus contributing to explain PAR decrease in OLE-fed Tg mice.

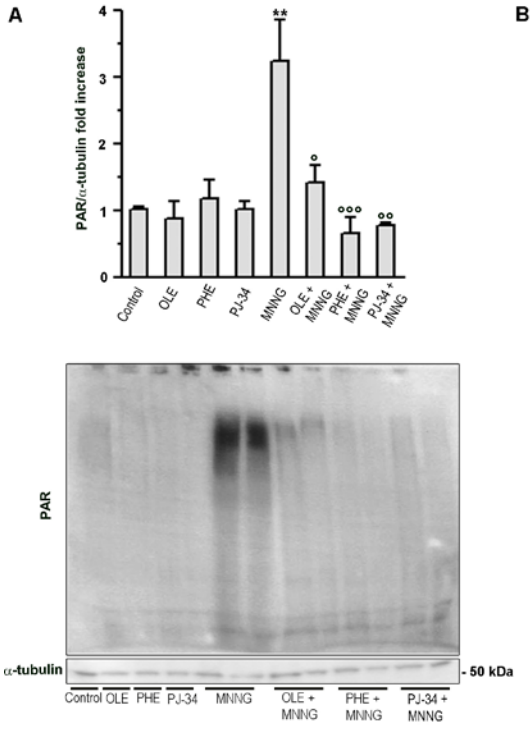

Fig. 20 OLE inhibits PARP1 activation and attenuates NAD+ levels in N2a cells exposed to MNNG. *(A) N2a cells were exposed to MNNG for 15 min then, PARylated proteins were detected by immunoblotting. B) NAD+ levels in N2a cells treated with MNNG for 90 min alone or in combination with PJ-34 or OLE, 24 h prior to MNNG exposure. A) One way ANOVA plus Bonferroni post hoc test °p < 0.05, °° p < 0.01, and °°°p < 0.001 versus MNNG treated cells; **p < 0.01 vs controls. B) One way ANOVA plus Bonferroni post hoc test *p < 0.05 vs control cells.*

1.3 OLE reduces PARP1 expression and modulates the PARP1-SIRT1 interplay in 6-month-old TgCRND8 mice

Since PARP1 accounts for over 85% of nuclear PARP activity (Kauppinen T.M. and Swanson R. A.., 2007), we investigated PARP1 expression levels in 6-month-old Tg mice at a normal diet or at a diet supplemented with OLE by determining PARP1 mRNA levels. We found that PARP1 mRNA levels in the cortex of Tg mice were over 4 times higher than in the cortex of wt mice but were fully rescued to those of wt mice following OLE treatment (Fig. 21 A; one way ANOVA plus Bonferroni post hoc test ***p < 0.001 vs Tg mice). These data match those relative to the PARP1 protein levels; in fact, in the cortex of TgCRND8 mice we found a roughly 100% increase of the 116 kDa PARP1 (the uncleaved protein) and over a 100% increase of the 89 kDa PARP1 (the caspase 3-cleaved fragment) as compared to wt mice (Fig. 21 B; One way ANOVA plus Bonferroni post hoc test * p < 0.05 vs Tg mice). However, in OLE-fed Tg mice the levels of the 116 kDa and 89 kDa proteins were maintained to the values found in wt mice.

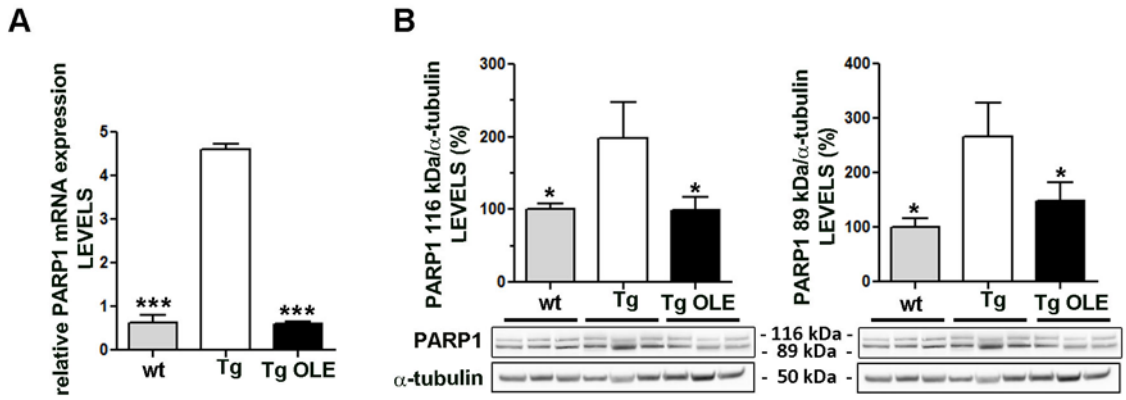

Fig. 21 OLE decreases PARP1 expression and increases SIRT1 expression in 6-month-old TgCRND8 mice. *A) PARP1 mRNA levels and (B) western blotting analysis of PARP1 protein levels in the cortex of 6-month-old OLE-fed Tg mice and untreated wt and Tg mice. n = 5-6 mice/group. A) One way ANOVA plus Bonferroni post hoc test ***p < 0.001 vs Tg mice. B) One way ANOVA plus Bonferroni post hoc test * p < 0.05 vs Tg mice.*

Next, we investigated the expression of SIRT1 in the cortex of our TgCRND8 mice. We found that SIRT1 immunoreactivity was strongly reduced in the cortex of TgCRND8 mice as compared to wt mice and that, similarly to the effect of other polyphenols (Chung S. et al., 2010). OLE treatment increased its expression in Tg mice (Fig. 21 C). These data were confirmed by western blotting analysis showing a significant reduction of SIRT1 in Tg mice as compared to wt mice while it appeared markedly increased in OLE-treated Tg mice (Fig. 21 D; one way ANOVA plus Bonferroni post hoc test ** p <

0.01 and *** p < 0.001 vs OLE-fed Tg mice) and suggesting the existence of a link between PARP1 and SIRT1 modulation in Tg mice. To assess any PARP1/SIRT1 relation in our Tg mice, we investigated the colocalization between PARP1 and SIRT1 and their modulation by OLE treatment. We performed a double labeling fluorescent immunohistochemistry with PARP1 (green) and SIRT1 (red) antibodies plus DAPI (blue) that revealed co-localization between PARP1 and SIRT1 (Fig. 21 E, yellow color) in all groups of animals, irrespective of treatment and genotype; however, as reported for PAR, PARP1 was expressed in Tg mice at higher levels than in wt animals, whereas its expression was reduced in OLE-treated mice. Altogether, these data indicate that OLE treatment increases the expression of SIRT1 and reduces that of PARP1, confirming the ability of this polyphenol to interfere with the functional link between PARP1 and SIRT1.

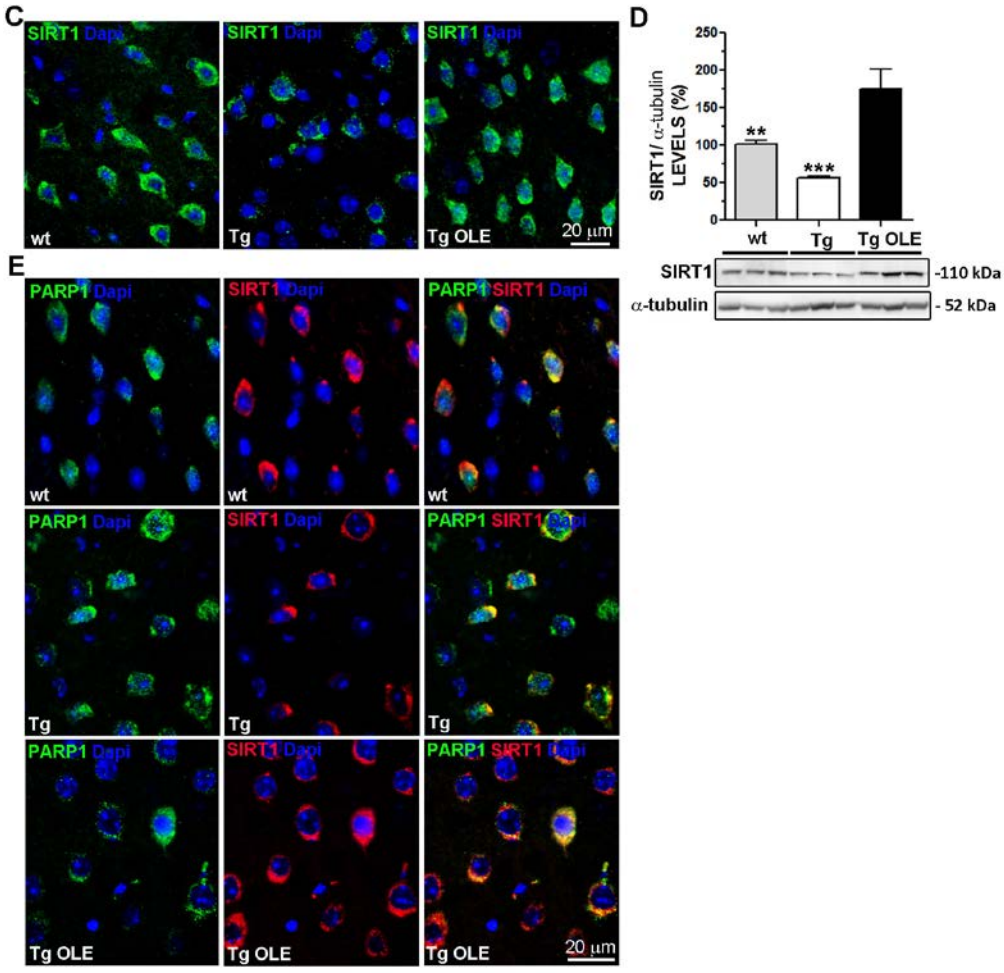

Fig. 21
(C and D) OLE decreases PARP1 expression and increases SIRT1 expression in 6-month-old TgCRND8 mice. *C) Immunofluorescence with SIRT1 (green) antibody plus DAPI (blue). D) Western blotting analysis of SIRT1 protein levels in OLE-fed Tg mice and untreated wt and Tg mice. E) Double immunofluorescence with PARP1 (green) and SIRT1 (red) antibody plus DAPI (blue) in wt, Tg and OLE-fed Tg mice. n = 5-6 mice/group D) One way ANOVA plus Bonferroni post hoc test ** p < 0.01 and*** p < 0.001 vs OLE-fed Tg mice.*

1.4 OLE increases SIRT1 expression in N2a cells

Our previous studies in TgCRND8 mice and in cultured cells showed that OLE administration results in the activation of the autophagy-lysosome system (Grossi C. et al., 2013; Rigacci S. et al., 2015) and decreases the expression of HDAC2, with ensuing increase of histone acetylation (Luccarini I. et al., 2015). On the other hand, it is known that many polyphenols activate SIRT1 which, in turn, stimulates autophagy and thereby mimics caloric restriction-mediated lifespan extension (Fulco M. and Sartorelli V., 2008). It is also known that PARP1, besides playing a role in cell death, can also induce cell survival via autophagy stimulation (Huang Q. et al., 2009). So we investigated in N2a cells (I) the effect on SIRT1 expression of cell treatment for 24h with 100 μM OLE (Fig. 22 A) and (II) the effect on Beclin1 levels, a protein involved in the initiation and execution of autophagy, induced by cell treatment with 100 μM MNNG for 15min or of cell treatment for 24h with 100 μM OLE prior to exposure to 100 μM MNNG for 15 min (Fig. 22 B). We found that, in OLE-treated cells, SIRT1 levels were increased by over 250%, as compared to control cells, and that Beclin1 levels were increased by around 100% upon MNNG exposure and by roughly 200% in cells treated with OLE prior to MNNG exposure. Altogether, these findings indicate that PARP1 activation by MNNG results in protein PARylation and increased autophagy, in agreement with previous data on lifespan extension (Fulco M. et al., 2008) and that OLE reinforces the MNNG effect of autophagy activation. We have not assessed whether Beclin1 increase by OLE and MNNG results from the same or a different action, for example by interfering with the Beclin1/Bcl2 protein interaction. These data also indicate that autophagy stimulation by OLE also results from SIRT1 upregulation.

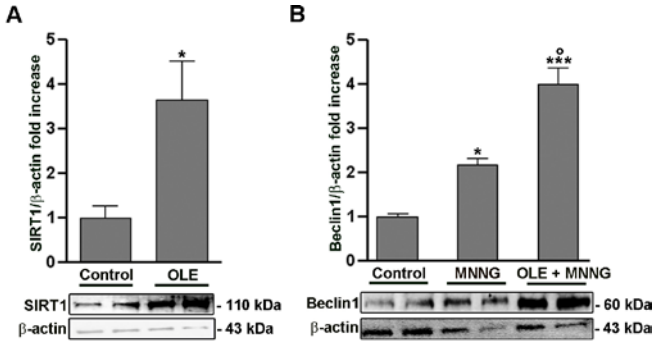

Fig. 22 OLE promotes SIRT1 and Beclin1 expression in N2a cells. *A) N2a cells were treated with 100 μM OLE for 24h, SIRT1 expression was detected by immunoblotting. B) Beclin1 protein levels in N2a cells exposed to 100 μM of OLE for 24h plus MNNG 100 μM for 15 min. A) One way ANOVA plus Bonferroni post hoc test *p < 0.05 OLE treated cells vs control cells; B) One way ANOVA plus Bonferroni post hoc test *p < 0.05 MNNG treated cells vs control cells; ***p < 0.001 OLE + MNNG-treated cells vs control cells; °p < 0.01 OLE + MNNG-treated cells vs MNNG-treated cells.*

1.5 OLE reduced NF-κB phosphorylation in TgCRND8 mice

PARP1 activation can influence brain injury and neuronal death through modulation of the brain inflammatory response; these effects are largely mediated by its interaction with the nuclear tran- scription factor NF-κB (Hassa P. O. et al., 2001; Chiarugi A. et al., 2003), whose abnormal activation is involved in AD (Lian H. et al., 2015). Thus, we investigated in TgCRND8 mice whether a reduction of the phospho (p)-NF-κB, the protein form that migrates to the nucleus, matched OLE-induced PARP1 inhibition. Double immunofluorescent staining with anti-p-NF-κB (red) and anti-PAR (green) antibodies plus DAPI (blue) showed that the high expression of p-NF-κB, detected in the cortex of 6-month-old TgCRND8 mice mainly at the perinuclear and nuclear levels fully co-localized with PAR-immunopositive polymers (Fig. 23 A). However, in OLE-fed TgCRND8 mice p-NF-κB immunoreactivity was drastically reduced and no, or very low, p-NF-κB immunoreactivity was detected, similarly to the expression found in wt mice. The western blotting quantitative analysis confirmed these data, and showed a significant increase of p-NF-κB in the cortex of TgCRND8 mice as compared to wt mice, whereas it was significantly reduced to the value of wt animals in OLE-treated Tg mice (Fig. 23 B; one way ANOVA plus Bonferroni *post hoc* test *p<0.05), in agreement with the reported anti-inflammatory effect of OLE in mouse brain (Grossi C. et al. 2013).

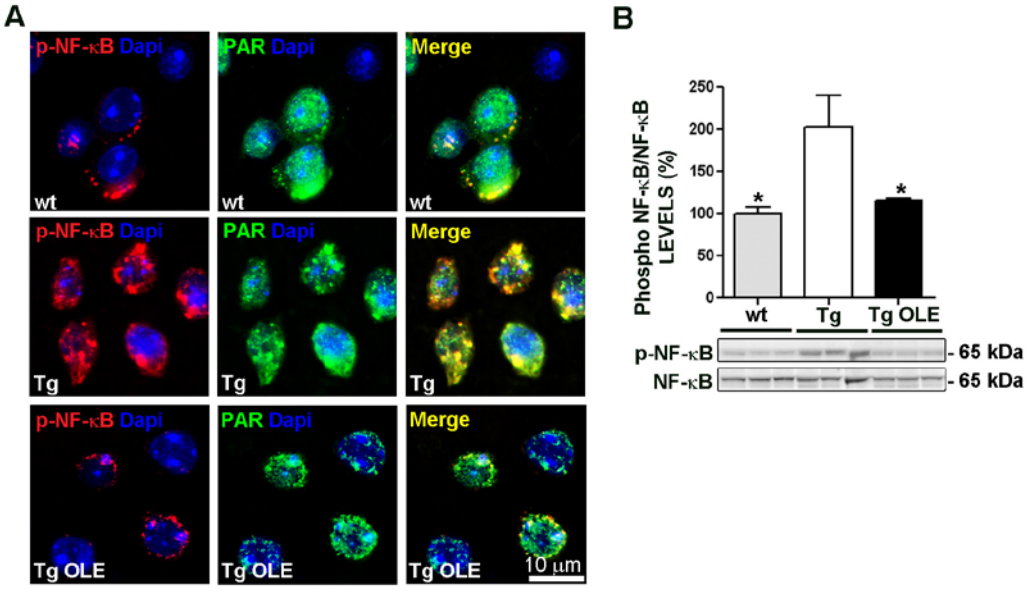

Fig. 23 OLE reduces p-NF-κB phosphorylation in 6-month-old TgCRND8 mice. *A) Double immunofluorescence with p-NF-κB (red) and PAR (green) antibodies plus DAPI (blue). B) Western blotting analysis of p-NF-kB levels versus total NF-kB levels in OLE-fed Tg mice and untreated wt and Tg mice. n = 5-6 mice/group. One way ANOVA plus Bonferroni post hoc test *p<0.05 versus Tg mice.*

1.6 OLE reduces p53 phosphorylation in the cortex of 6-month-old TgCRND8 mice

Modifications of the cellular levels of p53, a transcriptional activator that senses cellular stresses and is up-regulated during DNA damage, hypoxia and oxidative stress, have been associated with neuronal damage both in *in vivo* and *in vitro* models (Morrison R. S. and Kinoshita Y., 2000) as well as with neurodegenerative disorders (Kruman I. I. et al., 2000). p53 is phosphorylated at multiple sites and its phosphorylation at Ser46 regulates its pro-apoptotic power (Oda K. et al., 2000). We therefore investigated in TgCRND8 mice the relation, if any, between PARP, apoptosis and phosphorylated p53 and any interference of OLE administration with these processes.

In double immunofluorescence staining with antibodies to p53 phosphorylated at Ser 46 (p53 S46) (red) and to PAR (green) plus DAPI (blue) we found a strong co-localization between PAR and p53 S46 immunoreactivity in the neurons of cortex of TgCRND8 mice, as compared to wt mice (Fig. 24 A). Remarkably, the intense p53 S46 immunoreactivity and its colocalization with PAR staining detected in TgCRND8 mice was significantly reduced in OLE-fed Tg mice. These immunohistochemical data were confirmed by the analysis of p53 S46 levels; in fact, we found that the significant increase of p53 S46 levels detected in the cortex of TgCRND8 mice respect to wt mice, dropped to the wt values in OLE-fed Tg mice (Fig. 24 B; one way ANOVA plus Bonferroni *post hoc* test $^{**}p < 0.01$ vs Tg mice). These findings indicate that in our model OLE protects neuronal cells against apoptosis by interfering with the PARP/apoptosis/phosphorylated p53 relation.

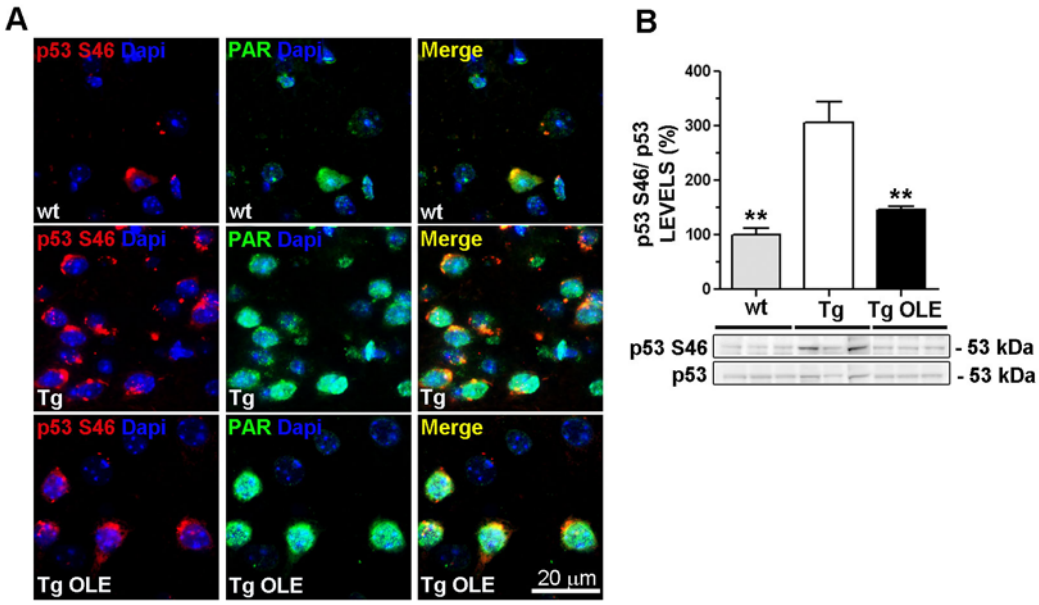

Fig. 24 OLE reduces p53 S46 levels in 6-month-old TgCRND8 mice*. A) Double immunofluorescence with antibodies to p53 S46 (red) and to PAR (green) plus DAPI (blue). B) Western blotting analysis of p53 S46 protein levels vs total p53 levels in OLE-fed Tg mice and untreated wt and Tg mice. n = 5-6 mice/group One way ANOVA plus Bonferroni post hoc test **p < 0.01 vs Tg mice.*

The conclusion from this part of the study is that OLE protects the TgCRND8 mouse model of Aβ deposition by increasing autophagy and concomitantly reducing the inflammatory and apoptotic response as shown in Fig. 25. It reaches these results by interfering with a number of cell responses/pathways; in addition to those previously described the effects on the PARP/SIRT1 system, NF-κB and p53 are also of importance. These results were confirmed in a simplified model as it is the N2a cell line.

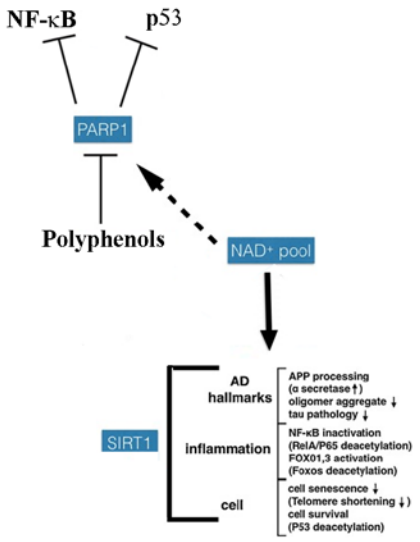

Fig.25 OLE modulates PARP1-SIRT1 pathway

2. POLYPHENOLS FROM OLIVE MILL CONCENTRATED WASTE WATER AND HYDROXYTYROSOL TREATMENT

In this study we investigated the *in vivo* effect on behavioural performance and neuropathology of both a polyphenolic extract from olive mill waste water and pure hydroxytyrosol (HT), both supplemented at 50 mg kg^{-1} of diet, corresponding to the dose of OLE found to be protective in previous experiments (Grossi C. et al., 2013). Our aim was to define whether the healthy effects of EVOO consumption are due to the content of specific phenols, such as OLE, or they could also be provided by a mix of olive polyphenols or other phenols or their metabolites, such as HT.

2.1 Behavioural performances: Polyphenols from olive mill concentrated waste water and HT ameliorate cognitive functions in TgCRND8 mice

Following our data on the beneficial effects of OLE on AD-like pathological signs, we sought to define whether the healthy effects of EVOO consumption are due to the content of specific phenols, such as OLE, or they could also be provided by a mix of olive polyphenols or HT. Thus, TgCRND8 mice were fed for 8 weeks with a polyphenolic extract from olive mill waste water at 50 mg/kg of diet or HT at 50 mg/kg of diet, corresponding to the highest dose of OLE found to be protective in our previous experiments.

During this period, HT and polyphenols treatments was well tolerated and no evident side effects were revealed, as shown by the body weight trend graph (Fig 26) and no animals died.

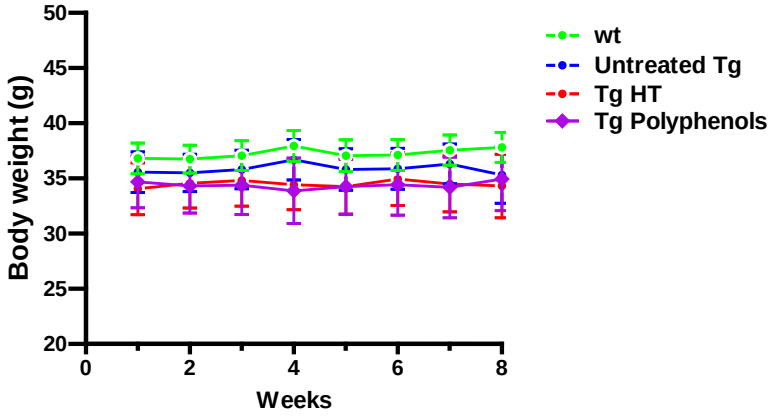

Fig. 26 Body weight graph of HT- and Polyphenols-fed TgCRND8 mice: *HT and polyphenols did not affect body weight parameters.*

During the last week of diet treatment memory performance was assessed firstly with MWM for HT-fed Tg mice only and then with step down inhibitory avoidance and ORT

tests for polyphenols and HT-fed Tg mice.

Tg and wt mice from each experimental group were trained for 4 days in the MWM task to learn where the hidden platform was located. Untreated Tg mice required significantly more time to find the platform at day 2 (*P<0.05) and 3 (**P<0.01) compared to wt mice (Statistical analysis: two way ANOVA plus Bonferroni *post hoc* test).

Interestingly, the acquisition phase of HT-fed Tg mice was much improved, they were good swimmers and showed an appropriate swim-search response after being placed in water and the escape latency during the 2- and 3-day reached the level displayed by control mice. Furthermore, the escape latency on day 2 of HT-fed Tg mice was significantly improved compared to untreated Tg mice (Fig. 27A; °p<0.05). These findings indicate that the transgene-induced early impairment in the spatial memory for platform location, as compared with control group, was restored by the HT administration. At day 4 of acquisition phase, untreated Tg mice shortened the escape latency with no significant differences when compared to HT-fed Tg or wt mice (Fig. 27 A), likely suggesting that in this experimental set-up the spatial memory impairment for platform location in untreated Tg mice is restored over sessions.

Memory performance was then investigated in the step down inhibitory avoidance task for both groups of animals (polyphenols- and HT-fed Tg-mice). We observed no significant differences between wt and treated or untreated Tg mice during the TT, as previously reported in experiments with OLE-fed Tg mice. However, in the 24 h RT step-down the latencies recorded for Tg mice were significantly reduced respect to wt mice (°°°P<0.001) and not significantly different from training latency. The administration to Tg mice of HT or olive waste water polyphenol extract significantly improved memory performances (Fig. 27 B and 28 A, respectively; one way ANOVA plus Bonferroni post hoc test °P<0.05). The animals were also tested for ORT with a retention interval of 60 min. In T1, the exploration time of the familiar object was comparable among all groups and no deficiencies were recorded in exploratory activity, directional movement towards the objects and locomotor activity. However, in T2 novel object preference was impaired in untreated Tg mice compared with wt mice, as shown by the significant reduction of the discrimination score (Fig. 27 C and 28 B). HT-fed (Fig. 27C) and polyphenol-fed (Fig. 28B) Tg mice displayed significantly improved ability to discriminate between the familiar and novel object with respect to untreated Tg mice, reaching a performance comparable with that of wt mice (Statistical analysis:

one way ANOVA plus Bonferroni *post hoc* test). These data indicate that HT and olive polyphenol extract treatment prevents/rescues efficiently cognitive impairment in our Tg model, as previously reported for OLE treatment (Grossi C. et al. 2013).

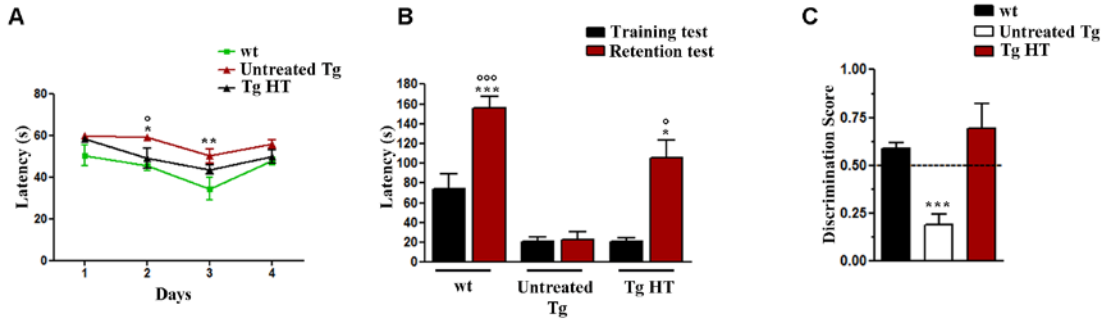

Fig. 27 HT improves cognitive performance in 6-month-old TgCRND8 mice*. A) MWM test: on day 2 the escape latencies to find the platform during the training sessions of HT-fed Tg mice were significantly different from those of untreated Tg mice (°P<0.05) and no significantly different from those of control group. Untreated Tg mice required significantly more time to find the platform compared to wt mice (Two way ANOVA plus Bonferroni post hoc test *p<0.05, day 2 and **p<0.01, day 3). Each point represents the mean daily values of four trials per day; data are reported as mean ± S.E.M. B) Step down test: one-way ANOVA plus Bonferroni's post comparison test show a statistically significant increase in the mean retention latencies in wt and in HT-treated Tg mice, as compared to their respective training latencies (*P<0.05, ***P<0.0001). In untreated Tg mice, the retention latencies were significantly reduced respect to controls (°°°P<0.001) and to HT-fed Tg mice (°P<0.05) and not significantly different from training latencies. C) ORT test: in the T2 trial the discrimination index of untreated Tg mice significantly differed from that of all other groups (One way ANOVA plus Bonferroni post hoc test ***P<0.0001). The dotted line indicates chance level performance. Number of animals: n = 6/group.*

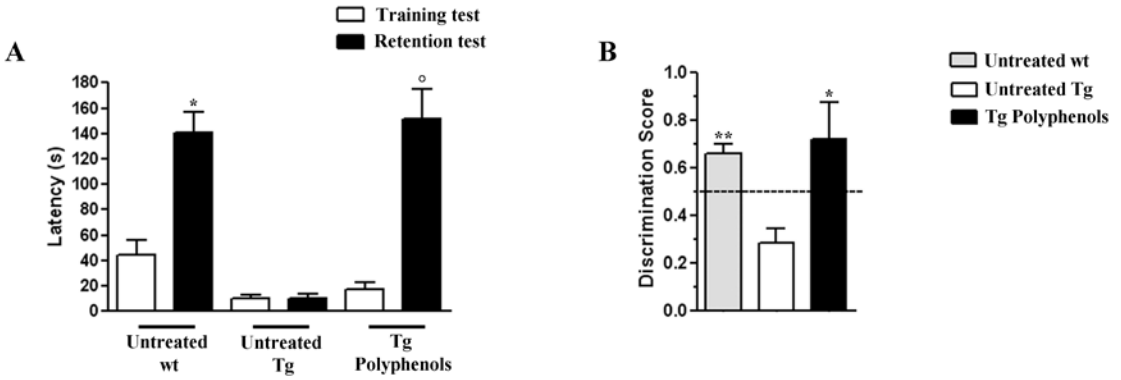

Fig. 28 Polyphenols from olive mill concentrated waste water restore cognitive performance in 6-month-old TgCRND8 mice*. A) Step down test: a statistically significant increase in the mean retention latencies in untreated wt mice (*P < 0.0001) and in polyphenol-treated Tg mice (°P < 0.0001), respect to the training latencies. The retention latencies of untreated Tg mice differed from those of untreated wt mice (*P < 0.0001) and polyphenol-treated Tg mice (°P < 0.0001). (□training test, ■retention test), B) ORT: in T2, the discrimination index of untreated Tg mice differed from that of untreated wt mice (**P < 0.0001) and polyphenols-fed Tg mice (*P < 0.001). The dotted line indicates chance level performance: Number of animals: n = 6/group. (□untreated wt, □untreated Tg, ■ Tg polyphenols) Statistical analysis: One way ANOVA plus Bonferroni post hoc test.*

2.2 Polyphenols from olive mill concentrated waste water and HT reduce Aß burden in Tg mice

At the end of the behavioural performance the brains of olive mill waste water and HT-fed Tg mice were analyzed for Aß42 and pE3-Aß plaque load. We found that the differently shaped and sized Aß42-positive plaques, detected in the cortex and hippocampus of untreated Tg mice, were markedly reduced in the same brain areas of polyphenol- and HT-fed Tg mice (Fig. 29A and 30A). The quantitative analysis (Statistical analysis: *t* test) confirmed these data indicating a significant reduction of total Aß42 plaque area and number in the cortex and hippocampus of polyphenols treated mice (Fig. 29 B) and in the cortex of HT-treated mice (Fig. 30A, right). In the hippocampus of HT-fed Tg mice an apparent trend towards a reduction of Aß42 was seen. pE3-Aß load was also significantly affected by polyphenols and HT treatment. Immuhistochemical (Fig. 29C) and quantitative analysis (Figure 29 D; Statistical analysis: *t* test) revealed a marked reduction of pE3-Aß load in the cortex and hippocampus of polyphenol-fed Tg mice. In the HT-fed animals, plaque load (plaque number and total plaque area) was significantly reduced in the parietal cortex while in the hippocampus a significant reduction in the plaque number and an apparent trend towards reduction of total plaque area was seen, as compared to untreated animals (Fig. 30B).

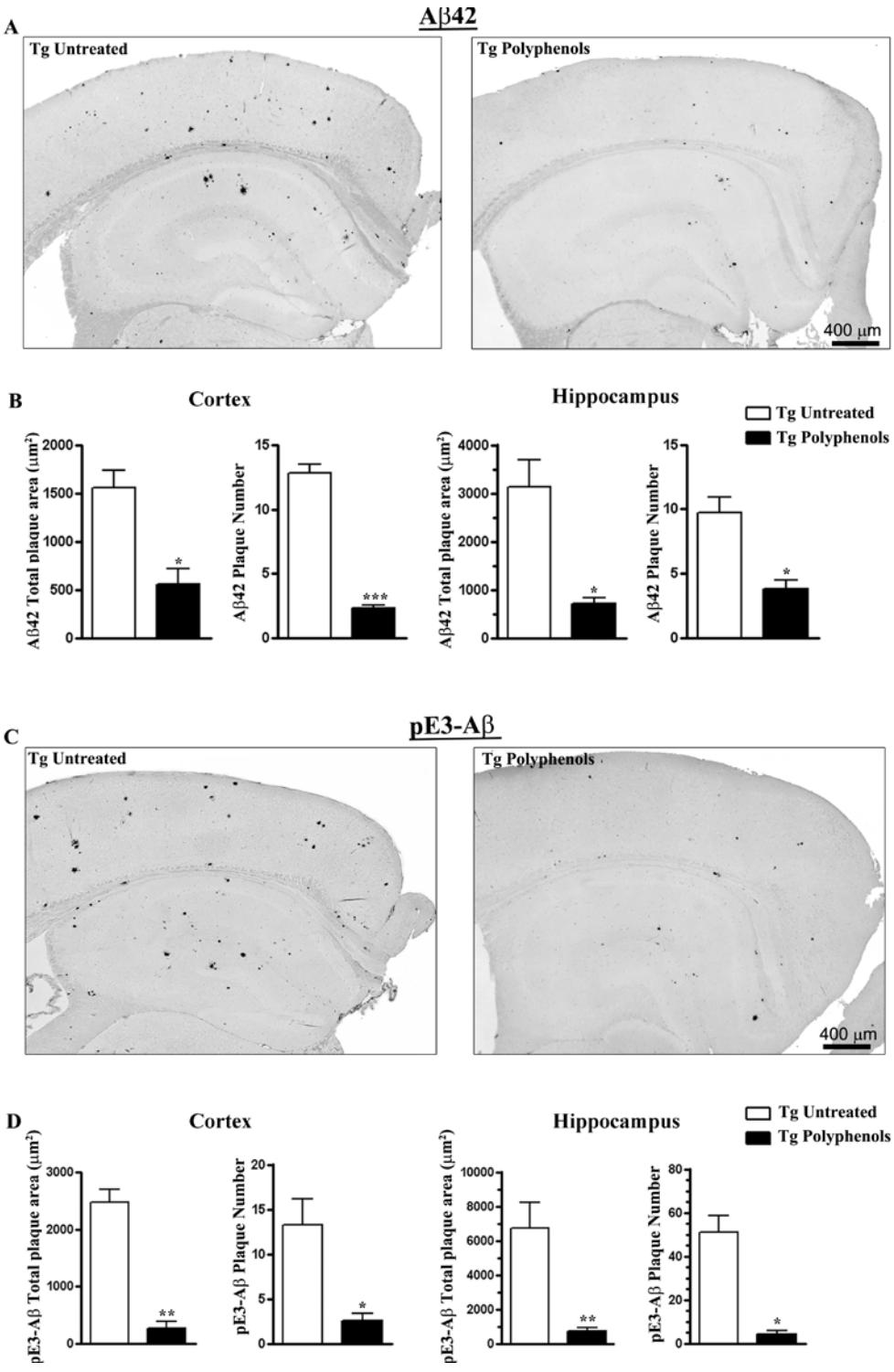

Fig. 29 Polyphenols from olive mill concentrated waste water reduce Aß42 and pE3-Aß burden in the brain of TgCRND8 mice*. (A and C) representative photomicrographs of Aß42 and pE3-Aß respectively, immunopositive deposits. (B and D) quantitative analysis of Aß42 and pE3-Aß, respectively, plaque area and plaque number in the parietal cortex and hippocampus of 6-month-old untreated and polyphenols-fed Tg mice. t test *P < 0.01, **P < 0.001 and ***P < 0.0001 vs. untreated Tg mice. Scale bar = 400 μm applies to all images.*

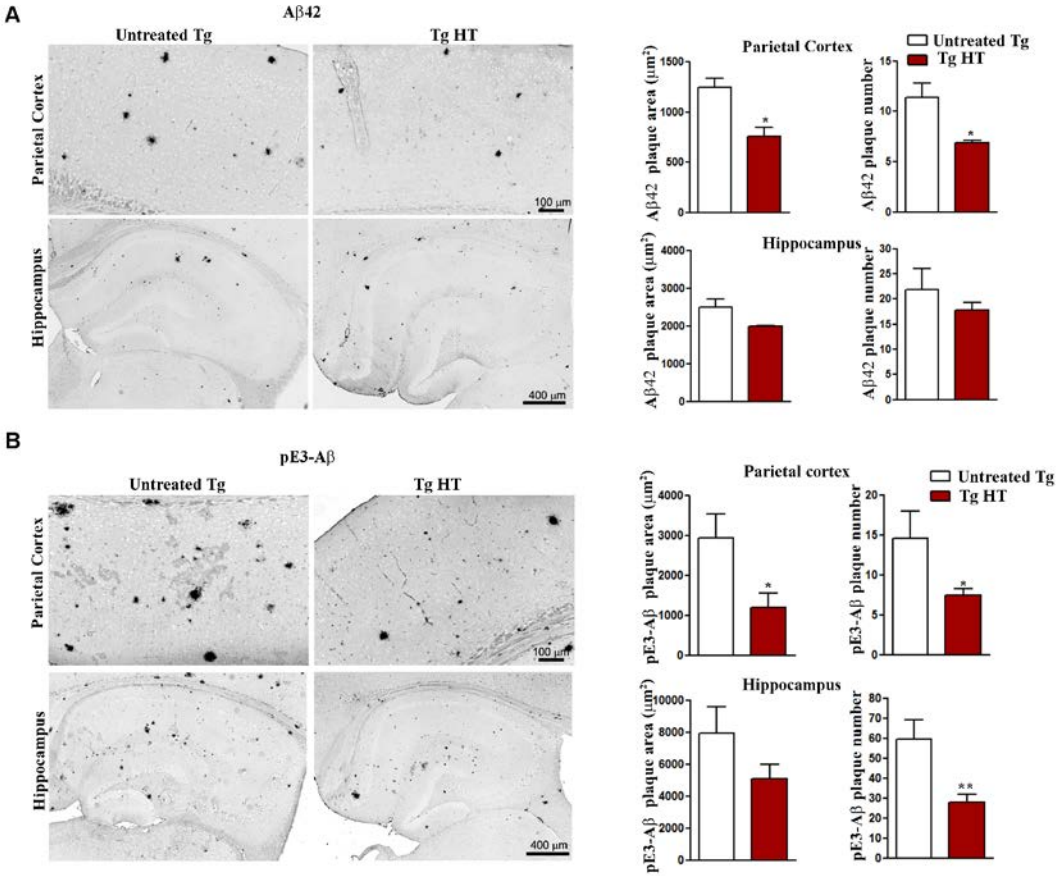

Fig. 30 HT reduces Aß42 and pE3-Aß plaque load in the brain of TgCRND8 mice*. Representative photomicrographs of Aß42 and pE3-Aß immunopositive deposits, left images (A and B) and their quantitative analysis on the right histograms (t test *P < 0.05 and **P < 0.001 vs. untreated Tg mice). Scale bars = 100 µm applies to all images of parietal cortex and 400 µm applies to all images of hippocampus.*

2.3 Olive mill concentrated waste water polyphenols and HT induce autophagy in TgCRND8 mice

We recently reported an intense activation of the autophagosome-lysosome system in the cortex of 3.5-, 6- and 12-month-old TgCRND8 mice fed with OLE at 50 mg kg^{-1} of diet. We found the same intense activation of autophagy in the cortex of polyphenol- or HT-fed Tg mice. A bright and punctate immunoreactivity for LC3, involved in the initiation and execution of autophagy, was found in the soma, perikarya and dendrites of neurons in different layers of somatosensory/parietal cortex, with respect to untreated mice for both treatments (Fig. 31 and Fig. 32).

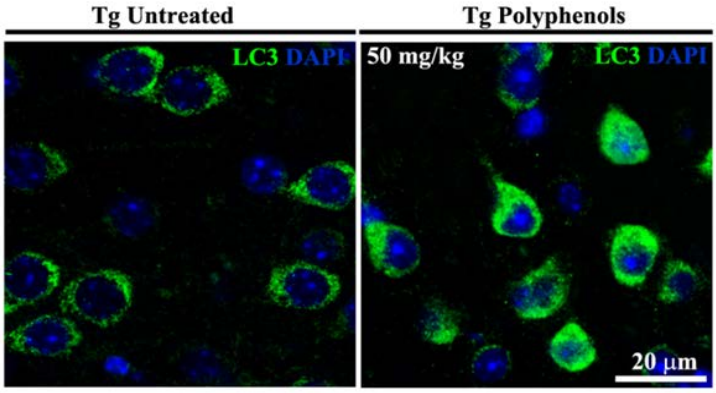

Fig. 31 Polyphenols from olive mill concentrated waste water induce autophagy in the cortex of TgCRND8 mice. *Representative images of LC3 immunoreactivity showing a strong and bright LC3 puncta in the neuronal cell bodies and processes of neurons in the somatosensory/parietal cortex of Tg mice fed with polyphenols, as compared with untreated Tg mice. Scale bar =20 μm applies to all images.*

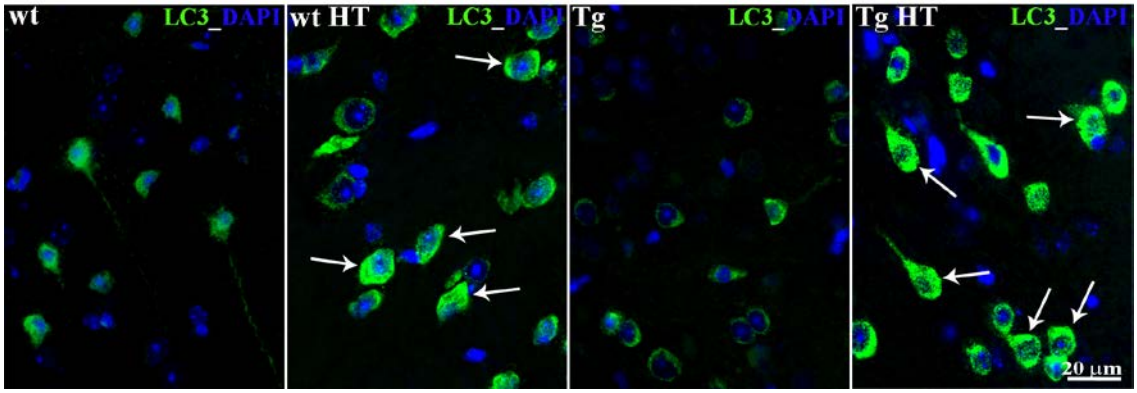

Fig. 32 Autophagy induction in the cortex of HT-fed TgCRND8 mice. *Bright and punctate LC3 immunoreactivity (white arrows) is shown in the somatosensory/parietal cortex of Tg and wt mice fed with HT, as compared with respective untreated mice. Scale bar =20 μm applies to all images.*

Then, we investigated whether the expression of the lysosome-associated membrane protein type 2A (LAMP-2A), a chaperone-mediated autophagy (CMA) receptor at the lysosomal membrane, and the levels of the heat shock protein of 70 kDa (hsp70), involved in CMA, are affected by the transgene and by the HT-supplemented diet. Western blotting analysis did not reveal any difference in the cortical hsp70 levels due both to transgene and to HT (data not shown). Similarly, LAMP-2A did not reveal any difference among groups (Fig. 33). These data seem to exclude an effect of the transgene on the CMA system and that the HT-induced Aß disaggregation and reduction in our experimental model is not related to CMA activities.

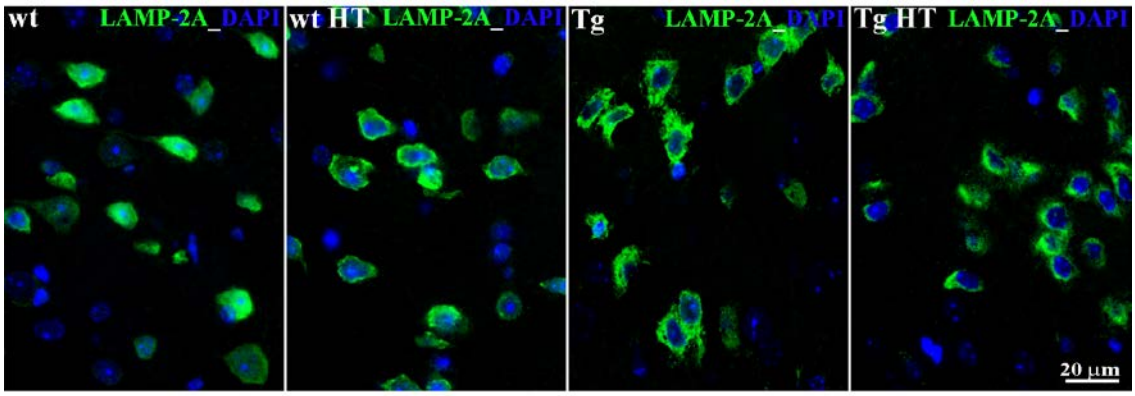

Fig. 33 HT does not activate CMA in TgCRND8 mice. *Representative images of LAMP-2A immunoreactivity showing the lack of differences among groups. Scale bar =20 μm applies to all images.*

2.4 HT reduces TNF-α expression and astrocytes reaction

A large body of evidence indicates the presence of a number of inflammatoty markers in the AD brain, such as elevated inflammatory cytokines and chemokines and an accumulation of activated glial cells in the damaged regions (Hong J. T., 2017). We therefore investigated the expression and cell localization of the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) in hippocampal areas of Tg mice and whether its expression could be reduced by HT treatment.

In immunofluorescence analysis with anti TNF-α antibody, a strong TNF-α immunoreactivity (TNF-α IR) (red) was found throughout hippocampal areas of untreated Tg mice (Fig. 34A), that was markedly diminished in hippocampal areas of HT-fed mice and almost undetectable in hippocampal areas of untreated and HT-fed wt mice. Next, we investigated TNF-α expression levels in Tg mice normally fed or supplemented with HT by checking TNF-α mRNA levels. As expected, the TNF-α mRNA levels in the hippocampus of Tg mice were higher than in the hippocampus of wt mice while were significantly reduced close to those of wt mice upon HT treatment (Fig. 34B; one way ANOVA plus Bonferroni *post hoc* test).

Finally, we searcherd the type of cells displaying TNF-α espression in double immunofluorescence staining using anti TNF-α (red) and anti GFAP (green) antibodies plus DAPI (blue). In Tg animals TNF-α immunoreactivity was found mainly within astrocytes, as revealed by the co-localization between TNF-α and GFAP staining (yellow color indicated by arrows in the merged images of Fig. 34 C). However, TNF-α

and GFAP expression detected in TgCRND8 mice was strongly reduced in HT-fed Tg mice. These data confirm our previous findings showing that activation of astrocytes in the brain of TgCRND8 mice is strongly attenuated in OLE-fed Tg mice (Grossi C. et al., 2013, Luccarini I. et al., 2015).

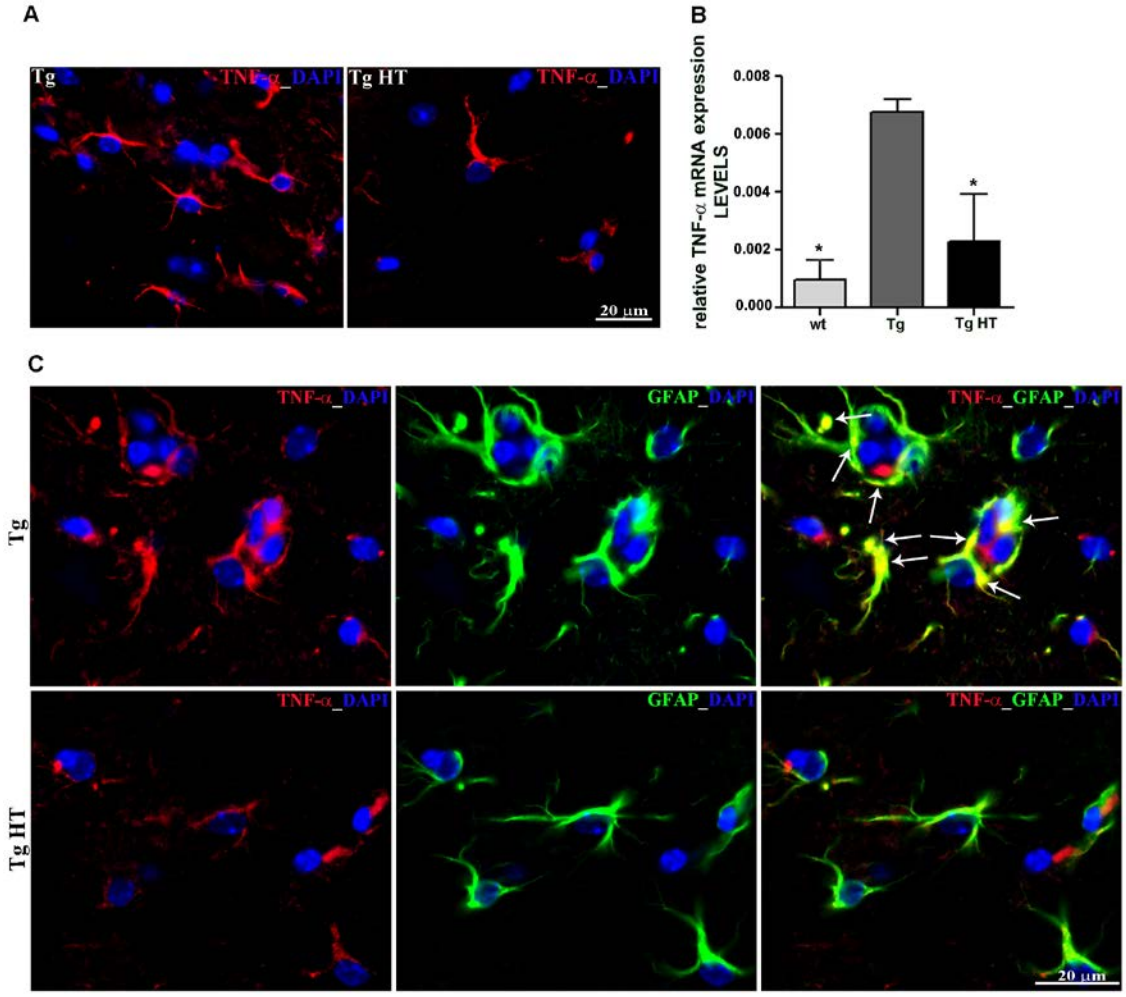

Fig. 34 HT reduces TNF-α expression and attenuates astrocyte reaction in TgCRND8 mice. *A) Representative images of TNF-α immunoreactivity and B) TNF-α mRNA levels. C) Double immunofluorescence with TNF-α (red) and GFAP (green) antibodies plus DAPI (blue) in HT-fed Tg mice and untreated Tg mice. Statistical analysis: one way ANOVA plus Bonferroni post hoc test *P < 0.05 vs Tg. Scale bar =20 µm applies to all images.*

The conclusion of the second part of my study is that OLE protection against Aß plaque pathology is obtained also by supplementation to the Tg mice of either HT, the main OLE metabolite that is detected in mouse brain (Luccarini I. et al., 2015), or a concentrated olive mill waste water enriched in a mixture of olive polyphenols and their metabolites. Even in this case protection underlies the same cellular and molecular mechanisms, supporting the similarity of action of this class of plant molecules.

3 CHARACTERIZATION OF CHOLINERGIC NEURONS IN HUMAN FETAL NUCLEUS BASALIS MAGNOCELLULARIS (hfCNs)

Prof.ssa Vannelli and colleagues, University of Florence, have isolated NBM tissue that was dissected from two 12-weeks old human female fetuses (Permit Number: 678304) and incubated with 1.0 mg/ml collagenase type IV (Sigma-Aldrich Corp., St. Louis, MO). The cell suspensions were mechanically dispersed by pipetting and cultured in Coon's modified Ham's F12 medium (Euroclone, Milan, Italy) supplemented with 10% FBS (Hyclone, Logan, UT). Cells were used within the 26^{th} passage. One week after plating, the cells started to emerge from aggregates and grew as a population of adherent cells in monolayer. At this stage, the cells were harvested and re-plated into fresh medium generating a primary cell culture of hfCNs neurons that was propagated, characterized and used for successive experiments.

3.1 hfCNs improve memory functions in NBM-lesioned rats

After quisqualic acid (QA) injection into the right NBM of rats we analyzed the ability of hfCNs to regenerate impaired cholinergic function. As previously reported (Bartolini et al. 1996), 21 days after NBM lesioning the number of magnocellular ChAT-positive neurons in the QA-injected side was significantly reduced (-64%), compared to saline-injected NBM (Fig. 35 A-B, *t* test $P<0.001$). hfCNs ($1.5x10^6$ cells) were i.v. administered in subgroups of QA-injected and saline-injected rats.

To evaluate whether the intravenously administered cells reached the lesioned NBM, hfCNs were labeled with the red fluorescent dye PKH26 before administration (Fig. 35 C). As shown in Fig. 35 D, the immunohistochemical analysis of astrogliosis revealed the presence of reactive GFAP-positive astrocytes with enlarged cell bodies and long processes in NBM sections from QA- but not saline-injected rats (Fig. 35 D). The presence of PKH26-labeled hfCNs was detected both in the QA- and saline-injected NBM (Fig. 35 D). By immunofluorescent staining with ChAT antibody of NBM sections from the experimental rats the hfCN-PKH26 cells resulted ChAT-positive both in QA-injected rats, at day 1, 7 and 21, and in saline-injected rats, as exemplified by the 21 day data (Fig 35 E).

The effect of hfCNs administration on cognitive functions were assessed by both MWM and step down inhibitory avoidance tests. At day 21, rats from each experimental group were trained for 4 days in the MWM task to learn where the hidden platform was located. The rats were naive to the water maze and showed no deficiencies in swimming

abilities, directional swimming toward the platform, or climbing onto a hidden platform during training trials. As shown in Fig. 35 F, QA-injected rats required more time to find the platform compared to controls. Interestingly, rats from the QA-injected + hfCNs group were good swimmers and showed an appropriate swim-search response after being placed in water, significantly shortening the escape latency during the 2- and 3-day acquisition phase compared to the QA-injected group (Fig. 35 F: two way ANOVA plus Bonferroni *post hoc* test). These findings indicate that the QA-induced early impairment in the spatial memory for platform location, as compared with control group, was restored by hfCNs administration. At day 4 of the acquisition phase, QA-injected rats shortened the escape latency with no significant differences when compared to QA+ hfCNs or control rats (Fig. 35 F), suggesting a compensatory intervention over sessions of the un-injected contralateral NBM in QA animals. No differences were detected in the escape latency in all acquisition phase sessions between control and saline injected + hfCNs rats and, thus, these data are not shown. Then, the same animals were tested for the Step down inhibitory avoidance. No significant differences were observed between QA, QA+ hfCNs or control rats during the training test of the step down test. However, in the 24 h retention test step-down latencies recorded for QA rats were significantly reduced respect to controls and not significantly different from training latency, indicating that QA rats were unable to memorize the punishment and to perform the inhibitory avoidance. Administration of hfCNs to QA-injected rats significantly improved their performance that reached the level displayed by control rats (Fig. 35 G; one way ANOVA plus Bonferroni *post hoc* test).

No differences were detected during retention test between control and saline injected + hfCNs rats and, thus, these data are not shown.

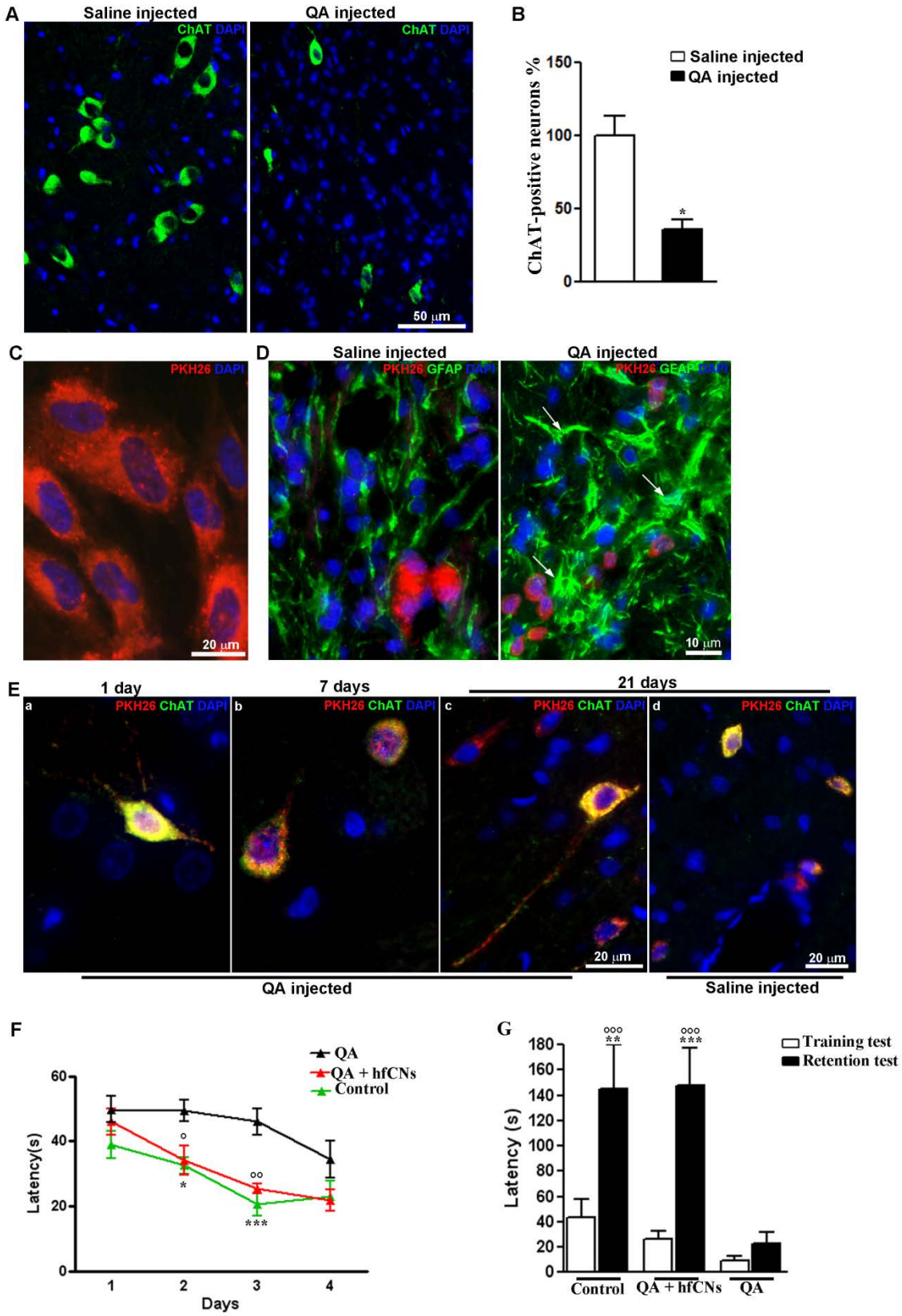

Fig. 35 Quisqualic acid injection into the nucleus basalis magnocellularis of rats and hfCNs treatment.

*A: representative photomicrographs of ChAT-positive cells (green) plus DAPI (blue) in the NBM of saline- and QA-injected rats; B: number of ChAT-positive neurons in the saline- and QA-injected NBM (t test *P<0.001), data are reported as per cent mean ± S.E.M; C: histochemical analysis of PKH26-labeled hfCNs(red); D: PKH26-labeled hfCNs (red) within*

*GFAP-positive astrocytes (green) plus DAPI (blue) in the NBM injected with saline or QA. Note the reactivity of astrocytes (arrows) in the QA-injected NBM; E: NBM injected with QA at day 1, 7 and 21 and injected with saline at day 21 after i.v. administration of PKH26-labeled hfCNs (red): note that some of them are ChAT-positive (yellow), DAPI in blue. F: Morris Water Maze test, effect of hfCNs on the escape latencies to find the platform during the training sessions. Each point represents the mean daily values of four trials per day; data are reported as mean ± S.E.M. On day 2 and 3 the escape latencies of QA injected animals were significantly different from QA + hfCNs treated rats (Two way ANOVA plus Bonferroni post hoc test °P<0.05, °°P<0.01) and from control rats (Two way ANOVA plus Bonferroni post hoc test *P<0.05, ***P<0.001); G: Step down inhibitory test, the mean retention latencies was increased in QA + hfCNs treated rats (***P<0.001) and in controls (**P <0.01), as compared with the respective training latencies. The retention latencies of QA injected rats differ from control and QA + hfCNs treated rats (°°°P<0.001), whereas they are very similar to the latencies of training test.Statistical analysis: one way ANOVA plus Bonferroni post hoc test.*

4 TREATMENT OF CELLS AND Tg MICE WITH ANDROGRAPHOLIDE CONJUGATED WITH FLUORESCENT ALBUMIN NANOPARTICLES (NPs) AND TREATMENT OF RATS WITH FLUORESCENT SOLID LIPID NANOPARTICLES (SLNs)

The aim of this study was to evaluate the use of fluorescent solid lipid nanoparticles (SLNs) and albumin fluorescent nanoparticles (NPs) to deliver andrographolide (ANDRO) through the central nervous system. We studied *in vivo* the distribution and fate of SLNs after intravenous administration in healthy rats and of NPs after intraperitoneal injection in Tg mice and we evaluated the ability of the delivery system to cross the BBB and reach brain tissues and the effect of andrographolide conjugated to albumin nanoparticles (NPs-ANDRO) *in vivo* on behavioural performance of TgCRND8 mice. SLNs present many advantages such as long-term stability, increased bioavailability of encapsulated active ingredient, possibility to obtain a controlled or targeted release, versatility in encapsulating both lipophilic and hydrophilic drugs, high efficiency of encapsulation. Furthermore NPs represents an attractive strategy, since a significant amount of drug can be incorporated into the particle matrix because of the different drug binding sites present in the albumin molecule.

4.1 Andrographolide does not affect N2a viability

To exclude intrinsic toxicity of ANDRO and NPs-ANDRO we investigated their effects on N2a cultured cells performing the MTT test. The cells were seeded into 96 well plates and after 24h cells were treated with ANDRO or NPs-ANDRO for 48h, 24 h or 4h at three differents concentrations (1.0µM, 2.0µM or 3.0µM). We found that the viability of each group of treated cells was comparable to that of control cells (Fig 36 and Fig. 37).

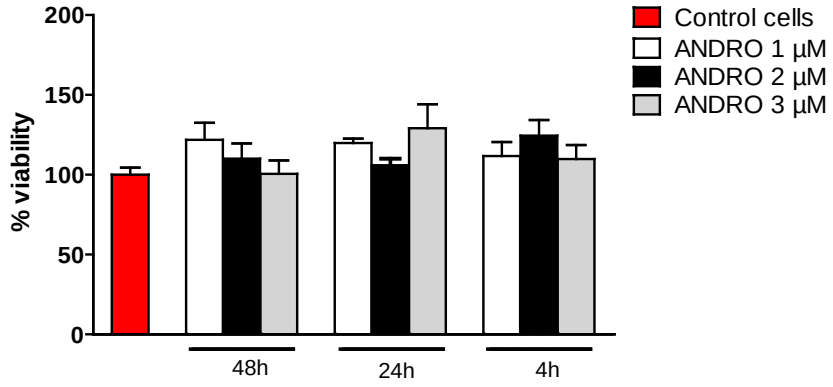

***Fig. 36 MTT in ANDRO-treated cells**: N2a cells were seeded into 96well plates and exposed for 48h, 24h or 4h to 1.0 µM, 2.0µM or 3.0µM ANDRO. Viability is expressed as a percentage respect to that of control untreated cells. Data are reported as mean ± S.E.M.*

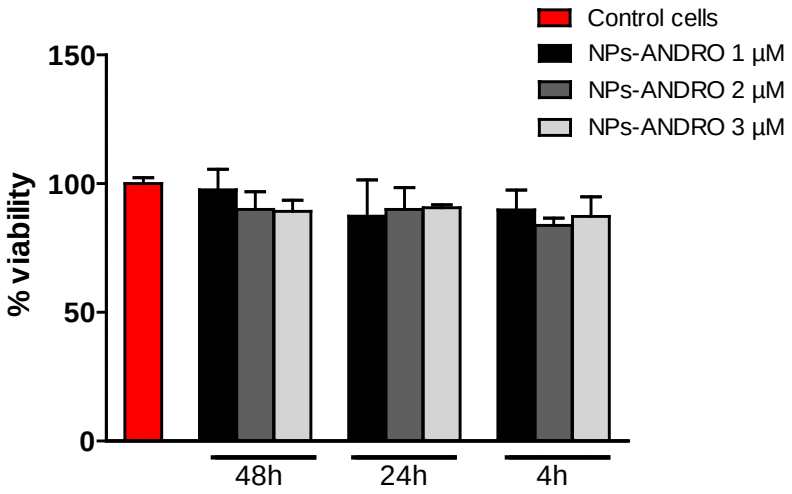

***Fig. 37 MTT in NPS-ANDRO-treated cells**: N2a cells were seeded into 96well plates and exposed for 48h, 2 h or 4h to 1.0µM, 2.0µM or 3.0µM of NPs-ANDRO. Viability is expressed as a percentage respect to control untreated cells. Data are reported as mean ± S.E.M.*

4.2 SLNs and NPs-ANDRO crosses the BBB

The presence of SLNs and NPs was evaluated by histochemical analysis.

SLNs were evaluated in groups of rats sacrificed at 3, 24 and 72 h after injection (Fig. 38 A). A high presence of SLNs (green) was observed in the brain of animals sacrificed 24 h after injection. In animals sacrificed 3 h and 72 h after injection, a fewer number of SLNs was identified probably due to the fact that in few hours after administration, the "long circulating" nanocarriers are located predominantly into the blood stream and after three days they are mostly degraded. In the control group (saline) no green fluorescence was observed, thus confirming the presence of SLNs in the other groups and excluding a possible autofluorescence of the tissue.

Subsequently, in the group of rats sacrificed at 24 h and characterized by an abundant presence of nanoparticles, the localization of SLNs was further evaluated. By immunohistochemical analysis with CD34 antibody, an endothelial cell marker, nanoparticles were found both at the vessel level (white arrows) and in brain parenchyma (yellow arrows) (Fig. 38 B). In the saline-injected rats (Fig. 38 B) no fluorescence was detected in either the vessels or brain tissue, further excluding the presence of nonspecific tissue autofluorescence in the SLNs-administered rats. Then, by means of IBA1 antibody that recognizes microglial cells, we evaluated whether the administration of SLNs leads to any microglia activation. As shown in Fig. 38 C, the morphology of microglial cells (red) in different brain areas of rats, sacrificed 24 h after

intravenous injection of SLN (green), is typical of that at rest and not different from that of saline-injected rats. This lack of microglia activation likely indicates that the brain macrophages do not recognize SLN as foreign bodies.

NPs were evaluated in the brain of TgCRND8 mice 4 weeks treatment and we found an high presence of NPs-ANDRO (green) in the brain parenchyma (Fig.39). Also in this case in the control group (untreated animals) no green fluorescence was observed. These data indicate that nanoparticles cross the BBB and reach brain tissues, suggesting that the developed nanocarriers may represent a new brain delivery system to increase the efficacy of natural molecules and their potential use in neurodegenerative diseases.

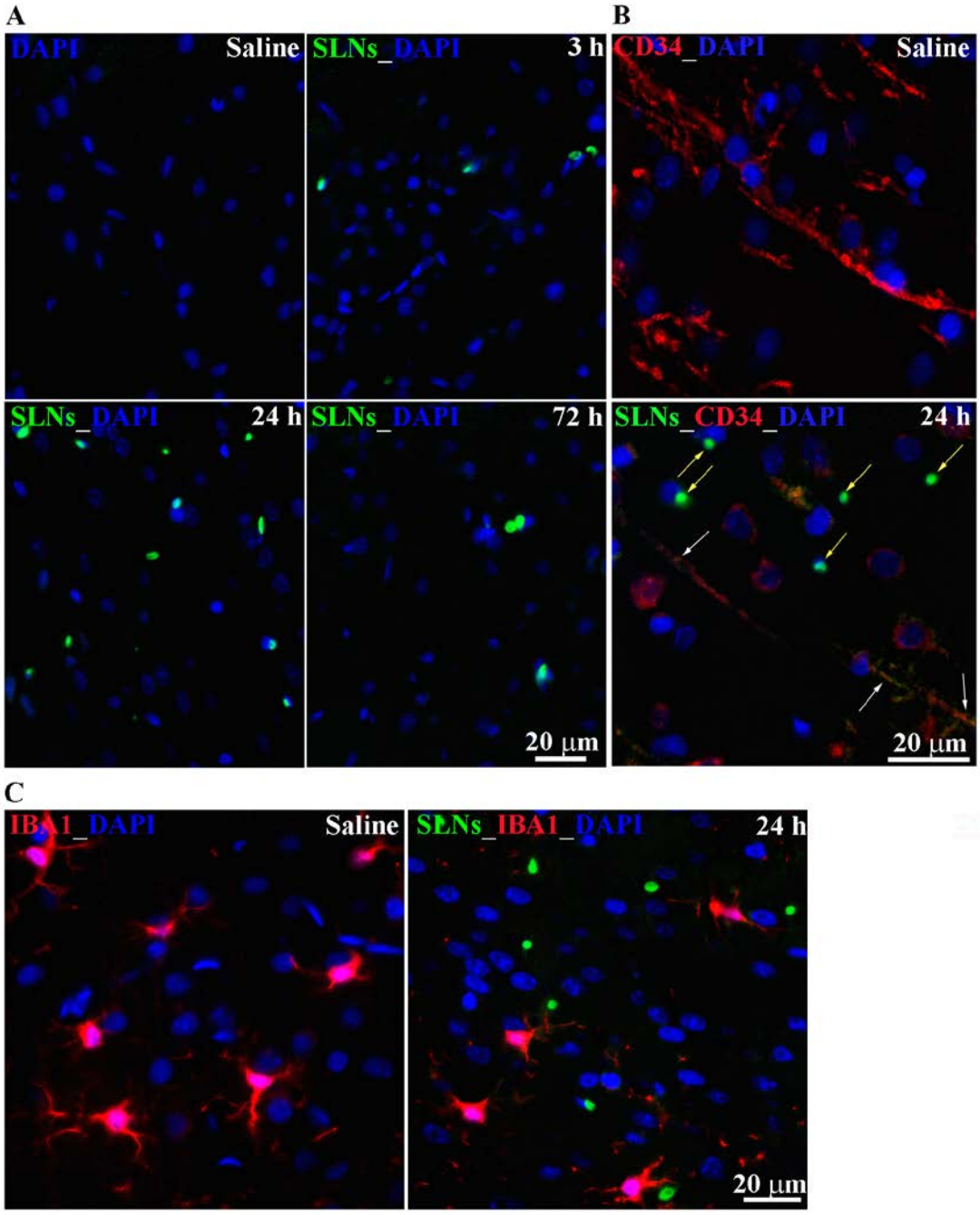

Fig. 38 A) Intravenous administration of fluorescent SLNs*: representative photomicrographs showing the presence of SLNs (green) in the brain parenchyma of rats 3, 24 and 72 h after injection. Note the high presence of fluorescent SLNs at 24 h after injection. Scale bar = 20 µm applies to all images. B) Immunohistochemical detection of SLNs (green) with the endothelial marker CD34 (red) in the brain of rats 24 h after injection. SLNs are detected both within blood vessels (white arrows) and brain parenchyma (yellow arrows). A-B) No SLNs are detected in the brain of rats injected with saline. Scale bar = 20 µm applies to both images. C) Immunohistochemical detection of SLNs (green) with the microglia antibody IBA1 (red) in the brain of rats 24 h after injection. SLNs are not detected within microglial cells and no microglia activation is detected compared to saline injected rats. Scale bar = 20 µm applies to both images. A-C) DAPI is in blue.*

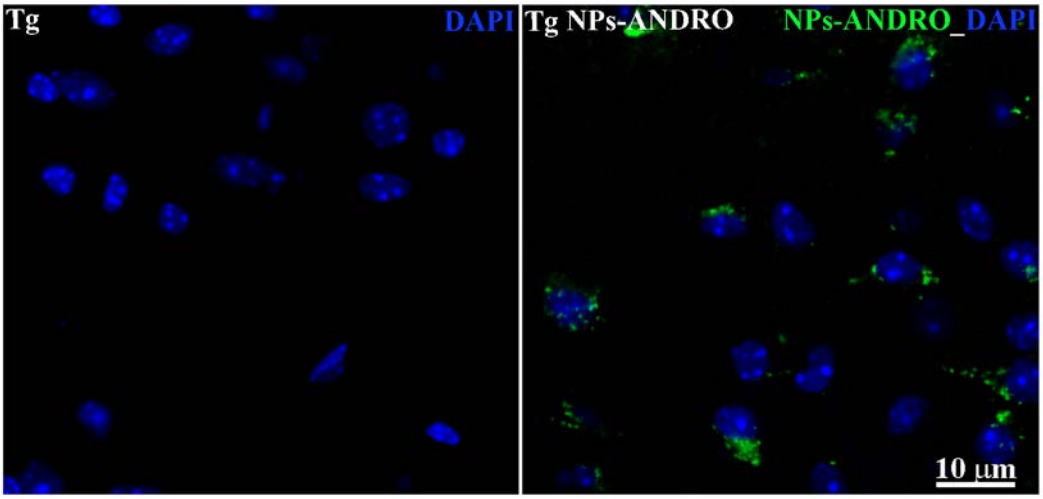

Fig. 39 Intraperitoneal administration of fluorescent NPs-ANDRO: *Representative photomicrographs showing the presence of NPs-ANDRO in the brain parenchyma of Tg mice as compared to control Tg mice. Scale bar =10 µm applies to all images.*

4.3 Behavioural performance: NPs-ANDRO ameliorate cognitive functions in TgCRND8 mice

NPs-ANDRO treatment was well tolerated and no evident side effects were revealed as shown in body weight graph in Fig. 40.

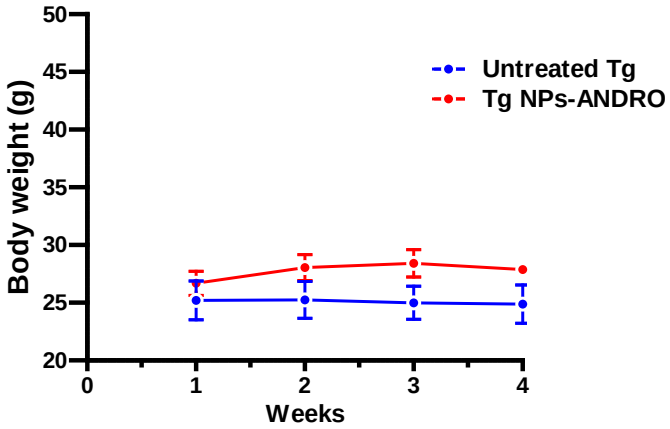

Fig. 40 Body weight graph of Tg NPs-ANDRO: *NPs-ANDRO did not affect body weight parameters.*

Memory performance was investigated with the tests described above in olive oil polyphenols experiments. In the step down inhibitory test (Figure 41 A: one way ANOVA plus Bonferroni *post hoc* test) the administration of NPs-ANDRO to Tg mice significantly improved their performance, that reached the level of wt mice. In the ORT, in the T2 trial we found that NPs-ANDRO treated Tg mice displayed significantly improved ability to discriminate between the familiar and novel object with respect to

untreated mice, reaching the performance of wt mice, as shown by their discrimination score below 0.5 (Fig. 41 B: one way ANOVA plus Bonferroni *post hoc* test). These data suggest that this treatment ameliorates cognitive impairment in our Tg model.

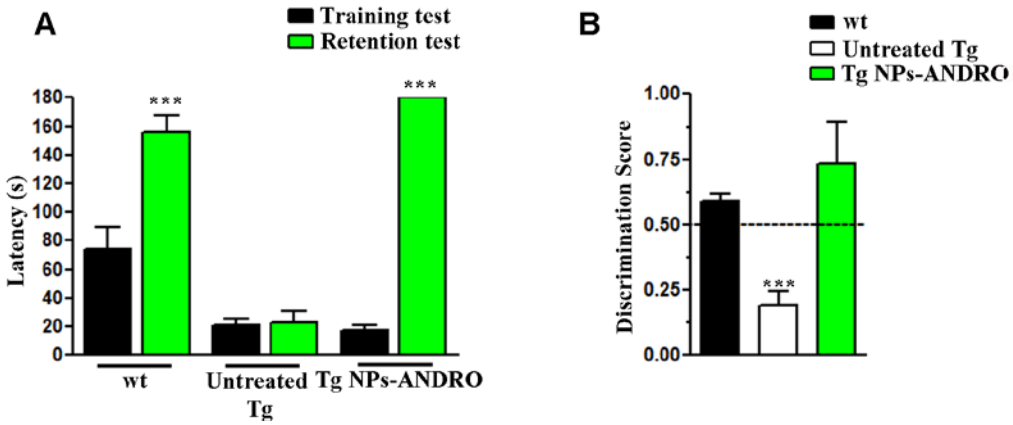

Fig. 41 NPs-ANDRO improve cognitive performaces in TgCRND8 mice: *A) Step down test: a statistically significant increase in the mean retention latencies in NPs-ANDRO-treated Tg mice, as compared to their respective training latencies (***P<0.0001). Untreated Tg mice did not show significant differences between training and retention latencies. B) ORT test: in the T2 trial the discrimination index of untreated Tg mice significantly differed from the discrimination index of all other groups (***P<0.0001). The dotted line indicates chance level performance. Statistical analysis: one way ANOVA plus Bonferroni post hoc test. Number of animals: n = 6/group.*

We conclude that NPs-ANDRO are harmless to cultured N2a cells, penetrate the BBB and are found inside neuronal cells of Tg mice, where they exert protection against cognitive impairment similarly to the results obtained with olive polyphenols, even though at lower concentrations. We have not investigated the molecular mechanisms underlying the improved cognitive performace of treated Tg mice.

DISCUSSION

Histone acetylation has been reported to improve cognitive deficits in animal models of AD and this indication has been proposed as a novel therapeutic strategy against AD (Adwan L. and Zawia N. H., 2013). Luccarini and colleagues (2015) have recently shown that OLE administration down regulates the expression of histone deacetylase 2 (HDAC2), an enzyme known to be up regulated in AD (Graff et al., 2012), both in the hippocampus and in the cortex of TgCRND8 mice and that such decrease matches a significant increase of histone H3 and H4 acetylation (Luccarini I. et al., 2015). Epigenetic involvement in some of the positive effects resulting from OLE administration is further supported by the modifications of the expression levels of effector proteins such as the glutaminyl cyclase, involved in the generation of pE3-Aβ, a particularly sticky Aβ derivative considered involved in plaque generation, and some autophagic players such as beclin-1 and LC3. Overall, these data agree with the reported ability of HT to restore the expression of a number of genes involved in the regulation of memory and survival in transgenic mice (Arunsndar M. et al., 2015).

We previously showed that dietary supplementation of OLE (50 mg kg^{-1} of diet) to TgCRND8 mice results in a significant reduction of both soluble and aggregated Aß and of the astrocyte reaction in AD relevant brain areas with a remarkable attenuation of Aß-mediated cognitive deterioration, LTP restoration and reduction of pE3-Aβ (Grossi et al. 2013; Luccarini et al. 2015). In addition, in aged mice displaying increased pE3-Aß in the brain deposits, we found that OLE is active against glutaminylcyclase-catalyzed pE3-Aß generation by reducing enzyme expression and that it interferes with both Aß42 and pE3-Aß aggregation (Luccarini I. et al., 2015). Similarly, here we report that feeding TgCRND8 mice with a mix of polyphenols from olive mill waste water or with HT at the same dose of 50 mg/kg of diet resulted in a remarkable improvement in both non-spatial episodic memory and working memory that correlates with the loss of Aß42 and pE3-Aß load in the brain (Pantano D. et al., 2017; Nardiello P. et al., 2017). Brain inflammation was also strongly reduced by EVOO polyphenols; in particular, we previously reported that OLE reduces astrocyte activation in Tg mice; here we found that OLE reduced NF-κB-mediated inflammation in the same mice and that HT treatment also reduced the inflammatory response by lowering the expression of TNF-α, a cytokine that plays a central role in directing the inflammatory state in the brain and is the only cytokine shown to be consistently implicated as detrimental in AD (Janelsins M. C. Et al., 2005). Altogether, these data show that olive polyphenols are beneficial either when administered as pure components, metabolites or as a mixture and that their effects are comparable.

Histone acetylation has been reported to improve cognitive deficits in animal models of AD and this indication has been proposed as a novel therapeutic strategy against AD. Then, in line with previous

observation indicating that polyphenols such as resveratrol, quercetin, and catechins are able to activate sirtuin 1 (SIRT1), a class III HDAC involved in the pathogenesis of different chronic pathologies, including neurodegenerative diseases (Chung S. et al., 2010), we have extended our previous epigentic study to SIRT1. SIRT1 requires NAD+, similarly to PARP1, that plays a role in many processes related to neurodegeneration (Martire S. et al. 2015). The normal function of PARP1 is the routine repair of DNA damage by adding poly (ADP ribose) polymers in response to a variety of cellular stresses by its ability to convert NAD+ in chains of PAR polymers. A significant accumulation of PAR polymers matching a significant increase of PARP1 activation following its enhanced expression was detected in the cortex of our Tg mice at the early (3.5 months) and intermediate (6 months) stage of Aβ deposition. However, OLE treatment for 8 weeks to 6-month-old TgCRND8 mice rescued both PARP1 activation and the accumulation of its product PAR to the values found in wt mice. Also in N2a neuroblastoma cells, PARP1 activation and PAR formation induced by cell exposure to the alkylating agent MNNG were abolished by 24 h pretreatment with either OLE or PARP inhibitors.

Our previous studies both *in vitro* with cultured cells (Rigacci S. et al., 2015) and in TgCRND8 mice (Grossi C. et al., 2013; Casamenti F. et al., 2015), together with the present study in N2a cells, indicate that OLE is a strong activator of autophagy. Autophagy is a basic mechanism that is activated to support cellular homeostasis under stress conditions as well as a survival strategy; however, autophagy can also be associated with programmed cell death (Chen Y. et al., 2008; Xu Y. et al., 2006). Furthermore, it has been reported that autophagy might be cytoprotective in response to DNA damaging agents and that PARP1 activation is involved in the regulation of this process (Munoz-Gamez J. A. et al., 2009). Accordingly, in N2a cells we have found a remarkable increase of the expression of Beclin1, an early autophagosome marker, by cell pretreatment with OLE for 24 h prior to exposure to MNNG.

The crosstalk between PARP1 and SIRT1 suggests that the increased activity of one protein might interfere with the activity of the other (Chung S. et al., 2010). Moreover, PARP1 was shown to enhance the transcription of NF-kB, while SIRT1 was found to inhibit NF-kB activity (Hassa P. O. et al., 2003; Yeung F. et al., 2004). The functional activity of p53, similarly to NF-kB, has been shown to be regulated oppositely by PARP1 and SIRT1 (Langley E. et al., 2002; Luo J. L. et al., 2001). Our experiments agree with this view, indicating that the activities of the two proteins are also oppositly regulated by OLE. Thus, the increased SIRT1 expression following OLE treatment might underlie the decreased PARP1 expression and hence the decreased PARP1-mediated expressions of NF-kB and p53, thus opposing to the inflammatory and apoptotic response to plaque pathology.

In conclusion, our *in vitro* and *in vivo* study highlights the existence of a complex interplay between EVOO polyphenols, apoptosis, autophagy, SIRT1, and PARP1 activity. In particular, we have reported for the first time that, in addition to autophagy, OLE and olive polyphenols interfere with the PARP1-mediated cascade of events leading both to apoptotic neuronal cell death via p53, to the inflammatory response via NF-kB and TNFα induction, and to the autophagic response via increased SIRT1 activity, further favoring cell survival.

The degeneration of the NBM represents a pathological correlate of the cholinergic derangement, in AD patients, as the impairment of the NBM cholinergic system is a crucial event in the progression of memory and cognitive decline associated to neurodegenerative disorders, such as AD. It therefore represents the main target for therapeutic approaches (Ferreira-Vieira et al., 2016).
We then investigated the ability of a primary cell culture of human fetal cholinergic neurons (hfCNs), isolated from the nucleus basalis of Meynert of 12-week old fetuses and labeled with PKH26 (PKH26-hfCNs), to migrate to the quisqualic acid (QA) injected NBM and to improve memory functions in rats. Our data show that i.v administered PKH26-hfCNs migrate to the QA-injected NBM and along the needle tract. Some of these PKH26-hfCNs were choline acetyltransferase (ChAT)-positive in saline and QA-injected rats.
In AD patients, the general consensus of memory impairment is that the working memory system is already compromised at the early stages of disease development (Desgranges et al., 1996). Thus, the working and spatial memory impairments of QA NBM-injected rats might correspond to early clinical stages of the disease. In the NBM-QA injected rats the cognitive impairments were significantly ameliorated 21 days after i.v. administration of hfCNs as indicated by their reduced escape latencies to find the platform during the training sessions in the MWM test and their improved performance, that reached the control level, in the step down test. Overall, the improved behavioral performances likely reflect improved cortical cholinergic functions; accordingly, our findings indicate that hfCNs could be used in cell therapy to replace cholinergic neurons in the nucleus basalis of Meynert in the early stages of AD (Morelli A. et al., 2017).
In the last period of my experimental work we have examined the encapsulation technique to deliver to the brain plant extracts and/or polyphenolic compounds in consideration of their reduced intestinal absorbance and bioavailability. Our preliminary data indicate that the formulation containing “stealth” SLNs is able to overcome the BBB and to reach the brain tissue that does not recognize them as a foreign agent. Furthermore, our previous *in vivo* study shows the ability of NPs to reach the brain after an acute systemic administration in rats and the lack of their toxicity in C57/B6 mice NPs-administered for two weeks. Our *in vivo* study confirmed that SLNs and NPs could cross the BBB and reach the brain tissue further supporting the possibility that these

nanocarriers represent a new potential brain delivery system to treat neurodegenerative disease (Graverini G. et al., 2018).

As an example, we used andrographolide, a labdane diterpene isolated from the stem and leaves of *Andrographis paniculta*. The compound was encapsulated in nanoparticles of albumins, a preparation displaying high drug loading capacity in combination with good biodegradability and biocompatibility. Our results show that these nanoparticles are able to cross the blood brain barrier, to reach the brain tissue and to ameliorate cognitive impairments in our Tg model, as reported for EVOO polyphenols. Altogether, these findings suggest that the developed nanocarriers can represent a new potential brain delivery system to be used to increase the efficacy of andrographolide, and possibly olive polyphenols, against neurodegenerative diseases.

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