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CHARACTERIZATION OF BETA AMYLOID DEPOSITION PROCESS IN THE BOVINE BRAIN: NEUROPATHOLOGICAL, IMMUNOBIOCHEMICAL AND GENETIC FEATURES

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Chiara PORCARIO
Nr. R08627

Tutor: Prof. Giorgio POLI and Dr. Cristina CASALONE

Coordinator: Prof. Fulvio GANDOLFI

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Chapter 1: Amyloidosis: definition and mechanisms

The word *amyloid*, meaning cellulose-like, was firstly introduced by Virchow in 1851. The pathologist detected a positive iodine-staining reaction during the histopathological examination of some human livers, which actually appeared very similar to that displayed by the extracellular material of wood and starch. The positive iodine-staining in the deposits detected was likely due to the presence of sulphate proteoglycans, now recognized as a common constituent of amyloid deposits. The term amyloid was hence conserved, though a few years later it was demonstrated that amyloid had a protein nature (Friedreich and Kekule, 1859). Amyloid is constituted by insoluble fibrils with a β- pleated sheet conformation, mostly deriving from the proteolysis of a normally soluble protein precursor (Sipe, 1994). Following cleavage of such precursor, the proteolytic fragments produced undergo pathological conformational changes into a β-sheet structure, which reduces their solubility, inducing their polymerization and subsequent aggregation. Fibrils are rigid, fine, non-branching, 8-10 nm wide and indeterminate in length, with the β-sheet conformation presenting a green birefringence under polarized light when stained with Congo Red or green fluorescence when stained with thioflavin T (Forloni et al., 1996). *Amyloidoses* can therefore be considered pathologic conditions caused by protein conformation disorders, i.e. "misfolding" diseases. The deposits of amyloid substance differ in protein composition depending upon the type of amyloidosis and the different clinical forms which may be encountered. This indicates that amyloid is a biochemically heterogeneous substance, although displaying similarities in properties and staining characteristics. At present 20-25 different types of proteins with the ability to aggregate, insolubilize, and deposit in tissue as amyloid have been identified (Murphy et al., 2001; Gruys, 2004; Woldemeskel, 2012) (Table 1).

Although amyloidosis is heterogeneous with regard to the biochemical composition and anatomical distribution of amyloid fibrils there are some common factors at the basis of the process of amyloid formation. Firstly, as formerly introduced, the involvement of a precursor protein that encompasses an amyloidogenic motif. Precursor proteins may undergo conformational changes following prior proteolysis, as happens in most cases, or even avoiding such cleavage. Secondly, the elevation of serum/tissue levels of the amyloid precursor protein, reflecting its overproduction, or impaired clearance, or both. Thirdly, the abnormal processing of precursor proteins, which is probably the most complex and not yet

fully disclosed issue in amyloidogenesis, in which genetic predisposition and disfunctions of the immune system seem to be involved. In some cases, the aberrant processing might be determined by mutant genetic variants of the precursor itself; in others, the precursors are normal wild type proteins, but impaired cellular functions or post-translational modifications may cause their incomplete degradation. Normally, misfolded proteins are degraded intracellularly in proteasomes or extracellularly by macrophages, which in amyloidosis fail to occur. The concept of processing also involves a group of amyloid associated proteins (chaperones) such as amyloid P component, glycosaminoglycans, apolipoprotein E and J (Castano et al., 1991). Further, the importance of tissue-related enzymes and their inhibitors may account for some of the regional selectivity of amyloid deposition, for example in vessel walls, brain parenchyma or peripheral nerves. Regarding humans, amyloidosis appears to be associated with a range of medical disorders including cancer, rheumatoid arthritis, chronic renal dialysis, familial amyloid polyneuropathy and diabetes. Thus, amyloid is supposed to be involved in ageing, lipid metabolism, acute phase response, peripheral nerve function, neuronal degeneration and infection with non-classical agents.

The clinical presentation of amyloidosis is also quite variable depending on the protein types and tissues involved and on the extent of functional disruption of the affected organs in the different species.

1.1 Amyloid nature and its distribution: the basis for the classification of amyloidosis

According to the WHO-IUIS Nomenclature Subcommittee (WHO-IUIS, 1993) on the nomenclature of amyloid and amyloidosis, amyloidosis is classified based on the amyloid fibril protein, followed by a designation of the fibril protein precursor. Therefore the capital letter A for amyloid is followed by the protein designation in abbreviated form (Table 1).

For example AL-amyloid refers to the amyloid derived from an immunoglobulin light chain or immunoglobulin heavy chain fragment (Gertz, 2004). In AL-amyloidosis unstable monoclonal immunoglobulin light chains, produced by a plasma cell discrasia, lead to the formation and deposition of fibrils. AL-amyloidosis is very rare in domestic animals, with few reports available describing systemic forms and few more cases reporting localized deposits. On the contrary, in humans the AL-amyloid type is the most common form of systemic amyloidosis.

The amyloid fibril protein in the immunoglobulin heavy chain has been given the designation AH.

AA-amyloid refers to the amyloid derived from serum A-amyloid protein (SAA), a high density lipoprotein with apoSAA as its precursor protein. AA-amyloidosis, besides being characterized by an increased level of SAA, which is common in inflammatory states, might be due to defects in the degrading monocyte-derived enzymes or to a genetically determined structural abnormality in the SAA molecule. AA amyloidosis is the most common form of amyloidosis in domestic animals. It is associated with chronic inflammatory or neoplastic diseases, or it may even be idiopathic, where no underlying disease is found. In AA amyloidosis the deposition in most species is in the central organs and tissues such as spleen liver, kidney and the arterial walls.

Some species of animals appear to have genetic predisposition to AA amyloidosis. Familial amyloidosis is reported in Siamese and Abyssinian cats (Boyce J.T. et al., 1984) and Shar Pei dogs (DiBartola S.P. et al., 1990) with AA-proteins differing in primary sequences and deposition patterns. The kidney is the main target organ for the deposition of amyloid in the Abyssinian cat and Shar Pei dogs, while the amyloid protein is mainly deposited in the liver in Siamese cats.

To give further insights into the classification, islet amyloid polypeptide is a precursor for AIAPP amyloid protein deposited in pancreatic amyloidosis. The deposition of islet amyloid polypeptide, a normal protein secreted by the β cells of the pancreas, is reported in pancreas of cats and macaques.

Eventually, another commonly recurring form of amyloidosis is due to the beta-amyloid (Aβ), which characterizes a variety of diseases such as Alzheimer's disease (AD), Down Syndrome (DS) and Dutch forms of hereditary cerebral hemorrhage with amyloidosis.

According to the extent of deposition in bodily tissues of man and animals, amyloidosis is classified as systemic or localized. Both the aforementioned types include inherited and non-inherited forms.

Amyloidosis involving several tissues and organs throughout the body is referred to as systemic amyloidosis, which can be due to AL-amyloidosis, AA-amyloidosis or familial forms of amyloidosis. Familial amyloidosis in humans could be due to mutations in fibrinogen, lysozyme, apolipoprotein AI, and transthyretin (with ATTR as amyloid protein).

Conversely, amyloid substance may also be confined at a given area in the body in the form of localized amyloidosis.

Various forms of local amyloid deposits are known in animals and humans. They include deposition of Aβ protein in AD, AIAPP in pancreatic islets and AANF in atrial amyloid deposit.

Regarding systemic amyloidosis, the affected organs may be enlarged and exhibit variable pallor grossly, or the amyloid deposit may be detected only after microscopic examination of the affected tissues without distinct grossly discernible lesions. In systemic amyloidosis amyloid appears as a pale eosinophilic homogenous extracellular deposit in tissues.

In localized forms of amyloidosis the amyloid fibrillar protein is deposited at a given site in an organ or tissue either as a grossly visible mass or as a microscopic deposit. For instance, localized AL-amyloidosis is characterized by limited growth of monoclonal plasma cells and restriction of amyloid deposits to the adjacent sites to those of the synthesis of the precursor (Merlini and Stone, 2006).

Amyloid protein [a,b]	Protein precursor	Protein type	Clinical diagnosis
AA	apoSAA		Reactive (secondary) amyloidosis, familial mediterranean fever, familial amyloid nephropathy with urticarial and deafness (Muckie-Wells' syndrome)
AL	*κλ*, for example, *κ*III	A*κ*, A*λ*, for example, A*κ*III	Idiopathic (primary) amyloidosis associated with myeloma/macroglobulinaemia
AH	IgG1 (γ^1)	Aγ^1	
ATTR	Transthyretin	For example, Met 30[c] For example, Met III TTR or IIe 122	Familial amyloid polyneuropathy, Portuguese Familial amyloid cardiomyopathy, Danish Systemic senile amyloidosis
AApoAI	apoAI	Arg 26	Familial amyloid polyneuropathy, Iowa
AGel	Gelsolin	Asn 187[d] (15)	Familial amyloidosis, Finish
ACys	Cystatin C	Gin 68	Hereditary cerebral hemorrhage with amyloidosis, Icelandic
Aβ	*β* protein precursor for example, *β*PP 695[e]	Gin 618 (22)	Alzheimer's disease, Down syndrome, and hereditary cerebral hemorrhage with amyloidosis, Dutch
A*β* 2M	*β*2-microglobulin		Associated with chronic dialysis
AScr	Scrapie protein precursor	Scrapie protein 27–30	Creutzfeldt-Jakob disease, and so forth
	33–35[f] cellular form	For example, Leu 102	Gerstmann-Straüssler-Scheinker syndrome
ACal	(Pro)calcitonin	(Pro)calcitonin	In medullary carcinomas of the thyroid
AANF	Atrial natriuretic factor		Isolated atrial amyloid
AIAPP	Islet amyloid polypeptide		In islets of Langerhans, diabetes type II, insulinoma
AIns[g]	Insulin		Islet amyloid in the degu (a rodent)
AApoAII[g]	apoAII (murine)	Gin5	Amyloidosis in senescence, accelerated mice

Table 1: Nomenclature and classification of amyloid and amyloidosis. Source: WHO/IUIS Nomenclature subcommittee.

[a]Nonfibrillar proteins, for example, protein AP (amyloid P-component) excluded.
[b]AA: amyloid A protein; SAA: serum amyloid A protein; apo: apolipoprotein; L: immunoglobulin light chain; H: immunoglobulin heavy chain.
[c]ATTR Met 30 when used in text.
[d]Amino acid position in the mature precursor protein. The position in the amyloid fibril protein is given in parentheses.
[e]Number of amino acid residues; [f]Molecular mass (kilodaltons); [g]Not found in humans.

1.2 Cerebral amyloidosis and related neurodegenerative disorders

Within the framework of localized forms of amyloidosis several neurodegenerative disorders are encountered.

In the human population Aβ-amyloid is involved in various neuropathological affections, the most common of which is AD. This disorder, which will be further described more in details, can present as a sporadic or familial form, with a presenile or senile esordium. Familial forms with a presenile esordium may be due to mutations in the gene encoding the precursor of Aβ protein, defined as amyloid precursor protein (APP), or in the genes encoding for presenilins, enzymes involved in the processing of APP. Furthermore, a risk factor for the senile esordium of the disease is represented by the presence of at least one allele encoding for the ε4 isoform of apolipoprotein E.

In AD deposition of Aβ fibrils occurs mainly extracellularly in the form of plaques but also intracellularly within neurons (LaFerla et al., 2007), and, in variable proportions, inside the blood vessels of the cerebral cortex and leptomeninges, where it gives origin to a neuropathological trait designated as congophilic angiopathy.

Neuropathological features similar to those aforementioned characterize nearly all the patients affected by DS reaching the adulthood. This finding is likely to be ascribed to an increase of Aβ protein amount in Down patients, due to hyperdosage of the gene encoding for APP, given the presence of a supernumerary chromosome 21.

Massive deposition of Aβ in the vessels of the cerebral cortex and leptomeninges, and, to a lesser extent, of cerebellum, basal ganglia and brainstem is the morphological substrate of a disease known as "Hereditary cerebral hemorrhage with amyloidosis, Dutch type", which is a dominant autosomal disease due to a point mutation responsible for the substitution of the amino acid at position 22 of Aβ. The disease, identified in several Dutch families, is characterized by recurrent and often lethal phenomena of ictus, caused by hemorrhages and strokes, occurring between 45 and 60 years of age.

In Iceland a familial form of cerebral amyloidosis due to the deposition of cystatin C in the small arteries has been identified. This severe congophilic angiopathy causes often lethal cerebral hemorrhages usually appearing between 20 and 40 years of age. The disease transmits by an autosomal dominant mechanism and is caused by a mutation at codon 68 of the gene encoding for cystatin C, determining a substitution of the amino acid leucine with glutamine. Unlike cerebral vascular amyloidosis due to deposition of Aβ, in the "Hereditary cerebral hemorrhage with amyloidosis, Icelandic" the amyloid deposition is systemic,

involving also blood vessel of extra cerebral tissues, such as salivary glands, spleen, testicles and skin.

Amyloid deposits in the central nervous system have been described in patients affected by familial amyloid polyneuropathy, a disorder due to mutations in the gene encoding for transthyretin. This disease is characterized by lacking of clinical neurological symptoms, though amyloid deposits of transthyretin occur inside leptomeninges and leptomeningeal vessels, in the subependymal cerebral tissue and inside choroid plexi, and eventually in the superficial layers of brain and spinal cord.

Another various group of neurological disorders categorized as cerebral amyloidosis is represented by prion diseases. Prion diseases are degenerative encephalopathies caused by protein misfolding disorders in which misfolding of the host-encoded prion protein (PrP) occurs. PrP may exist as a normal cellular prion protein, defined as PrP^{C} , and a pathogenic misfolded conformer, designated as PrP^{Sc}. Unlike PrP^{C}, PrP^{Sc} is insoluble and forms extracellular aggregates which can be amorphous or be structured as amyloid fibrils.

PrP^{Sc} are infectious, naturally transmissible misfolded proteins with neurotoxic properties and cause fatal neurological diseases in humans and a wide range of animal species (Savistchenko et al., 2011). Sixteen different variants of prion disease have been identified in humans and animals (Imran and Mahmood, 2011). Animal prion diseases include scrapie of sheep and goats, bovine spongiform encephalopathy (BSE), transmissible mink encephalopathy (TME), chronic wasting disease of cervids (CWD) and feline spongiform encephalopathy of felids (FSE).

In humans, prion diseases are traditionally classified into Creutzfeldt-Jacob disease (CJD), its variant form (vCJD) resulting from human infection by BSE prions, Gerstmann-Sträussler-Scheinker disease (GSS) and fatal familial insomnia (FFI) (Savistchenko et al., 2011).

Prion diseases can arise without any apparent cause as in sporadic Creutzfeldt-Jacob disease (sCJD) or due to genetic disorders linked to mutations in the endogenous PrP protein. The diseases can also be acquired by infection, through ingestion of contaminated products or through iatrogenic procedures and are in most cases experimentally transmissible (Imran and Mahmood, 2011). Infectivity and transmissibility of prion diseases has been much of a concern particularly since the outbreak of BSE in cattle. Cattle are infected with BSE when they ingest prion-contaminated meat and bone meal of ruminant origin contaminated with prions. Consumption of BSE-contaminated bovine tissues is associated with a fatal variant form of Creutzfeldt-Jacob disease (vCJD) in humans (Hill et al., 1997).

Amorphous deposits are the most common neuropathological feature of sCJD, which is mainly characterized by spongiosis of the cerebral and cerebellar cortices and of some subcortical nuclei. Only 10% of the patients affected by sCJD display deposits of PrP^{Sc}-amyloid, while the structured form of PrP^{Sc} fibrils as amyloid plaques is a neuropathological hallmark of vCJD, caused by transmission of the agent of BSE to man.

In sCJD affected patients PrP^{Sc} amyloid plaques are mainly confined to the cerebellar cortex; in vCJD patients, instead, PrP^{Sc} plaques are detected both in the cerebral and cerebellar cortices and in the adjacent neuropil they present a peculiar surrounding rim of spongiosis, on the basis of which the term "florid plaque" has been coined.

Cerebral PrP^{Sc}- amyloid plaques have been detected in 80% of the patients affected by Kuru, a prion disease nearly disappeared, which has been described in some tribes of New Guinea devoted to ritual cannibalism.

Eventually, PrP^{Sc}–amyloid is the neuropathological hallmark of another neurological disorder of man, the Gerstmann-Straüssler-Scheinker syndrome (GSS). It is an autosomic dominant familial disease which can be due to mutations at codons 102, 105, 117, 198, 212 and 217 of the PrP encoding gene (PRNP). In GSS PrP^{Sc} –amyloid accumulates in form of unicentric or multicentric deposits in the neuropil of cerebellum, cerebral cortex and in subcortical nuclei. It can be associated to spongiosis (as in case of mutation occurring at codon 102), or to alterations of the neuronal cytoskeleton (e.g. in case codon 198 and 217 are mutated).

In a rare GSS variant due to mutation at codon 145 of PRNP amyloid is mainly deposited in the vessels of cerebral and cerebellar cortices, thus displaying in the form of "PrP^{Sc} vascular cerebral amyloidosis".

Chapter 2: Aβ-amyloid in the brain: pathway of formation

2.1 Basic knowledge of the amyloid precursor protein (APP)

It was in the mid-1980s when brain amyloid plaques associated with AD were first purified and were found to consist of multimeric aggregates of Aβ polypeptide containing about 40 amino acid residues (Glenner and Wong, 1984). Subsequent cloning of the complementary DNA (cDNA) of Aβ revealed that Aβ is derived from a larger precursor protein (Tanzi et al., 1987). The full length DNA of the amyloid precursor protein (APP) was later isolated and sequenced and APP was predicted to be a glycosylated integral membrane cell surface receptor protein with 695 amino acids (Kang et al., 1987). The APP gene is located on chromosome 21 and contains 18 exons. Although alternative splicing of transcripts from the single APP gene results in several isoforms of the gene product, APP695, whose encoding cDNA lacks the gene sequence from exons 7 and 8, is preferentially expressed in neurons (Sandbrink et al., 1994). Two homologues of APP were also identified and named APP-like protein 1 and 2 (APLP1 and APLP2) (Coulson et al., 2000). APLP2, similarly to APP, is expressed ubiquitously while APLP1 is only expressed in the brain and is only found in mammals.

The APP protein is a type I integral membrane protein with a large extracellular portion, a hydrophobic transmembrane domain, and a short C-terminus designated the APP intracellular domain (AICD) (Fig. 2a). The extracellular portion of APP contains two domains, E1 and E2.

The precise physiological function of APP is not known and remains one of the vexing issues in the field.

Because of its highly similar structure to the developmental signaling molecule Notch, APP has been proposed to function as a cell surface receptor (Zheng and Koo, 2011). Several studies have reported that certain ligands, including F-spondin, Nogo-66, netrin-1 and BRI2, bind to the extracellular domain of APP, resulting in modulated APP processing and sequential downstream signals (Matsuda et al. 2009; Zheng and Koo 2011). However, the physiological functions of these interactions remain to be determined. Nevertheless, APP is more widely accepted as a protein contributing to cell adhesion via its extracellular domain. Studies have demonstrated that the E1 and E2 regions of APP can interact with extracellular matrix proteins (Small et al. 1999). Furthermore, the E1 and E2 regions of APP were found to interact with themselves, in parallel or anti-parallel, forming homo- (with APP) or hetero-

dimers (with APLPs) (Dahms et al., 2010). Recent studies also suggest APP/APLPs as synaptic adhesion molecules as silencing of APP led to defects in neuronal migration (Wang et al. 2009).

In most studies, APP overexpression shows a positive effect on cell health and growth. This effect is epitomized in transgenic mice that overexpress wild-type APP and have enlarged neurons (Oh et al. 2009). In transiently transfected cell lines, APP modulates cell growth, motility, neurite outgrowth, and cell survival. In adult animals, intracerebral injections of the APP ectodomain can improve cognitive function and synaptic density (Meziane et al., 1998).

2.2 APP processing

APP is produced in large quantities in neurons and it is metabolized very rapidly (Lee et al., 2008). After sorting in the endoplasmatic reticulum (ER) and Golgi, APP is delivered to the axon, where it is transported by fast axonal transport to synaptic terminals (Koo et al., 1990).

Crucial steps in APP processing occur at the cell surface and in the Trans-Golgi network (TGN) (Fig. 1). From the TGN, some of the newly synthesized APP can be transported down the axon to the cell surface, where it takes insertion. There one of the two ascertained post – translational APP processings, the so called "non-amyloidogenic pathway", may occur. According to this pathway APP is sequentially cleaved by α-secretase and γ-secretase (Fig. 2b). Although most APP must pass through the cell surface as part of its processing, this step is very rapid, as a small amount of APP is on the surface at any point in time. The cleavage by α-secretase releases the sAPP-α fragment, which diffuses away extracellularly, and a membrane-associated C-terminal fragment consisting of 83 amino acids (APP C83). APP C83 is further clived by γ-secretase to release P3 peptide and the AICD, both of which are degraded rapidly.

Cleavage of APP by α-secretase is attributed to the ADAM (a disintegrin and metalloproteinase) family of proteases (Asai et al., 2003; Jorissen et al., 2010) and takes place, to a large extent, on the cell surface. However, there is some α-secretase activity in the TGN.

The constitutively secreted sAPP-α has been found to be neuro-protective (Han et al. 2005; Ma et al., 2009). sAPP-α is thought to promote neurite outgrowth and synaptogenesis as well as cell adhesion (Mattson, 1997; Gakhar Koppole et al., 2008). Studies have found that sAPP-α is a growth factor (Herzog et al., 2004) that regulates the proliferation of embryonic and

adult neural stem cells (Ohsawa et al.,1999; Caille et al., 2004). sAPP-α alone is able to rescue most of the abnormalities of APP deficient mice (Ring et al., 2007), implying that most of the physiological functions of APP are conducted by its extracellular domain.

No biologically relevant roles are currently established for the carboxyterminal fragments APP C83 and P3 generated by α- and γ-secretase, respectively. Regarding AICD, as the molecule is quickly degraded after γ-cleavage, the biochemical features and physiological functions of AICD in vivo are difficult to study.

Some of the APP directly sorted from the Golgi apparatus, as well as some of the APP present on the cell surface, can be further transported into clathrin-coated pits to endosomal compartments, containing the enzymes β-secretase and γ-secretase. There the "amyloidogenic pathway" of APP processing occurs. Firstly, β-cleavage at the ectodomain of APP generates an sAPP-β domain and the membrane associated APP C-terminal fragment C99 (APP C99). Subsequently, APP C99 is cleaved by γ-secretase to release Aβ which has neurotoxic properties (Fig. 2c). β-site APP cleaving enzyme 1 (BACE1) is the major β-secretase in the brain (Vassar et al., 1999). BACE 1 can also cleave APP at a more carboxy-terminal position, resulting in CTF89 (and Aβ 11-40 after γ-secretase cleavage). A related protein, BACE2, also can exert β-secretase activity (Hussain et al., 2000), but it is expressed at very low levels in the brain and is mostly confined to glial cells (Laird et al., 2005). BACE 1 was first identified and characterized in 1999 (Sinha et al., 1999), as a type 1 transmembrane aspartyl protease with its active site on the luminal side of the membrane. The originally identified full-length BACE1 has 501 amino-acids (BACE1-501) and is predominantly expressed in the perinuclear post-Golgi membranes, vescicular structures throughout the cytoplasm (Huse et al., 2002), as well as on the cell surface (Ehehalt et al., 2002). Although BACE1 reaches the plasma membrane due to vescicle traffic, it is recycled quickly, and very little BACE1-mediated APP cleavage occurs at the plasma membrane; instead APP is cleaved by BACE primarily in endocytic vescicles.

The proteolytic fragment sAPP-β generated by β-secretase reportedly lacks most of the neuroprotective effects of sAPP-α (Furukawa et al., 1996). A recent study suggested that sAPP-β can be cleaved to generate an N-terminal fragment that is a ligand for death receptor 6, activating caspase 6 which further stimulates axonal pruning and neuronal cell death (Nikolaev et al., 2009). Regarding the carboxy-terminal fragments CTF99 and CTF89 a biological role still has to be disclosed.

The APP CTFs generated by both α-cleavage and β-cleavage are further processed by γ-secretase. Distinct from α-/β-secretases, γ-activity involves a large proteinase complex

consisting of at least four major components (Presenilin1 or Presenilin2, Presenilin enhancer 2 (PEN2), Anterior pharynx-defective 1 (APH1) and Nicastrin) (Vetrivel et al., 2006).

Presenilins (PSs) were identified and cloned in the mid-1990s (Levy-Lahad et al., 1995). PSs are multi-transmembrane proteins and can be cleaved at the cytoplasmic loop between the sixth and seventh transmembrane regions to generate an N-terminal and a C-terminal fragment during post-translational maturation (Thinakaran et al., 1996). The two fragments interact with each other and they are both necessary for γ-secretase activity.

Nicastrin, identified as a protein that interacts with PS in 2000, is a type I membrane glycoprotein with a large ectodomain (Fagan et al., 2001). Nicastrin undergoes a glycosylation/maturation process that causes a conformation change in its ectodomain, which is crucial for the assembly and maturation of the γ-secretase complex and γ-activity (Chavez-Gutierrez et al. 2008). Mature nicastrin can bind to the ectodomain of APP C-terminal fragments derived through α-/β-secretase cleavage and may act as a substrate receptor of γ-secretase (Shah et al., 2005).

PEN2 and APH1 are another two γ-secretase complex components that were originally identified as the enhancers of PSs (Francis et al., 2002). APH1 is a multiple transmembrane protein with seven transmembrane domains and a cytosolic C-terminus (Fortna et al., 2004). APH1 interacts with immature nicastrin and PS to form a relatively stable pre-complex which is then translocated to the trans-Golgi from the ER/cis-Golgi for further maturation (Niimura et al., 2005). PEN2 is a hairpin-like protein with two transmembrane domains and with both ends in the lumen (Crystal et al., 2003). PEN2 is found to mediate the endoproteolysis of PS (Luo et al., 2003).

The γ-secretase complex is assembled in sequential steps. Nicastrin and APH1 initially form a subcomplex and then PS binds to the Nicastrin-APH1 subcomplex. The joining of PEN2 results in a conformation-dependent activation of γ-secretase (Niimura et al., 2005). Nicastrin, PEN2, APH1 and PS interact with each other and also mutually modulate each other (Kaether et al., 2004).

γ-Secretase cleaves APP at multiple sites and in sequential steps to generate Aβ peptides of different lengths.

The majority of Aβ peptides produced are 40 amino acids long (Aβ 40), however, peptides ranging from 38 to 43 amino acids are found in vivo. A small proportion (approximately 10%) of Aβ forms is the 42 residue variant ($A\beta_{42}$). Some Aβ forms (e.g. Aβ 1-42, Aβ 3-40) are considered more hydrophobic and more prone to fibril formation than others (e.g. Aβ 1-40

or 1-38) (Jarrett et al., 1993). $A\beta_{42,}$ the more amyloidogenic species, is also the predominant isoform found in cerebral plaques (Perl D. P., 2010).

Besides the dominant γ-cleavage site at 40 and 42 residues, ζ-cleavage at 46 and ε-cleavage at 49 residues are also thought to be mediated by γ-secretase (Weidemann et al., 2002; Zhao et al., 2004). Accordingly, various AICDs (C50, C53, C57 and C59) can be generated during these multi-site cleavages by γ-secretase. Interestingly, as the substrate of γ-secretase, APP itself can regulate the intracellular trafficking and cell surface delivery of PS1 (Liu et al., 2009). In addition, APP has been found to possess a domain that negatively modulates γ-secretase activity in Aβ production by binding to an allosteric site within the γ-secretase complex (Zhang and Xu 2010). These results reveal a novel mutual regulation between γ-secretase and its substrate.

2.3 Aβ accumulation process

2.3.1 Intraneuronal Aβ accumulation process

Not long after the discovery of Aβ peptide as a component of extracellular amyloid plaques in the mid-1980s, descriptions of the presence of Aβ inside neurons began to appear in the literature. In the first study reporting the presence of intraneuronal Aβ, Aβ immunoreactive material was observed in neurons in the cerebellum, cerebrum and spinal cord of individuals either affected or not by AD. As the age of the partecipants in the study ranged from 38 to 83 years, these findings suggested that the occurrence of intracellular Aβ might not be an age-dependent event (Grunke-Iqbal et al., 1989). Since the original report, there have been a large number of studies on post-mortem AD, DS and transgenic mouse brains which have provided evidence for the presence of intracellular Aβ within neurons. Careful studies using C-terminal specific antibodies against $A\beta_{40}$ and $A\beta_{42}$ have established that most of the intraneuronal Aβ ends at residue 42, and not at residue 40 (Gouras et al., 2000). Furthermore, immunogold electron microscopy has been carried out to demonstrate that $A\beta_{42}$ is localized to the outer membrane of multivescicular bodies (MVBs) of neurons in the human brain, where it is associated with synaptic pathology (Takahashi et al., 2002). MVBs are considered late endosomes and are formed from the early endosome system.

Recent studies suggest that the buildup of intracellular Aβ may be an early event in the pathogenesis of AD and DS. In patients with mild cognitive impairment (MCI), intraneuronal Aβ immunoreactivity has been reported in brain regions that are more prone to the

development of early AD pathology, such as the hippocampus and the entorhinal cortex (Gouras et al., 2000). Similarly, it has been shown that the accumulation of intracellular Aβ precedes extracellular plaque formation in patients with DS (Gyure et al., 2001). These results suggest that the accumulation of intraneuronal Aβ is an early event in the progression of AD, preceding the formation of extracellular Aβ deposits. Indeed, it has been demonstrated that intraneuronal Aβ levels decrease as extracellular plaques accumulate (Mori et al., 2002). These conclusions are also consistent with results from transgenic mouse models, in which intracellular Aβ accumulation appears as an early event in the progression of the neuropathological phenotype, preceding the accumulation of extracellular Aβ plaques (Wirths et al., 2001). According to further studies on patients with AD and DS and controls it would seem that intraneuronal Aβ immunoreactivity appears in the first year of life, increases in childhood and stabilizes in the second decade of life, remaining high through adulthood even in healthy brains.

Although intracellular Aβ accumulation is now ascertained, how intracellular Aβ builds up is still being addressed. To investigate this issue, it is pivotal to focus on APP cleavage and releasing from its parent protein, APP. As mentioned before, APP localizes to the plasma membrane, but also to the TGN, ER and endosomal, lysosomal (Kinoshita et al., 2003) and mitochondrial membranes (Mizuguchi et al., 1992). As Aβ generation may potentially occur wherever APP and β- and γ- secretase are localized, it is likely that this occurs in several cellular compartments. In case Aβ cleavage occurs inside the cell, then Aβ would be intracellular; if liberation of Aβ occurs at the plasma membrane or in the secretory pathway, then it would be released into the extracellular fluid. Both the pathways are likely to occur, but the majority of Aβ is secreted out of the cell, suggesting that Aβ is predominantly produced as part of the secretory pathway, or, to a lesser extent, at the plasma membrane. Actually APP which is not cleaved at the plasma membrane could be re-internalized into the early/late endosome system by endocytosis (Golde et al., 1992). The endosomes are a likely site of intraneuronal Aβ generation owing to their acidic nature – BACE1 has optimal activity at acidic pH, and APP and BACE1 interactions have been observed by fluorescence resonance energy transfer (FRET) microscopy within the endosomes (Kinoshita et al., 2003). BACE1 cleavage of APP inside endosomes results in the 99 amino-acid fragment C99, which can be re-directed to different compartments. It can be shuttled back to the ER to be processed into Aβ by ER γ-secretase, shuttled back to the plasma membrane where the γ-secretase complex is also found, or processed to Aβ within the endosome/lysosome system. Therefore

the internalization of APP by endocytosis is one of the important pathway for the generation of Aβ.

Furthermore, besides the endosome system, strong evidence suggests that Aβ is also generated intracellularly along the secretory pathway (Busciglio et al., 1993). Actually it has been ascertained that $A\beta_{42}$ and $A\beta_{40}$ are produced in the ER (Wild-Bode et al., 1997) and in the TGN (Hartmann et al., 1997) respectively. Interestingly, these sites of Aβ production were limited to neurons, as in non-neuronal cells both $A\beta_{42}$ and $A\beta_{40}$ were produced at the cell surface rather than intracellularly.

In addition to Aβ being produced intracellularly, it is also possible that previously secreted Aβ, which forms the extracellular Aβ pool, may be taken up by cells and internalized into intracellular pools. Aβ can bind to various biomolecules of the plasma membrane, including lipids, proteins and proteoglycans. Consequently, it is likely that some intracellular Aβ is derived from extracellular Aβ pools, taken up into the cells through receptors or transporters. A number of putative Aβ transporters have been identified.

Aβ binds to the α7 nicotinic acetylcholine receptor (α7nAChR) with high affinity, and it has been shown that this binding results in receptor internalization and accumulation of Aβ intracellularly (Nagele et al., 2002).

Recent studies have also shown that apolipoprotein E (APOE) receptors, members of the low-density lipoprotein receptor (LDLR) family, modulate Aβ production and Aβ cellular uptake (Bu et al., 2006). Another member of this family, the LDL receptor related protein (LRP), binds to Aβ directly, or through ligands such as APOE, and undergoes rapid endocytosis, facilitating Aβ cellular uptake (Bu et al., 2006).

APOE*ε4 is the major genetic risk factor for AD, and it is notable that one of its functions appears to be to directly mediate the accumulation of intracellular Aβ.

In addition to LRP and nicotinic receptors, Aβ internalization has been reported through the scavenger receptor for advanced glycation end products (RAGE), in neurons and microglia (Deane et al., 2003). In addition to these downstream effects, it has been demonstrated that RAGE–Aβ complexes are internalized and that they co-localize with the lysosomal pathway in astrocytes in the brain of patients with AD (Sasaki et al., 2001).

The formyl peptide receptor-like 1 (FPRL1) is a G-protein-coupled receptor associated with inflammatory cells, including astrocytes and microglia, that binds to Aβ and mediates the chemotactic response to Aβ42. Internalization of Aβ is rapid and results in cytoplasmic Aβ aggregates that stain with Congo red in macrophages (Yazawa et al., 2001).

Neuronal Aβ uptake has also been shown to be mediated through NMDA (*N*methyl-d-aspartate) receptors. Blocking this NMDA receptor–Aβ internalization prevents pathogenicity, including increased microglial activation and cathepsin D levels (Bi et al., 2002).

It is plausible that intracellular Aβ has different roles in different cell types and that internalization in glial cells may be part of the regulatory system that seeks to control rising extracellular Aβ levels by taking the peptides up and degrading them. In neurons, the effects of intracellular Aβ are likely to be different.

2.3.2 Pathological role of intracellular Aβ *in vivo*

A recent study characterizing intracellular accumulation of Aβ in humans, including patients with AD, concluded that intracellular Aβ was abundantly present, and apparently independently of plaque load or formation of neurofibrillary tangles (NFT) (Wegiel et al., 2007). The investigation of the functional consequences of intracellular Aβ in the human brain is limited to correlational studies in post-mortem brains. To better address the consequences of intracellular Aβ, animal models provide significant advantages that extend beyond mere correlations.

Within neurons, Aβ42 appears to be predominantly localized to MVBs and lysosomes (Fig. 3). In mouse experimental models (Langui et al., 2004) Aβ-containing MVBs were most often located in the perinuclear region.

It has recently been shown that Aβ accumulation within MVBs is pathological, leading to disrupted MVB sorting via inhibition of the ubiquitin–proteasome system (Almeida et al., 2006). As the proteasome is primarily located within the cytosol, and as Aβ has been shown to inhibit the proteasome directly (Gregori et al., 1995), this observation suggests that intracellular Aβ within the MVBs is mechanistically linked to cytosolic proteasome inhibition. The proteasome inhibition has been demonstrated to lead to higher Aβ levels, both *in vivo* and *in vitro* (Oddo et al., 2006a; Tseng et al., 2005), suggesting that the proteasome degrades Aβ, and that Aβ must be within the cytosolic compartment for this degradation to occur. MVBs are also viewed as major transport organelles within neuronal processes (Weible and Hendry, 2004), and Aβ accumulation in their outer membranes may disrupt vital cargo transport. Endosomal vescicles such as MVBs are an ideal location for Aβ aggregation, since the lipid membranes and low pH within these vescicles favor Aβ aggregation. Actually Aβ

oligomerization was observed within endosomal vescicles with AD pathogenesis using oligomer specific antibodies (Takahashi et al., 2004).

In Tg2576 mice, accumulation of Aβ has also been observed in mitochondria (Manczak et al., 2006), organelles in which all subunits of the γ-secretase have been located. Progressive accumulation of intracellular Aβ in mitochondria is associated with diminished enzymatic activity of respiratory chain complexes III and IV, and a reduced rate of oxygen consumption (Caspersen et al., 2005). These observations may help to explain the multitude of mitochondrial defects described in AD and mouse models of the disease (Keil et al., 2006).

There is evidence for a role for intraneuronal Aβ in synaptic dysfunction, which could underlie cognitive deficits. The 3xTg-AD mouse model of AD develops intraneuronal accumulation of Aβ at 4 months of age, which is when cognitive deficits are first detected (Billings et al., 2005). Furthermore, the electrophysiological responses were recorded, and it was found that the appearance of intraneuronal Aβ led to a profound deficit in long-term potentiation (LTP) (Oddo et al., 2003), a form of synaptic transmission thought to underlie memory.

Aβ is produced as a monomer, but readily aggregates to form multimeric complexes. These complexes range from low molecular weight dimers and trimers to higher molecular weight protofibrils and fibrils. The oligomeric species of Aβ have been found to be the most pathological, from dimers disrupting learning and memory, synaptic function and long term potentiation (LTP) (Cleary et al., 2005; Walsh et al., 2002), to dodecamers affecting cognition and memory in transgenic mouse models (Lesne et al., 2006). It has been shown that in tissue derived from human brain, Aβ oligomerization initiates within cells rather than in the extracellular space (Walsh et al., 2000).

Aβ oligomerization has also been shown to occur during interactions with lipid bilayers, in particular cholesterol- and glycosphingolipid-rich microdomains known as lipid rafts (Kim et al., 2006) : it was found that Aβ fibrillogenesis was accelerated in the presence of plasma and endosomal and lysosomal membranes (Waschuk et al., 2001).

2.3.3 Accumulation process and role of extracellular Aβ

The intraneuronal Aβ deposition process does not counter the established evidence for a pathogenic role also for Aβ secreted extracellularly as part of the constitutive secretory pathway (Walsh and Selkoe, 2004). Furthermore, Aβ accumulating and aggregating in dystrophic and degenerating neuronal processes and synapses eventually leads to their dissolution and the subsequent extracellular localization of Aβ.

Aβ is thought to gradually increase in the extracellular space until it begins aggregating to form insoluble β-pleated amyloid plaques, which in turn may propagate Aβ toxicity to surrounding neurons and their processes. The fact that extracellular Aβ can influence intracellular Aβ suggests that Aβ may have prion-like properties.

It remains to be established whether plaque formation depends on extracellular- or previously released intracellular- Aβ propagation to surrounding neuronal processes by a mechanism of permissive templating (self-seeding), or on the damage inflicted to Aβ surrounding neuronal processes secondary to the occurrence of inflammatory mechanisms. Inflammation, including the recruitment and activation of microglia, could lead to digestion of most of the degenerated neuronal remnants, while leaving indigestible Aβ behind as plaques.

Extracellular Aβ oligomers have been shown to impair physiological processes involved in learning and memory, and injection of Aβ oligomers in rodent brains induced learning impairment (Cleary et al., 2005).

The currently predominant hypothesis states that Aβ forms soluble oligomers in the extracellular space and that these oligomers inhibit NMDA-mediated synaptic transmission and ultimately cause spine and synapse loss through mechanisms not yet fully understood (Selkoe, 2000).

2.3.4 Potential normal function of Aβ

The constitutive generation and normal presence of Aβ in the brain supports the hypothesis that Aβ may have a normal physiological function, rather than merely being a toxic by-product of APP processing. Neuronal excitation increases secretion of Aβ, which, in turn, depresses synaptic function decreasing neuronal activity. In this context, the Aβ peptide could have a negative feedback function preventing excitotoxicity (Kamenetz et al., 2003).

Although excessive Aβ causes neurotoxicity, some studies have shown that Aβ 40 protects neurons against Aβ 42- induced neuronal damage and is required for the viability of central neurons (Plant et al., 2003; Zou et al., 2003). Moreover, two groups recently reported that low doses (picomolar) of Aβ can positively modulate synaptic plasticity and memory by increasing hippocampal LTP (Morley et al., 2008; Puzzo et al., 2008), revealing a novel positive physiological function of Aβ under normal conditions. Picomolar levels of Aβ can also rescue neuronal cell death induced by inhibition of Aβ generation (by exposure to inhibitors of β - or γ-scretases) (Plant et al., 2003), possibly through regulating the potassium ion channel expression, hence affecting neuronal excitability (Plant et al., 2006).

Interestingly, according to other studies, Aβ has been shown to possess a number of trophic properties that emanate from the protein's ability to bind Cu, Fe and Zn (Atwood et al., 2003). Actually Aβ belongs to a group of proteins that capture redox metal ions, thereby preventing them from participating in redox cycling with other ligands. The coordination of Cu appears to be crucial for Aβ's own antioxidant activity that has been demonstrated both in vitro as well as in the brain, cerebrospinal fluid and plasma. Given that oxidative stress is one of the earliest pathological events in AD pathogenesis (Smith et al., 2000), as well as an increasing phenomenon during normal brain aging, in both cases promoting Aβ generation, the formation of Aβ diffuse amyloid plaques in the brain is likely to be seen as a compensatory response to remove reactive oxygen species. Within this perspective it has been proposed that Aβ deposition may act as a "sink" for trapping potentially harmful transition metal ions (particularly redox active metal ions) that can be released from metal-binding proteins by oxidative and mildly acidotic conditions, such as those accompanying acute brain trauma and AD, and that would otherwise catalyze adverse oxidation of biomolecules (Atwood et al., 1998; Kontush et al., 2001). Therefore Aβ generation would be aimed at reducing oxidative damage, thereby preventing ROS-mediated neuronal apoptosis, sealing vessels and promoting neurite outgrowth. The capture of metal ions in turn would promote the aggregation of Aβ that deposits as diffuse amyloid.

It has been postulated that, rather than Aβ itself, it may be the product of Aβ's antioxidant activity, that is hydrogen peroxide (H_2O_2), to mediate toxicity as the levels of this oxidant rise with the accumulation of Aβ in the AD brain.

2.3.5 Linking intracellular and extracellular Aβ

A number of factors have been shown to modulate intraneuronal Aβ in animal models of AD, subsequently affecting also the extracellular Aβ pool. One of the most interesting observations is the effect of aging. For example, young 3xTg-AD mice accumulate both soluble and oligomeric Aβ within neuronal cell bodies, but the intraneuronal pool decreases at ages in which extracellular plaques manifest (Oddo et al., 2006b). This finding also parallels studies in human brain tissue, including that from patients with AD and DS (Mori et al., 2002; Wegiel et al., 2007). These studies suggest that the brain of patients with early stage AD might have more abundant intraneuronal Aβ, which then becomes extracellular as the disease progresses and neuronal death and lysis occur. Actually it has been demonstrated that intraneuronal Aβ levels in AD decrease as extracellular plaques accumulate. It may be that the

relatively low levels of intracellular Aβ in AD (compared with relatively high extracellular Aβ levels) are vital at first for the seeding of toxic oligomers giving rise to pathological events and further for extracellular plaque formation by secretion of these oligomeric species into the extracellular space. Secreted oligomers may also facilitate other pathological events, such as disruption of synaptic transmission.

It is likely that both the intra- and extra- cellular Aβ pools contribute to cognitive decline in AD, and there is a complex relationship between these two pools and the various Aβ aggregation states. For example, 3xTg-AD mice that repeatedly learned to locate a hidden platform in the Morris water maze show improved cognition compared to animals that were not trained (Billings et al., 2007). More significantly, this learning alters the dynamics between intraneuronal Aβ, extracellular plaques and Aβ oligomerization. Learning increased intraneuronal and soluble Aβ, but decreased extracellular and oligomeric Aβ, with the net effect being improved cognition. Thus, the reduction in extracellular and oligomeric Aβ was highly beneficial, despite increases in intraneuronal Aβ in aged mice with established extracellular Aβ pathology.

Other studies turned to Aβ immunotherapy to determine whether the intracellular and extracellular Aβ pools are related. Aβ immunotherapy has been used in various mouse models and quickly and effectively leads to clearance of the extracellular plaque load and improved cognition (Janus et al., 2000). In the 3xTg-AD model, removal of extracellular Aβ plaques is shortly followed by the clearance of intraneuronal Aβ (Oddo et al., 2004). Notably, as the pathology re-emerges, intraneuronal Aβ appears first, followed by the extracellular plaques. These observations show that clearance of extracellular Aβ with immunotherapy also leads to the indirect reduction of intraneuronal stores. This finding indicates that extracellular Aβ may originate from intraneuronal pools and that a dynamic equilibrium exists between the two pools, such that when extracellular pools are removed, intraneuronal pools are sequestered out of the cell.

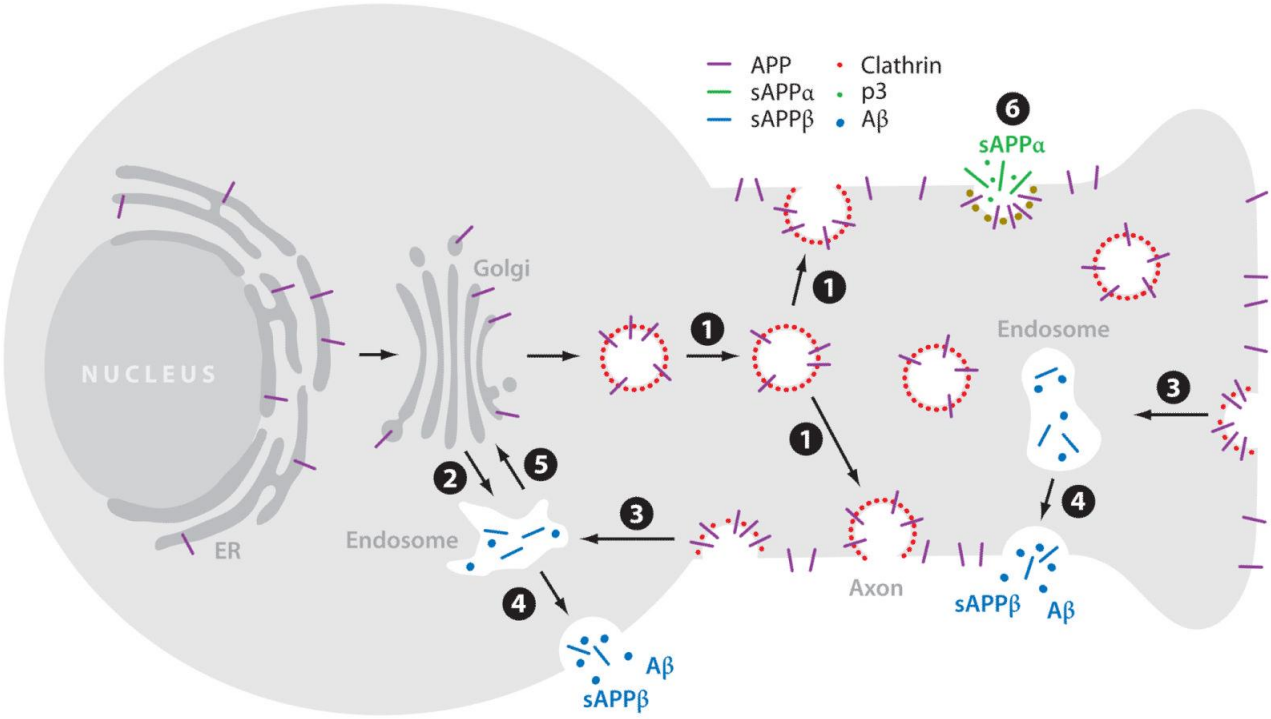

Fig. 1 : APP trafficking in neurons (from O'Brian et al., 2011). Newly synthesized APP (*purple*) is transported from the Golgi down the axon (1) or into a cell body endosomal compartment (2). After insertion into the cell surface, some APP is cleaved by α-secretase (6) generating the sAPPα fragment, which diffuses away (*green*), and some is reinternalized into endosomes (3), where Aβ is generated (*blue*). Following proteolysis, the endosome recycles to the cell surface (4), releasing Aβ (*blue*) and sAPPβ. Transport from the endosomes to the Golgi prior to APP cleavage can also occur, mediated by retromers (5).

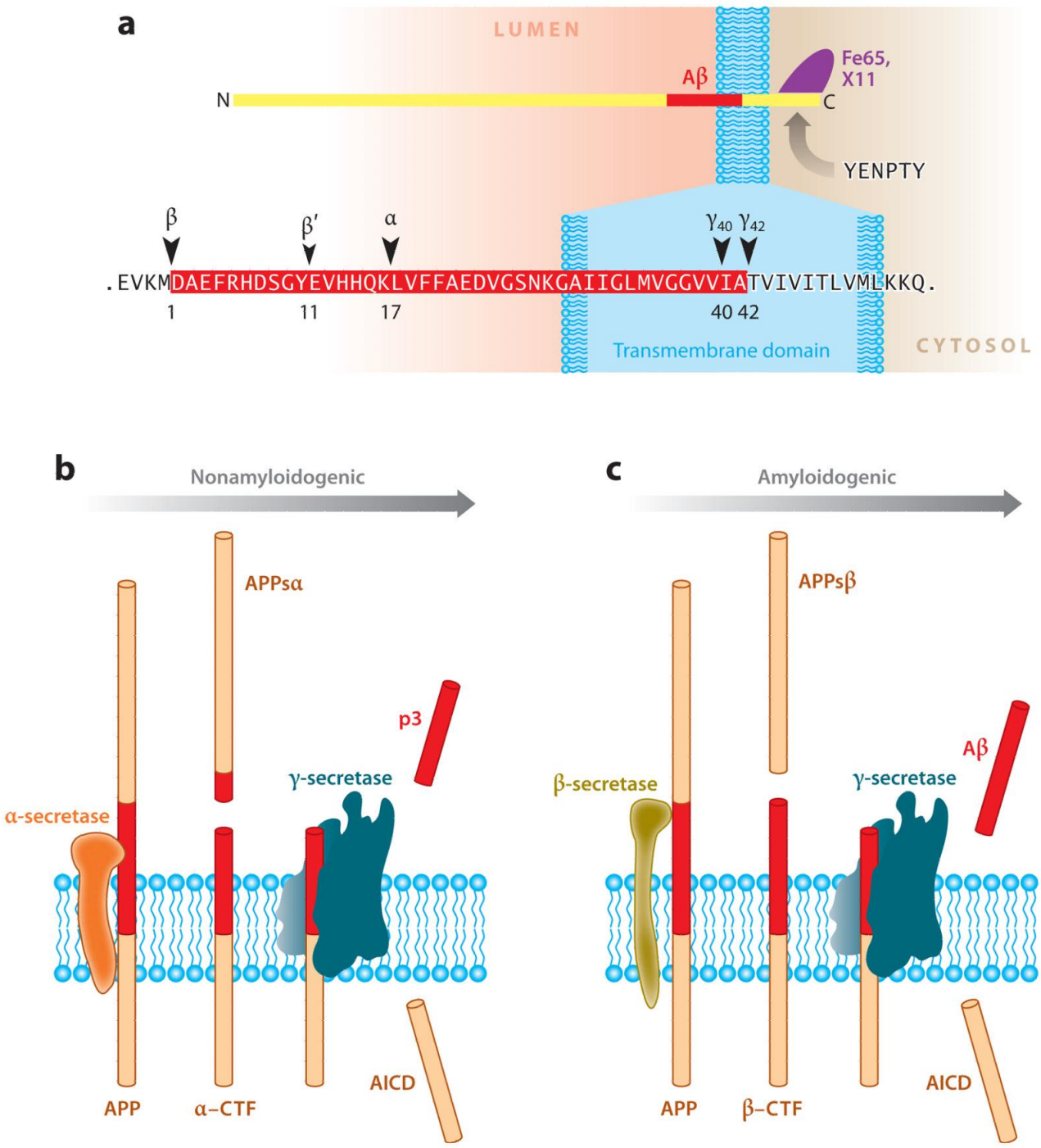

Fig. 2: Sequential cleavage of the amyloid precursor protein (APP) occurs by two pathways (from Thinakaran et al., 2008**)**

(*a*) The APP family of proteins has large, biologically active, N-terminal ectodomains as well as a shorter C-terminus that contains a crucial Tyrosine–Glutamic Acid-Asparagine-Proline-Threonine-Tyrosine (YENPTY) protein-sorting domain to which the adaptor proteins X11 and Fe65 bind. The Aβ peptide starts within the ectodomain and continues into the transmembrane region (*red*). (*b*) Nonamyloidogenic processing of APP involving α-secretase followed by γ-secretase is shown. (*c*) Amyloidogenic processing of APP involving BACE1 followed by γ-secretase is shown. Both processes generate soluble ectodomains (sAPPα and sAPPβ) and identical intracellular C-terminal fragments (AICD).

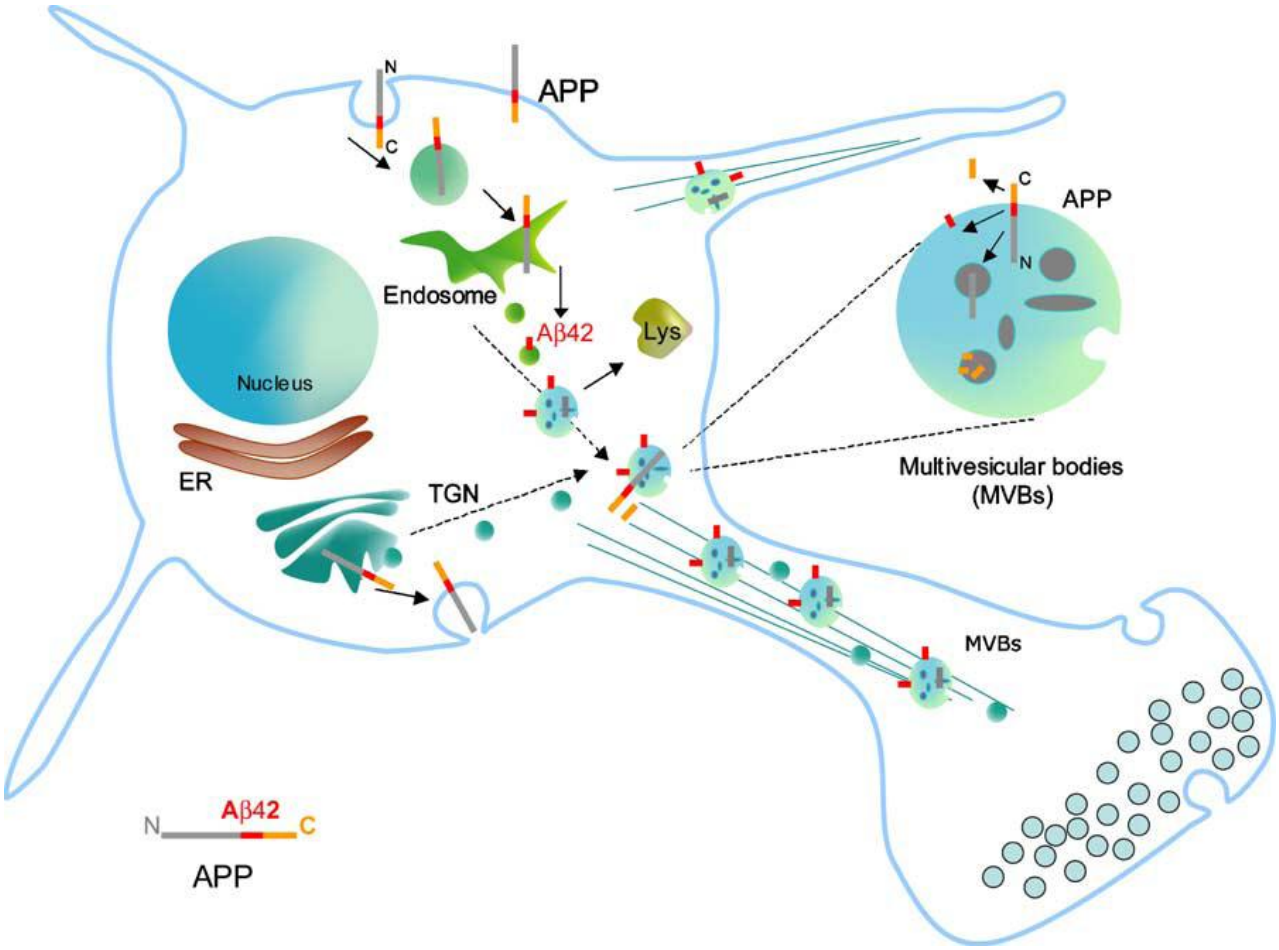

Fig. 3: Schematic diagram of APP and Aβ trafficking within a neuron (from Gouras et al., 2005).

APP is trafficked from the ER, where some Aβ may be generated, to the Golgi apparatus and then the plasma membrane (PM) where additional Aβ appears to be generated. Significant Aβ is produced in the trans-Golgi network (TGN). An important site of Aβ generation is in the endocytic pathway after APP internalization from the PM. Although APP localizes especially to the TGN, both APP and Aβ localize to vesicles within neuronal processes. In Alzheimer's disease Aβ42 accumulates within MVBs of vulnerable neurons, especially within distal neuronal processes and pre- and post-synaptic compartments.

Chapter 3: Aβ in human cerebral amyloidosis

3.1 Insights into Alzheimer's disease (AD)

3.1.1 Neuropathology of AD

It was over a century ago that Alois Alzheimer admitted a 51-year-old patient, Auguste D., for progressive cognitive decline. Alzheimer's histopathological observations of her brain following her death a few years later led to his realization that he was observing a unique clinical-pathological process, with a distinct and recognizable neuropathological substrate, that no long after was named Alzheimer's disease (AD).

Investigators at the beginning of the last century were intrigued by the origin of the deposition of a "peculiar substance" (also called "miliary necrosis"), eventually termed "plaques", that along with neurofibrillary tangles (NFTs) appeared to characterize presenile and senile dementia. Alzheimer's summary of his presentation in 1906 on the case of Auguste D. was published in 1907 and for the first time linked plaques and tangles with dementia.

It is now widely recognized that the two primary cardinal lesions associated with AD are the NFTs and the senile plaque.

NFTs are very difficult to be detected with the traditional morphological stain used by pathologists, hematoxylin and eosin. By and large, one of a variety of silver impregnation staining techniques, such as the modified Bielschowski or Gallyas technique, or the fluorochrome dye thioflavin S is typically employed to visualize neurofibrillary tangles. Additionally, there are a number of immunohistochemical approaches used to visualize neurofibrillary tangles. These have mostly employed antibodies directed against abnormally phosphorylated *tau*.

With these special stains, within neurons with a pyramidal shape to the perikaryon, such as those of the cornu ammonis 1 (CA1) sector of the hippocampus and the layer V neurons in areas of the association cortex, the neurofibrillary tangles appear as parallel, thickened fibrils that surround the nucleus and extend toward the apical dendrite. When the neurofibrillary tangle occurs within a neuron with a more rounded configuration (e.g., neurons within the substantia nigra and locus ceruleus), the inclusion appears as interweaving swirls of fibers, and here it is called a globoid neurofibrillary tangle.

Ultrastructurally, the neurofibrillary tangle is composed of abnormal fibrils measuring 10 nm in diameter that occur in pairs and are wound in a helical fashion with a regular periodicity of 80 nm (Wisniewski et al., 1976). On the basis of these observations, such structures are generally called paired helical filaments. The primary constituent of the neurofibrillary tangle

is the microtubule-associated protein tau. The tau within neurofibrillary tangles is abnormally phosphorylated with phosphate groups attached to very specific sites on the molecule. The best established functions of tau are thought to be the stabilization of microtubules and the regulation of motor-driven axonal transport (Götz et al., 2006). It is known that the phosphorylation of tau causes its detachment from microtubules (Ballatore et al., 2007) and that soluble hyperphosphorylated tau contributes to neuronal disfunction before its deposition, negatively interfering with mitochondrial respiration and axonal transport (Götz et al., 2006).

There are a number of other protein constituents associated with the neurofibrillary tangle, such as ubiquitin, cholinesterases and Aβ (Hyman et al., 1989) but tau is considered to be the critical constituent of most of these structures.

The pattern of distribution of neurofibrillary tangles present in cases of AD is, for the most part, rather stereotyped and predictable. Severe involvement is seen in the layer II neurons of the entorhinal cortex, the CA1 and subicular regions of the hippocampus, the amygdala, and the deeper layers (layers III, V, and superficial VI) of the neocortex (Morrison and Hof, 1997). Studies have shown that the extent and distribution of neurofibrillary tangles in AD cases correlate with both the degree of dementia and the duration of illness (Bierer et al., 1995). However, it is also clear that other factors contribute to the production of the clinical features of the disease. Although the neurofibrillary tangle is considered a cardinal histopathological feature of AD, this neuropathological lesion may also be encountered in association with many other disease states (Wisniewski et al., 1979), such as postencephalitic parkinsonism, dementia pugilistica, type C Niemann-Pick disease, and amyotrophic lateral sclerosis/parkinsonism dementia complex of Guam.

The other cardinal pathological lesion encountered in patients suffering from AD is the senile or neuritic plaque. Senile plaques are complex structures defined by the presence of a central core accumulation of a 4-kD protein with a β-pleated sheet configuration called Aβ. The predominant β-pleated sheet configuration of this protein confers its ability to bind the planar dye Congo red and produce birefringence when illuminated by polarized light; it thus conforms to the physical definition of an amyloid.

The brains of aged individuals and cases of AD may also contain several forms of Aβ-containing plaques. The senile or neuritic plaque has a central core of Aβ protein arranged in a radial fashion and is surrounded by a corona of abnormally formed neurites (or neuronal processes, either dendrites or axons). These abnormal or dystrophic neurites stain strongly with the same silver impregnation stains used to identify the NFTs, and ultrastructurally these structures contain dense bodies, membranous profiles, and packets of paired helical filaments.

In the periphery of the neuritic plaque, one commonly encounters reactive astrocytes of the macroglia, and, more frequently, one to several microglial cells.

Microglial cells, which derive from mesoderm and comprise 5-10% of the glial population in the nervous system, have the potential to develop into macrophages and in fact they represent the first line of defense against invading pathogens or other types of brain tissue injury. Under pathological situations, these cells become activated, migrate, and surround damaged or dead cells, and subsequently clear cellular debris from the area. In AD, microglia have a central role in the inflammation process. Actually inflammation in the AD brain is mediated by proinflammatory cytokines and would create a chronic and self-sustaining inflammatory interaction between activated microglia and astrocytes, stressed neurons, and Aβ plaques, as amyloid peptides and APP are potent glial activators. In some situations, the role of microglia has been found to be beneficial, since activated microglia can reduce Aβ accumulation by increasing its phagocytosis, clearance, and degradation (Frautschy et al., 1998; Qiu et al., 1998). Microglia can also secrete a number of soluble factors, such as the glia-derived neurotrophic factor (GDNF), which are potentially beneficial to the survival of neurons (Liu and Hong, 2003).

Astrocytes, besides maintaining extracellular and neuronal environment, and stabilizing cell-cell communications, also appear to be involved in the induction of neuroinflammmation. In AD they are known to be important for Aβ clearance and degradation, for providing trophic support to neurons, and for forming a protective barrier between Aβ deposits and neurons (Rossner et al., 2005). The presence of large numbers of astrocytes associated with Aβ deposits in AD suggests that these lesions generate chemotactic molecules that mediate astrocyte recruitment. Astrocytes are capable of accumulating substantial amounts of neuron-derived Aβ42 positive material and other neuron specific proteins as a consequence of their debris-clearing role in response to local neurodegeneration. Astrocytes overburdened with these internalized materials can eventually undergo lysis, and radial dispersal of their cytoplasmatic contents can lead to the deposition of a persistent residue in the form of small, GFAP-rich, astrocytic amyloid plaques (Nagele et al., 2004).

Under certain conditions related to chronic stress, however, the role of astrocytes may not be beneficial. A report suggests that astrocytes could also be a source for Aβ, because they overexpress β-secretase of APP (BACE1) in response to chronic stress (Rossner et al., 2005). On the basis of such a state-of-the-art it appears evident that the role of macroglia and microglia in AD still remains a matter of intense debate.

Through the use of immunohistochemical techniques with antibodies raised against portions of the Aβ molecule, it has been recognized that focal diffuse deposits of this amyloid protein may occur in the cerebral cortex in the absence of accompanying dystrophic neuritis (Yamaguchi et al., 1989). Such Aβ deposits will also stain with the aforementioned silver-based stains and are called diffuse plaques. Such plaques are very commonly encountered in the brains of elderly individuals and can be seen in relatively large numbers in the absence of any associated evidence of cognitive impairment (Morris et al., 1996).

A third form of plaque, consisting of a dense core of Aβ not showing any accompanying dystrophic neurites, has also been identified. Such plaques have been called burned-out plaques and end-stage plaques and are considered to be the remnants of what were once neuritic plaques (Wisniewski et al., 1982).

Senile plaques contain longer Aβ forms (having a total of 42 or 43 amino acids), while shorter Aβ forms (containing 40 amino acids) tend to accumulate within the leptomeningeal and cerebral cortical and cerebellar blood vessels, giving origin to a phenomenon of vascular amyloid deposition called congophilic angiopathy (Prelli et al., 1988). In this alteration Aβ accumulates in the walls of small arteries and arterioles of the leptomeninges and within the gray matter of the cerebral cortex. The accumulations of Aβ within the vessel walls do not appear to clog the vascular lumina or otherwise interfere with the vascular functions. However, when the degree of vascular involvement is severe, spontaneous vascular rupture may arise leading to focal accumulation of blood in the brain tissue. Hemorrhages tend to occur in the white matter of the frontal and/or occipital poles, are often small and multiple, and may be microscopic in size. If large (a relatively rare situation), they may be multiple and are commonly called lobar hemorrhages. Although rare, such lobar hemorrhages represent one of the few fatal intracerebral complications of AD.

Furthermore, in the cerebral cortex of AD and DS affected patients, as well as in non-demented individuals, Aβ extracellular deposits that were neither birefringent nor fluorescent nor associated with degenerating neurites, tangle-bearing neurons or congophilic vessels have been detected (Tagliavini et al., 1988). These deposits conceivably consisted of Aβ precursors lacking the molecular conformations of amyloid fibrils. Accordingly, they have been named preamyloid deposits, and they are likely supposed to be made up of APP itself or some of its early cleavage fragments (Giaccone et al., 1989). Preamyloid deposits have been reported to be ubiquitous in the brain grey substance (Ogomori et al., 1989), and are likely to give origin to senile plaques in the presence of vulnerable neurons (particularly at the level of the cortex and neostriatum) or to diffuse plaques in the brain regions where no permissive neurites are

available (Bugiani et al., 1989). In literature it has been reported that Aβ fibrillation intermediates, more than Aβ fibrils, seem to be involved in AD onset. Actually the number of plaques and the levels of insoluble Aβ in AD poorly correlate with the local extent of neuronal death and synaptic loss, or with cognitive impairment (McLean et al., 1999). On the other hand, the levels of soluble Aβ oligomers appear to strongly correlate with disease progression in animal models and AD subjects (Haass et al., 2007).

The major morphological counterpart to cognitive loss in AD is represented by a substantial loss in cerebral synaptic profiles. This aspect has been investigated by the quantification of immunohistochemical markers directed against synaptic proteins and by quantitative electron microscopy. A study reported a 45% loss of the extent of staining of presynaptic boutons in cases of AD in comparison with normal controls, finding that strongly correlates with the degree of functional impairment (Terry et al., 1991).

It is clear that AD is a slowly progressive disorder whose lesions accumulate in the brain over a period of many years.

It is not known long it may take before a sufficient extent of neuropathological damage occurs to produce a degree of functional impairment that might be clinically diagnosed, but it is likely to take many years for this to occur. Additional years are likely needed for the further progression from the early stages to the middle stages of the disorder. Within this perspective, researchers began to investigate the progressive stages of involvement of the brain in the course of AD and to speculate on what the earliest phases of the disease might look like. In 1991, neuroanatomists Eva and Heiko Braak published a proposed sequence of progression of the neuropathology of AD, breaking the disorder down into 6 stages with increasing involvement of the brain (the so-called Braak and Braak stages). Braak and Braak stages 1 and 2 show selective involvement by neurofibrillary tangles in the transentorhinal cortex. Stages I-II in the neuropathology of AD are considered preclinical and silent. They are followed by stages 3 and 4 with increasing limbic lobe involvement, with the final 2 stages (stages 5 and 6) showing the more typical widespread pattern of involvement in the neocortex. In stage VI, the process extends into the motor and sensory field. It is during stages III and IV that the first clinical symptoms of AD are frequently manifested. These Braak and Braak stages were initially developed by the evaluation of the pattern of neurodegenerative changes (predominantly neurofibrillary tangles) present in a series of 83 brain specimens derived from elderly individuals. They devised the stages by mapping out the extent and distribution of lesions in these brain specimens, but no associated clinical data were available from the patients from whom the samples were obtained.

Although it is clear that not all AD patients progress precisely along the stages they described, the Braak and Braak stages do represent a useful concept and have provided a format for neuropathologists to use in evaluating the relative stage of development of the disease.

3.1.2 Neuropathological changes in the normal human brain aging: blurring the divide with AD

The histological alterations upon which the neuropathological diagnosis of AD is made may also be seen, to some degree, in the brains of elderly individuals who during life had shown normal cognitive function. These changes are generally much fewer than in AD, and occur in restricted regions of the cerebral cortex, in the absence of significant cognitive decline. It is now well established that NFTs, senile plaques and synaptic loss, the main pathological hallmarks of AD, can often be found in the brains of non-demented elderly individuals (Tomlinson et al., 1968; Ulrich J., 1985). Actually it has been reported that the majority of elderly people display NFT formation in the hippocampal formation even in absence of cognitive impairment or with very mild memory impairment (West et al., 1994; Bierer et al., 1995). Furthermore, senile plaques may appear early in the neocortex of intellectually preserved individuals, whereas the hippocampus is relatively spared by senile plaque formation and by the onset of the degenerative process (Hof et al., 1990, 1992). Synaptic alterations and neuronal loss have been found in the neocortex of elderly non-demented individuals, suggesting an age-dipendent mechanism for the loss of synapses in the neocortex (Masliah et al., 1993).

The examination of a large sample of 1144 non-demented autopsy cases, reported by Hof et al. (1996), always evidenced the involvement of layer II of the entorhinal cortex with NFT formation, whereas the CA1 field of the hippocampus and the subiculum were less consistently affected. Moreover the inferior temporal cortex (Brodmann's area 20) appeared particularly prone to develop NFTs in intellectually preserved elders, and such a finding was supposed to prelude the emergence of the neuropsychological deficits characteristic of AD. Regarding the detection of senile plaques, Hof et al. (1996) described its occurrence with an increasing prevalence concurrently with increasing age in non-demented people. A positive correlation between age and SP prevalence was highlighted in the inferior temporal, superior frontal and occipital cortices. It has been shown that at least 20% of cognitively normal elderly evidence Aβ neuropathology upon autopsy (Price and Morris, 1999; Bennett et al., 2006).

With respect to senile plaque counts, several studies have indicated that there is no relationship between their amount, time of appearance and distribution and the phenomena of neurodegeneration or cognitive impairment, as is the case in younger populations (Delaère et al., 1993; Giannakopoulos et al., 1993). Actually, as fibrillary plaques seem to correlate poorly with neurodegeneration, investigators began to turn their focus to the importance of soluble oligomers and the role of preamyloid and cerebrovascular deposits. Extensive numbers of preamyloid lesions can be found in aged individuals, even with no reported clinical symptoms (Delaere et al., 1990; Crystal et al., 1993), in the form of scattered deposits of amyloid fibril precursor occurring in both cerebral cortex and subcortical grey structures (Bugiani et al., 1990). It has been demonstrated that the deposition of amyloid fibril precursors in the neuropil is closely related to presynaptic terminals, although whether the former precedes or follows the development of presynaptic terminal changes is still undetermined (Bugiani et al., 1990).

Preamyloid deposits are amorphous, roughly spherical areas immunoreactive with anti-Aβ antibodies, having irregular borders, and are associated with few or no dystrophic neurites. Preamyloid deposits, unlike amyloid, are not stained by Congo red or thioflavine S (Wisniewski et al., 1989; Yamaguchi et al., 1991). Ultrastructurally, these deposits are mainly nonfibrillar.

The apparent lack of associated cerebral dysfunction from preamyloid lesions can be correlated with *in vitro* studies using Aβ synthetic peptides, where it has been suggested that toxicity is dependent on the presence of a fibrillar, predominantly β-sheet conformation (Pike et al., 1993; Lorenzo et al., 1994). The Aβ 17-42 isoform has been reported to be a major component of preamyloid deposits in AD (Gowing et al., 1994) and DS (Lalowski et al., 1996).

Often the recognition of the extent of involvement in certain brain areas by AD characteristic lesions is required for the neuropathologist to declare that AD was the cause of a patient's cognitive impairment. At times, this may involve subtle distinctions that require considerable experience and expertise, and at other times, the honest neuropathologist may be required to state that this diagnosis can be rendered with only a degree of probability and not certainty. This is particularly true when one is examining the brains of subjects dying within the oldest-old age range. In individuals dying at an extremely old age, the overlap of neuropathological changes between those found with severe dementia and those with intact cognitive function is extensive, and the distinction may be very difficult.

Indeed, correlations between the extent and distribution of senile plaques and neurofibrillary tangles are currently undergoing detailed scientific study using brain specimens derived from individuals in large cohorts of advanced elderly subjects who underwent rigorous neuropsychological studies (Haroutunian et al., 2008).

3.1.3 Genetic aspects underlying AD

AD is the most common irreversible, progressive cause of dementia. It is characterized by a gradual loss of memory and cognitive skills.

AD accounts for over 50% of all dementia cases, and it presently affects more than 24 million people worldwide. The incidence increases from 1% between the ages of 60 and 70 to 6% to 8% at the age of 85 years or older and is likely to increase as a greater proportion of the population ages. So the prevalence and incidence of AD suggest that age is the most known risk factor. The disease is divided into 2 subtypes based on the age of onset: early-onset AD (EOAD) and late-onset AD (LOAD).

Early-onset AD accounts for approximately 1% to 6% of all cases and ranges roughly from 30 to 60 or 65 years. However, LOAD, which is the most common form of AD, is defined as AD with an age at onset later than 60 or 65 years. Both EOAD and LOAD may occur in people with a positive family history of AD. Approximately 60% of EOAD cases have multiple cases of AD within their families, and 13% of these familial EOAD are inherited in an autosomal dominant manner with at least 3 generations affected. With the exception of a few autosomal dominant families that are single-gene disorders, most AD cases appear to be a complex disorder that is likely to involve multiple susceptibility genes and environmental factors.

Overall, more than 90% of patients with AD appear to be sporadic and to have a later age at onset of 60 to 65 years of age (LOAD). Although some studies support the existence of a genetic component in LOAD, no causative gene has been yet identified, except the apolipoprotein E (APOE) gene. The risk allele is the isoform ε4, but many carriers live into their 90s suggesting that there many others genetics and environmental risk factors associated.

Around 1980s, three principal genes were identified in association with early onset AD, APP, presenilin 1 (PSEN1), and presenilin 2 (PSEN2). Identification of the APP gene was made possible by the seminal work of Glenner and Wong who, in 1984, determined a partial amino acid sequence of a peptide isolated from AD brain cerebrovascular amyloid.

3.1.3.1 EOAD and genes involved

The APP gene was then mapped to chromosome 21q. The predicted protein, designated APP, is an ubiquitously expressed, single-pass transmembrane protein that contains an internal 39–43 amino acid sequence. Aβ peptides are generated from APP by two endoproteolytic cleavages catalyzed by β- and γ-secretases; α-secretase catalyzes endoproteolysis of APP within the Aβ sequence.

Aβ peptides are the primary component of amyloid deposits that form in the brain parenchyma (called ‘‘plaques’’) and in the walls of cerebral vessels, in the latter case giving origin to the pathological condition called ‘‘cerebral amyloid angiopathy’’(CAA); both Aβ plaques and vascular deposits are key features of AD neuropathological changes. To date, 24 APP single nucleotide mutations are known to cause AD.

APP can encode multiple isoforms, the longest of 750 amino acids and all AD mutations are clustered within a 54 amino acids sequence near or within the segment that encode Aβ peptide. One of this is the Swedish mutation, a double sostitution changing the two amino acids immediately before the Aβ sequence (lysine-methionine is replaced by asparagine-leucine). The Swedish mutation is outside the Aβ sequence, so the peptide produced is normal, even if its quantity is two to threefold higher than that produced by non mutated APP, presumably because the efficiency of the β-secretase cleavage is affected. The implication is that an excess of Aβ production is sufficient to cause AD. This conclusion is supported by the longstanding observation that individuals with trisomy 21 (DS affected) develop extensive AD neuropathologic changes that can occur very early in life, until their 40s.

Other APP mutations are located near the C-terminal Aβ peptide, and the most common is p.Val717Ile. These mutation affects the activity of γ-secretase, the cleavage of which normally reduces APP to a 40 amino acid peptide (Aβ40), with smaller amounts of Aβ that is 42 amino acids long (Aβ42). APP mutations at the C-terminal end of Aβ shift proteolysis to produce more Aβ42 at the expense of Aβ40, resulting in an increased Aβ 42/ Aβ 40 ratio but not necessarily changing the total amount of Aβ peptides formed. Aβ42 is more amyloidogenic and more prone to aggregate than shorter Aβ forms. These data were the first to suggest that Aβ aggregation is a key event to AD pathogenesis.

Another mutation of note is p.Glu693Gly, also known as the Arctic mutation. This amino acid change within Aβ does increase the aggregation rate of the mutant peptide, adding to the evidence that APP mutations can result in Aβ peptides with altered aggregation properties and that this process is critical to AD pathogenesis.

Several studies have demonstrated variation in the most characteristic AD neuropathological features among patients with APP mutations. At the time of autopsy, patients with APP mutations tend to have greater amounts of neocortical senile plaques than patients affected by ‘‘sporadic’’AD. The ratio of accumulated Aβ42/Aβ40 may be higher in cases of autosomal dominant AD in respect to APP mutated cases, but there is a limited number of examples and a wide variation in the results. In addition to variation in the amount of the peculiar neuropathological traits of AD, APP mutations are also associated with morphologic variants.

Several APP mutations have been associated with variation in the structure of Aβ deposits. These include the p.Ala692Gly (Flemish) APP mutation that is associated with large, dense plaques and the p.Glu693Gly (Arctic) APP mutation with ring-like plaques. CAA is a common feature of AD caused by mutations in APP. The p.Asp694Asn (Iowa) and p.Ala713Thr mutations are associated with exceptionally severe CAA in the context of the core neuropathological features of AD. The p.Glu693Gln (Dutch) APP mutation produces CAA without significant accumulation of the core features of AD; in the affected individuals, progressive cognitive impairment is linked to vascular brain injury. Several cases of AD caused by mutations in APP also have Lewy bodies dementia (LBD), which is detectable by histochemical stains of pigmented brainstem neurons and can be assessed also in other brain regions, including the limbic structures and the neocortex, by α-synuclein immunoreactive Lewy bodies and Lewy neuritis.

The other two genes involved in EOAD are presenilin 1 (PSEN1) and presenilin 2 (PSEN2), located on the chromosome 14q.

Over 180 mutations in PSEN1 are known to cause autosomal dominant AD. Penetrance of PSEN1 mutations is complete by 60–65 years of age, meaning that all mutation carriers develop early-onset AD. Fewer than 15 known mutations in PSEN2 can also cause early-onset autosomal dominant AD, but penetrance is more variable than PSEN1.

PSEN1 and PSEN2 encode closely related proteins that are part of the γ-secretase complex. Either PSEN1 or 2, together with nicastrin, APH1, and PEN2, form a complex that catalyze the cleavage of a number of different membrane proteins at sites embedded in the lipid bilayer. In the case of APP, the presenilin-containing γ-secretase catalyzes endoproteolysis at the C-terminal end of the Aβ-peptide sequence. This proteolytic event, along with a second cleavage at the N-terminal end of this sequence, is required for production of Aβ-peptide from APP.

The γ-Secretase containing mutation-altered presenilin still catalyzes cleavage of APP, but the proteolytic site is altered. Normal γ-secretase yields predominantly Aβ40 with smaller

amounts of Aβ42. Mutant γ-secretase produces more Aβ42, a result consistently obtained across multiple studies. As noted previously, Aβ42 is more amyloidogenic and more prone to aggregate than Aβ40. While the mutated genes that cause early-onset AD inform about molecular mechanisms, they account for only a fraction of AD cases. As with APP mutations, individuals who died from AD caused by mutations in PSEN1 or PSEN2 tend to have a greater amount of neocortical senile plaques and a shift toward a higher Aβ42/Aβ40 ratio than patients who had ‘‘sporadic’’ AD, although again these data derive from relatively few examples with wide variation even within the same family.

The relationship between PSEN1 or PSEN2 mutations and neurofibrillary degeneration is more complex. Actually there is no difference between the amount of neurofibrillary tangles present in AD resulting from PSEN1 or PSEN2 mutations and in ‘‘sporadic’’AD. A PSEN1 mutation, given by a substitution in exon 8 (leucine271valine) that results in transcripts lacking exon 8, has been associated with lack of neuritic change in senile plaques. In contrast, increased accumulation of tau species other than neurofibrillary tangles has been reported in some cases caused by a PSEN1 mutation.

PSEN1 mutations also are associated with a morphologic variant in Aβ plaques: the so-called cotton wool plaque. Cotton wool plaques occur in the same regions of brain as senile plaques and have been associated with multiple PSEN1 mutations. Cotton wool plaques typically have limited fluorescence following staining with thioflavin S and tend to be more immunoreactive for Aβ42 than for Aβ40. It is important to stress that although highly characteristic of PSEN1 mutation, cotton wool plaques also have been reported in apparently sporadic AD.

3.1.3.2 LOAD and genes involved

The majority of AD patients are late-onset AD (LOAD) cases with risk approximately doubling every 5 years after age 65. Except for a handful of PSEN2 mutation carriers, LOAD very often results from multiple genes contributing to susceptibility or risk.

To identify these multiple interacting genes, new technologies have been very important, especially a method called genome-wide association studies (GWAS). This method has led to an explosion in the discovery of genetic susceptibility for a large number of diseases, disease-related traits, and associated phenotypes. For neuropathological affections, successful GWAS results have been reported for AD, Parkinson's disease, amyotrophic lateral sclerosis and multiple sclerosis.

Late generation genotyping platforms permit interrogation of most of the genome for disease-associated variation in a single experiment, thanks to arrays containing assays to screen from 600,000 to 2.5 million single nucleotide polymorphisms (SNPs). Thus genotyping platforms provide key informations on linkage disequilibrium or correlation between neighboring SNPs, and are very useful in comparing the different genetic profile of case and controls. Thanks to this new technique the ApoE and later Sorl1 genes have been identified as key genes in LOAD.

The apolipoprotein E (ApoE), the gene of which is located on chromosome 19q, is a protein originally extensively studied for its crucial role in lipid metabolism, but then recognized as a pleiotropic molecule with important functions in lipid transport, Aβ trafficking, synaptic function, immune regulation, and intracellular signaling. ApoE, like other apolipoproteins, is a protein component of lipoprotein particles that binds to the cell surface receptors. In humans ApoE is present in three isoforms, given by the ε2, ε3, and ε4 alleles encoding 299 amino acid-long protein isoforms. This polymorphism is unique to man and it has been proposed to have evolved as a result of adaptive changes to diet. In 1991, Pericak-Vance and colleagues, using family-based methods, identified a genetic linkage between AD and a region of chromosome 19 that harbors ApoE.

The key polymorphism associated to AD is in the three allele isoforms ε2, ε3, and ε4. These alleles represent haplotypes of two SNPs in the coding region of ApoE. The ε2 allele encodes a cysteine (Cys) both at amino acid position 112 and at position 158. Allele ε3 encodes a Cys at 112 and an arginine (Arg) at 158, while the ε4 allele encodes an Arg both at 112 and at 158. The ε4 allele is the ancestral and high-risk form, the ε3 allele is the most common in humans and the neutral allele, and the ε2 allele is associated with decreased risk of AD.

On a population level, people with an ε4 allele have lower average onset age and those with an ε2 allele have a higher average onset age for AD. The AD risk associated with these alleles is additive. A person with a genotype of ε4/ ε4 is at higher risk than someone with an ε3/ ε4or ε2/ ε4 genotype. Likewise, ε2/ε2 genotype is more protective than genotypes where only one ε2 allele is inherited. Many publications reported the risk associated with this pattern, associated with all ethnic groups and observed that amyloid plaques had ApoE immunoreactivity and that ApoE expression is up-regulated in activated astrocytes in AD brain; it was the genetic discovery that clearly linked ApoE isoforms to AD pathogenesis. Although the risk associated with the isoform ε4 is clear, the pathogenetic mechanism still is not well determined. As the major apolipoprotein of the chylomicron in the brain, ApoE binds to a specific receptor and works through receptor mediated endocytosis to rapidly remove chylomicron and VLDL remnants from circulation; this process is essential for the normal catabolism of triglyceride-rich lipoprotein constituents. In the brain, lipidated ApoE binds aggregated Aβ in an ApoE isoform-specific manner, with Apoε4 being much more effective than the Apoε3 isoform.

Researchers have also proposed that the more efficient binding process of Apoε4 enhances the deposition of the Aβ peptide. ApoE genotypes also influence onset ages in carriers of PSEN1 or PSEN2 mutations. Less compelling evidence suggests that ApoE genotypes also affect age of onset in subjects carrying APP mutations. Alternatively, ApoE might also bind Aβ and act as a chaperone to influence the rate of Aβ fibrillogenesis.

Another hypothesis is that ApoE lipid particles bind Aβ and clear the extracellular peptide by endocytosis and subsequent degradation. In each of these proposed mechanisms, the functional difference between the ε2-, ε3-, and ε4-encoded isoforms is the basis of risk associated with ApoE. Early work suggested that polymorphisms within the promoter of ApoE influence risk, though these findings were not replicated in large data sets. A recent publication, using a modest sample size, suggests that a poly-T track in TOMM40, the gene adjacent to ApoE affects AD onset age. The poly-T track in TOMM40 is variable in length; long poly-T length alleles associated with the ε3 ApoE allele were reported to have an earlier onset age than short poly-T track alleles. In subsequent work using substantially larger samples, this relationship between poly-T track length and onset age was not replicated, and when ApoE genotypes were accounted for, there was no significant association to TOMM40 (Schellenberg et al., 2012).

Studies in knock-out mice also confirm the importance of ApoE in AD pathogenesis. Brain cells from APOE knockout (APOE_/_) mice are more sensitive to excitotoxic and age-related

synaptic loss, and Aβ-induced synaptosomal dysfunction in these mice is also enhanced compared to control animals. When human ApoE isoforms (Apoε3 and Apoε4) are expressed in APOE_/_ mice, the expression of Apoε3, but not Apoε4, is protective against age-related neurodegeneration and Aβ toxicity. In addition, astrocytes from APOE _/_ mice that express human Apoε3 release more cholesterol than those expressing Apoε4 (Buttini et al., 1999); this suggests that ApoE isoforms may modulate the amount of lipid available for neurons.

There are also clinical evidences of the correlation between Apoε4 genotype and Aβ accumulation, as showed in many clinical trials where this correlation was identified; in one of these (Christensen et al., 2010) 20 patients with AD and 10 controls were examined to correlate age, sex and ApoE genotype with accumulation of Aβ peptides; the clear result was that Apoε4 genotype was highly correlated with the intensity of the Aβ stainings.

Chapter 4: Evidence of β-amyloid and NFTs accumulation in the brain of animal species

A wide range of changes has been described in the ageing brain of many species with the most detailed investigations reported about dogs, cats and monkeys. Among the age-associated changes in animal brains, the detection of senile plaques and β-amyloid deposits has frequently been reported in the framework of retrospective neuropathological studies.

Regarding dogs, canines have always represented a good model in neurological research, as the detection of their age-related behavioural changes is rather easy for both pet owners and veterinarians, they are very easy to handle and their life span is moderate, from about 12 to 20 years. Furthermore, canines share both the same environment and often the same food as man, therefore they may provide a unique model for studies of human aging. There are many evidences in the canine brain that support the hypothesis that β-amyloid plays a central role in age-related cognitive dysfunction and neuropathology. Amyloid appears to build up initially in and around neurons and is present within apical and basal dendrites. Furthermore β-amyloid is deposited uniformly in some synaptic terminal fields that show a predilection for extensive plaque formation in AD. Probably in the dog β-amyloid accumulation precedes plaque formation. With time, early diffuse plaques form within these terminal fields and around β-amyloid laden neurons. At this early stage, glial cells do not appear to be associated with the developing plaque. Thus in the canine model neurons appear to be the initial source of β-amyloid deposition (Cummings et al, 1995).

More recent studies have clarified the role of β-amyloid in the neurodegenerative phenomena of dog brains. In this species three major types of plaques have been identified, depending on their stage progress: diffuse (non-β-sheet), primitive (β-sheet lacking a central core of amyloid) and neuritic (β-sheet containing a central core of amyloid and extensive reactive astroglia). It is believed that the progression of plaque formation proceeds through specific identifiable stages, from diffuse to the neuritic subtype, that is characteristic of the end-stage AD in humans. Extensive immunohistochemical and fluorescence staining analyses demonstrate that the plaque most consistently observed in the canine brain is of the diffuse subtype, although occasional occurrence of mature (primitive or neuritic) plaques have been shown in some studies (Aristotelis et al, 2002).

As in humans, the different plaque subtypes have a different location in the dog brain areas, giving rise to an uneven distribution. The distribution is uneven both at the immediate cellular level and the cerebrocortical surface level. In the former instance, at a cellular level a site that

has been known to selectively accumulate Aβ deposits is the axonal synaptic field of neurons, whereas at a neuroanatomical level the first site of Aβ accumulation in the dog brain is the prefrontal cortex, followed by the parietal, the entorhinal and the occipital. The observed cognitive decline in the aged dog might be probably ascribed to the phenomena of Aβ accumulation in these regions. The forms of Aβ detected in the plaques are of different lengths, depending on the cleavage of APP. The most common form found in the dog brain seems to be Aβ1–42, that is constitutive of the diffuse plaques, whereas mature (primitive or neuritic plaques) are predominantly formed by the Aβ1–40. This hypothesis is supported by another work (Borras et al, 1999) reporting a fewer amounts of amyloid plaques detected in aged dogs brains compared to other studies, as the anti-Aβ antibody employed recognized the Aβ40 peptide but not the Aβ42 isoform. This finding likely undirectly confirms that the diffuse plaques are the predominant type in canines.

Another notable feature of diffuse plaque deposition in the dog brain in comparison to man relates to their spread and number: interestingly, in those cases where the canine cognitive decline can only be regarded as pre-AD stage, the amount of cerebral plaques detectable equals or might even exceed that characterizing the most severe cases of AD in humans.

An additional difference between dogs and human beings regards glial reactivity within brain plaques: unlike AD affected patients, canines are devoid of any infiltrating hyperactive glia within diffuse plaques; instead, astrocytic reactivity in close relation to plaques has been reported (Aristotelis et al, 2002).

A surprising characteristic is the integrity of the intra-plaque neurons in the canine brain. This is probably due to the fact that Aβ exerts its neurotoxic effects only when found in its aggregated β-pleated-sheet form, whereas it is neurotrophic when not aggregated.

Regarding the presence of NFTs in the dog brain, they have not been reported in literature even using several antibodies against abnormally phosphorylated tau. This could be an interesting point for understanding the association between the accumulation of β-amyloid with selective behavioral dysfunction in the absence of NFTs.

Cats have been proposed as another candidate animal model of AD. A comparison of the β-amyloid deposition processes between dogs and cats of different ages has been reported by Takeuchi et al. (2008). Cerebral Aβ deposition was first detected at an age of 7 years and 9 months in the dog and at 10 years in the cat; quantity and frequency of Aβ deposits seemed to increase with age in both species. Whereas Aβ deposits were observed in the cerebral cortex, hippocampus, and meningeal vessel walls in dogs, in the cats examined Aβ deposits affected the cerebral cortex and the hippocampus, but not the meningeal vessel walls. In both dogs and

cats Aβ deposition in the brain parenchyma occurred as diffuse plaques, unlike the well developed and circumscribed plaques that are typical of humans.

β-amyloid accumulation in cats has been reported also in a study by Head et al. (2005) which demonstrated that Aβ1–40 isoform was not present in any of the cat brains examined immunohistochemically. In contrast, 4G8 revealed the presence of plaques of the diffuse type. Aβ deposits in the cat brain were large and even more diffuse than those described in dogs. Interestingly two of the five aged cats examined in that study showed hyperphosphorylated tau. The tau isoform detected did not present the phosphorylation at residue Thr181 and Thr205 as in humans, suggesting the presence of a different tau isoform compared to man. However NFTs have not been described in those cat brains, however the aforementioned report could be an example of a possible pre-tangle formation process occurring in older cats.

Other animal models that have been particularly studied because of their great genetic similarities with man are the non-primate species. Senile plaques and vascular amyloid have been mainly described in chimpanzees and macaques (Gearing et al., 1994), in the gorilla (Kimura et al., 2001), in baboons (Ndung'u et al., 2011) and squirrel monkeys (Walker et al., 1990).

In a study by Gearing et al. (1994) comparing the neuropathological findings of aged chimpanzees and rhesus monkeys both the species displayed amyloid deposition in meningeal and cortical vessels walls and amyloid plaques in the brain parenchyma. Whereas in chimpanzees amyloid plaques were mainly of the diffuse type and neuritic plaques were seldom discovered, neuritic plaques were the predominant subtype in the two oldest rhesus monkeys examined.

Cerebral amyloid deposits were reported by Kimura et al. (2001) also in a male middle-aged (44-year-old) Western Lowland gorilla. The senile Aβ-42 positive plaques found in the cerebral neocortex were of the diffuse type, showing filamentous and amorphous structures with an irregular margin. NTFs were investigated by immunostaining for tau2, but were not detected. It might be hypothesized that the diffuse plaques described in such middle-aged case would have evolved into senile plaques with time, as happens in man.

Aβ peptides deposition has been investigated also in the brain of different subspecies of baboons ranging from 18 to 28 years (Ndung'u et al., 2011). Similarly to the aforementioned species, brain plaques detected were of the diffuse non-fibrillar type. Neocortical plaques were distributed in layers 3-5 frequently involving a microvessel. In the hippocampus they were distributed largely in the pyramidal cell layers. Amyloid plaques in baboons were of

similar size and regional distribution as in the rhesus monkey. In addition to amyloid deposits, a faint immunolabelling of hyperphosphorylated tau in the oldest baboons was found. This finding reflects the situation in humans, where NFTs develop in the last stages of the diffuse plaque formation process.

Squirrel monkeys represent a peculiar species as in their brain amyloid is associated primarily with both intracerebral and meningeal capillaries, and it occurs to a lesser degree than the other non-primates as parenchymal deposits. The latter consist mainly of diffuse plaques, but also senile plaques have been reported to occur in squirrel monkeys (Walker et al., 1990).

In literature few but interesting case reports about β-amyloid and NFTs brain deposition are available also regarding some wild animal species, among which even bears and the wolverine are encountered. Diffuse and senile plaques were detected in the cerebral cortex of an American black bear (Uchida et al., 1995). Senile plaques were mainly localized in the molecular layer of the cortex, while diffuse plaques were also described in the caudatum, hippocampus and olfactory bulbs. Additionally, a supposed specific neuropathological feature of AD, i.e. the presence of diffuse plaques in the cerebellar molecular layer, was described in the case examined. Despite the severity of amyloidosis, NFTs were not detected.

In a study on the brain of a wild old aged carnivore, the wolverine (*Gulo gulo*), phenomena of β-amyloid deposition in the form of vascular deposits as well as both diffuse and neuritic cerebral plaques were described. NFTs appeared in large amounts at the cerebral cortex and hippocampal level (Roertgen et al., 1996).

The findings of Aβ and NFTs cerebral deposition reported in literature to date are mainly related to domestic carnivores and wild omnivores, and few data are available at present about domestic and wild large herbivores. Within this latter category of animals limited descriptions have been published regarding the horse (Capucchio et al., 2009), the sheep (Nelson et al., 1994), the elephant (Cole et al., 1990) and the camel (Nakamura et al., 1995). Among these species the presence of NFTs has been reported in sheep, whereas it was not shown in the brain of the elephant and the camel. In the horse, clear evidences of Tau accumulation were detected, but Tau-positive hippocampal neurons observed in the study by Capucchio et al. (2009) did not express hyperphosphorylated Tau (AT8), indicating that the accumulated Tau was not hyperphosphorylated at position S202. However, phosphorylation at other amino acid residues could not be ruled out. So the conclusion was that non-phosphoryled-S202 Tau accumulates in some hippocampal neurons in a non-neurofibrillary tangle manner, perhaps due to axonal transport deficiencies occurring in ageing.

The sheep and the elephant appeared to be spare of cerebral β-amyloid deposition; instead, diffuse β-amyloid plaques were found in the brain of horses (Capucchio et al., 2009), even if sporadically, characterized by the accumulation of the N-truncated Aβ42 isoform and no Aβ40, similarly to the dog.

On the histopatological examination of the brain of an aged 20-year-old camel (Nakamura et al., 1995) senile plaques were detected. They were mostly of the diffuse type, but with a more clearly demarcated border than those seen in aged dogs. Only a few primitive plaques were detected using an Aβ-40 antibody. Plaques were mainly distributed throughout the cerebral cortex, whereas they were not detected in the hippocampus and in the cerebellar cortex.

The camel case represents a very interesting case as it is the first herbivorous animal in which the presence of β-amyloid plaques has been detected on the basis of proper histopathological and immunohistochemical examinations.

Hence dietary habits were supposed to be important factors in plaques formation. However, evidence had already been provided that herbivorous species, such as cows and sheep, could develop senile plaques, since the amino acid sequence of their β-protein is identical to that of animals usually affected by senile plaques, although their nucleotide sequence is different (Nakamura et al., 1995).

Chapter 5: Materials and Methods

5.1 Animal and tissue collection

Brain sections of frontal cortex, hippocampus, cerebellum and brainstem samples obtained at necroscopy from 102 cattle of various breeds (Piemontese, Podolica, Friesian and mixed breed), ranging from fetuses to cattle 240 months of age, were investigated (see Table 2 for details). Fifty cattle were healthy at death while fifty-two had shown neurological signs *in vita* and they had undergone neuropathological examination at Istituto Zooprofilattico of Turin (CEA): in 23 of these animals no abnormalities had been detected on neuropathological examination while 29 of them presented a neuropathological framework attributable to different categories of diseases: the majority belonged to the group of inflammatory diseases, the remaining to the groups of toxic-metabolic or others diseases, like food poisoning. Because of their age at death, some of them were tested with screening routine test for PrP^{Sc}, but resulted negative.

At necropsy, the brain was removed and then a paramedian cut was done.

The small part was frozen at - 80°C until biochemical studies were performed, and the other was fixed in 10% buffered formaldehyde solution for histological and immunohistochemical analysis.

ID animal	age (months)	Breed	Health status	Neuropathological alterations
101685/09	**8 m**	**Frisona**	**healthy**	**absent**
102648/09	**8 m**	**Frisona**	**healthy**	**absent**
bov 13	**8 m**	**Frisona**	**healthy**	**absent**
bov 14	**8 m**	**Frisona**	**healthy**	**absent**
bov 15	**8 m**	**Frisona**	**healthy**	**absent**
bov 17	**8 m**	**Frisona**	**healthy**	**absent**
bov 18	**8 m**	**Frisona**	**healthy**	**absent**
bov 19	**8 m**	**Frisona**	**healthy**	**absent**
bov 20	**8 m**	**Frisona**	**healthy**	**absent**
bov 12	**8 m**	**Frisona**	**healthy**	**absent**
bov 22	**8 m**	**Frisona**	**healthy**	**absent**
bov 16	**8 m**	**Frisona**	**healthy**	**absent**
101609/09	**8 m**	**Frisona**	**healthy**	**absent**
101670/09	**8 m**	**Frisona**	**healthy**	**absent**

102487/09	**8 m**	**Frisona**	**healthy**	**absent**
102639/09	**8 m**	**Frisona**	**healthy**	**absent**
102448/09	**8 m**	**Frisona**	**healthy**	**absent**
16685	**180 m**	**Podolica**	**healthy**	**absent**
16686	**192 m**	**Podolica**	**healthy**	**absent**
16560	**180 m**	**Podolica**	**healthy**	**absent**
16476	**156 m**	**Podolica**	**healthy**	**absent**
16609	**120 m**	**Podolica**	**healthy**	**absent**
16561	**132 m**	**Podolica**	**healthy**	**absent**
16513	**156 m**	**Podolica**	**healthy**	**absent**
16674	**120 m**	**Podolica**	**healthy**	**absent**
16530	**132 m**	**Podolica**	**healthy**	**absent**
16713	**120 m**	**Podolica**	**healthy**	**absent**
16532	**144 m**	**Podolica**	**healthy**	**absent**
16559	**180 m**	**Podolica**	**healthy**	**absent**
16531	**168 m**	**Podolica**	**healthy**	**absent**
16489	**156 m**	**Podolica**	**healthy**	**absent**
16474	**156 m**	**Podolica**	**healthy**	**absent**
16687	**132 m**	**Podolica**	**healthy**	**absent**
15795	**240 m**	**Podolica**	**healthy**	**absent**
15745	**180 m**	**Podolica**	**healthy**	**absent**
16047	**180 m**	**Podolica**	**healthy**	**absent**
15903	**120 m**	**Podolica**	**healthy**	**absent**
15747	**180 m**	**Podolica**	**healthy**	**absent**
16475	**192 m**	**Podolica**	**healthy**	**absent**
16610	**168 m**	**Podolica**	**healthy**	**absent**
16048	**180 m**	**Podolica**	**healthy**	**absent**
16050	**36 m**	**Podolica**	**healthy**	**absent**
15851	**180 m**	**Podolica**	**healthy**	**absent**
16673	**13 m**	**Podolica**	**healthy**	**absent**
16991	**199 m**	**Podolica**	**healthy**	**absent**
17013	**112 m**	**Podolica**	**healthy**	**absent**
17014	**194 m**	**Podolica**	**healthy**	**absent**
17407	**184 m**	**Podolica**	**healthy**	**absent**
17408	**89 m**	**Podolica**	**healthy**	**absent**
17463	**123 m**	**Podolica**	**healthy**	**absent**
113516/04	**84 m**	**Pezzata Rossa**	**diseased**	**present**
16602/10	**84,5 m**	**Piemontese**	**diseased**	**present**
110308/07	**60 m**	**Piemontese**	**diseased**	**present**

142364/04	**72 m**	**cross-breed**	**diseased**	**present**
116461/08	**foetus**	**Piemontese**	**diseased**	**present**
685/03	**36 m**	**Valdostana**	**diseased**	**present**
121982/1/04	**7 m**	**Pezzata Rossa**	**diseased**	**present**
147096/05	**48 m**	**Frisona**	**diseased**	**present**
6323/05	**22 m**	**cross-breed**	**diseased**	**present**
106425/06	**3 m**	**Piemontese**	**diseased**	**present**
34986/09	**49 m**	**cross-breed**	**diseased**	**present**
35120/09	**12 m**	**cross-breed**	**diseased**	**present**
78122/10	**44 m**	**cross-breed**	**diseased**	**present**
12894/10	**24 m**	**Piemontese**	**diseased**	**present**
42432/10	**3,5 m**	**cross-breed**	**diseased**	**present**
93342/09	**36m**	**Piemontese**	**diseased**	**present**
56472/10	**18 m**	**Piemontese**	**diseased**	**present**
61201/10	**32 m**	**Piemontese**	**diseased**	**present**
124791/09	**8 m**	**cross-breed**	**diseased**	**present**
66095/6/10	**3m**	**Piemontese**	**diseased**	**present**
84580/10	**84 m**	**Pezzata Rossa**	**diseased**	**present**
83090/10	**48 m**	**Piemontese**	**diseased**	**present**
49557/1/2/09	**foetus**	**cross-breed**	**diseased**	**present**
13145/11	**132 m**	**Piemontese**	**diseased**	**present**
40973/09	**1 m**	**Frisona**	**diseased**	**present**
116716/02	**132 m**	**cross-breed**	**diseased**	**present**
66741/09	**26 m**	**Pezzata Rossa**	**diseased**	**present**
36700/09	**4 m**	**cross-breed**	**diseased**	**present**
105805/02	**108 m**	**Piemontese**	**diseased**	**present**
59587/2/04	**120 m**	**Piemontese**	**diseased**	**absent**
49164/10	**108 m**	**Piemontese**	**diseased**	**absent**
20561/10	**72,5 m**	**Frisona**	**diseased**	**absent**
30132/08	**84 m**	**cross-breed**	**diseased**	**absent**
25001/10	**3,5 m**	**frisona**	**diseased**	**absent**
139943/07	**75 m**	**Frisona**	**diseased**	**absent**
126354/07	**6 m**	**Piemontese**	**diseased**	**absent**
58389/09	**15 m**	**Piemontese**	**diseased**	**absent**
109298/07	**23 m**	**cross-breed**	**diseased**	**absent**
112142/09	**10 m**	**Piemontese**	**diseased**	**absent**
102290/09	**42 m**	**Pezzata Rossa**	**diseased**	**absent**
79272/09	**36 m**	**Frisona**	**diseased**	**absent**
5966/08	**65 m**	**Piemontese**	**diseased**	**absent**

2196/08	**68 m**	**Frisona**	**diseased**	**absent**
131662/07	**80 m**	**Piemontese**	**diseased**	**absent**
54528/07	**foetus**	**Piemontese**	**diseased**	**absent**
49560/09	**5 gg**	**cross-breed**	**diseased**	**absent**
34593/10	**120,5 m**	**cross-breed**	**diseased**	**absent**
72163/04	**120 m**	**cross-breed**	**diseased**	**absent**
90201/04	**29 m**	**Piemontese**	**diseased**	**absent**
125883/09	**2 m**	**Piemontese**	**diseased**	**absent**
64541/09	**45 m**	**Frisona**	**diseased**	**absent**
45143/2/10	**8 m**	**Frisona**	**diseased**	**absent**

Table. 2: Animals considered in the study

5.2 Single-labelling immunohistochemistry (IHC)

Following formaldehyde fixation, brain sections (5 mm thick) from each animal were coronally cut. Slices of the frontal cortex, hippocampus, cerebellum and brainstem were sampled, embedded in paraffin wax, sectioned coronally at a thickness of 5 μm and mounted on a glass slide. The tissues were then immunostained to detect Aβ. The slides were dewaxed and rehydrated by routine methods and then immersed in 98% formic acid for 10 min. To enhance Aβ immunoreactivity, after washing in distilled water, the sections were then boiled in citrate buffer (pH 6.1) for 10 min. Endogenous peroxidase activity was blocked in 3% hydrogen peroxide for 20 min at room temperature (RT). To block nonspecific tissue antigens, the sections were incubated with 5% normal goat serum for 20 min at RT. The primary monoclonal antibodies were incubated with the tissue overnight at 4°C. Two antibodies were used as primary antibodies: polyclonal APP (Abnova, Taipei City, Taiwan; 1:100 dilution) directed against APP, and monoclonal antibody 4G8 (Signet - Covance, Emeryville, California; 1:500 dilution) directed to the epitope in residues 17–24 of Aβ. After rinsing, a biotinylated secondary antibody (1:200 dilution; Vector Laboratories, Burlingame, CA) was applied to the tissue sections for 30 min at RT, followed by the avidin-biotinperoxidase complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA), according to the manufacturer's protocol. The immunoreactivity was visualized using 3,3'-diaminobenzidine (Dakocytomation, Carpinteria, CA) as a chromogen; the sections were then counterstained with Meyer's hematoxylin. For controls, the primary and secondary antibodies were omitted. A human brain tissue affected with AD and provided by Institute Carlo Besta (Milan, Italy) was used as positive control.

All four neuroanatomical areas (frontal cortex, hippocampus, cerebellum and brainstem) of each animal were observed under a light microscopy and evaluations for APP, and 4G8 immunoreactivity were done. The classification for APP positivity was assessed only by presence or absence of the signal while for 4G8 positive-deposits two different morphological types were evaluated: those types included intracellular (intraneuronal) and extracellular (glia-associated) accumulations of Aβ and they was scored quantitatively at all sites examined. The scoring system assessed the "amount and/or intensity" of 4G8 positive-deposits on the following scale: 0, absent; 1, slight; 2, moderate; 3 marked.

5.3 Lipofuscin staining

Some of the brain areas with the major Aβ deposition belonging to cattle older than 108 months were selected for a double immunohistochemistry, to highlight lipofuscin deposition. Tissue sections were dewaxed, rehydrated, formic acid treated, and boiled as in the IHC protocol described above. After unmasking, the sections were washed in distilled water and processed according to a dual-immunohistochemistry protocol. Endogenous peroxidase activity was blocked in 3% hydrogen peroxide for 20 min at RT. To block nonspecific tissue antigens, the sections were incubated with 5% normal goat serum for 20 min at RT. Primary monoclonal antibody 4G8 (SIGNET-Covance, Emeryville, California; 1:500 dilution), was applied overnight at 4°C. After rinsing, a biotinylated secondary antibody (1:200 dilution; Vector Laboratories, Burlingame, CA) was applied to the tissue sections for 30 min at RT, followed by the avidin-biotinperoxidase complex (Vectastain ABC peroxidase kit; Vector Laboratories, Burlingame, CA), according to the manufacturer's protocol. Aβ immunoreactivity was visualized using Vector VIP (Peroxidase Substrate kit, Vector Laboratories, Burlingame, CA) as a chromogen; the sections were then incubated with primary monoclonal anti-Dityrosine antibody (JaICA, Japan; diluted 1:100 in 5% normal goat serum), a specific marker for protein oxidation, for 1h at RT. After rinsing, a biotinylated secondary antibody (1:200 dilution; Vector Laboratories, Burlingame, CA) was applied to the tissue sections for 30 min at RT, followed by the avidin-biotin phosphatase complex (Vectastain ABC-AP kit; Vector Laboratories, Burlingame, CA), according to the manufacturer's protocol. Lipofuscin immunoreactivity was visualized under light microscopy using Blue AP (Alkaline phosphatase substrate kit III, Vector Laboratories, Burlingame, CA) as a chromogen.

5.4 Immunofluorescence (IF)

Brain sections that stained positive for Aβ by immunohistochemistry, were selected for immunofluorescence studies.
Immunofluorescence staining was performed according to the protocol described below.

- **ß-amyloid and glia detection**. Double labelling to evaluate the relationship between astrocytes and microglia cells with Aβ deposits was performed, according to the protocol here described: tissue sections were dewaxed, rehydrated, formic acid treated, and boiled in citrate buffer (pH 6.1) as in the IHC protocol described above. After unmasking, the sections were washed in distilled water and processed according to a dual-immunofluorescence protocol. To block nonspecific tissue antigens, the sections were incubated with 5% TBST-diluted normal goat serum for 20 min at RT. Primary antibodies 4G8 (Signet - Covance, Emeryville, California; 1:100 dilution) and monoclonal Iba-1 (Wako Chemicals, Richmond, VA, USA; 1:100 dilution) to visualize microglial cells or polyclonal GFAP (Dako Cytomation, Glostrup, Denmark; 1:100 dilution) were applied at RT for 1 h. Tissue sections were then incubated at RT with a Alexa fluor 555 anti-mouse or Alexa fluor 488 anti-rabbit secondary antibodies (Invitrogen, Life technologies, diluted 1:200) respectively, for 15 min. After rinsing in distillate water, the sections were mounted with Mounting Medium with DAPI (Vectashield, Vector Laboratories, Burlingame, CA) and examined under a Nikon Video Confocal fluorescence microscope (ViCO, Nikon Instruments, Florence, Italy). Dark-field fluorescence digital images were collected with a DS-U1 camera (Nikon Instruments, Florence, Italy) using fluorescein isothiocyanate and tetramethyl rhodamine isocyanate filters. The specificity of the secondary antibodies was tested by applying these antisera without the primary antibodies. No Aβ or glia immunolabeling signals were seen after omitting the primary antisera.

- **X34** Some of the brain areas with the major Aβ deposition were selected for X34 staining. Fixed brain tissues were first washed three times with PBS 10 mM for two minutes each. Sections were then stained with methoxy-X34 diluted 1:250 in 40% ethanol in a humid chamber for 10 min at RT and subsequently washed five times with distilled water. Therefore, after one passage in 0,2% NaOH in 80% ethanol for two minutes, sections were washed ten minutes in distilled water and then mounted

on a slide glass and cover-slipped with glycerol. Fluorescent staining was visualized using Nikon Video Confocal fluorescence microscope (ViCO, Nikon Instruments, Florence, Italy) and analyzed using NIS Elements imaging software (Nikon Instruments, Florence, Italy).

5.5 Western blot (WB)

Twenty seven frozen brains (from which 25 cortex, 8 cerebella and 7 brainstems were analysed) were weighed and homogenized in brain tissue lysis buffer containing 0,5% deoxycholic acid, 0,5% NP-40 and 10 mM EDTA diluted in Phosphate buffered saline (PBS) at a ratio of 1:10 (w/v) for 1 minute. Homogenates were centrifuged at 5000 x *g* for 3 minutes (Optima TLX ultracentrifuge, rotor TLA 110; Beckman Coulter, Fullerton, CA), and supernatants were collected. Sample buffer 3X containing 0.36 M Bistris, 0.16 M Bicine,1,5% wlv SDS, 15% w/v sucrose, 2.5% vlv 2-mercaptoethanol, 0.004% w/v bromophenol blue, was added to samples, after which samples were sonicated and then placed in boiling water for 5 min and then immediately chilled on ice. Aliquots (10 μL) of each sample and 5 μL of markers (β-amyloid proteins 1-38, 1-40 and 1-42) were loaded into urea gels and subjected to electrophoresis. The gels were run at room temperature at 12mA/gel for 10 minutes followed by 24 mA/gel for 1 h. Proteins were then transferred into polyvinylidine difluoride membranes (Immobilion P; Millipore, Billerica, MA) using a Trans-Blot Semi Dry transfer at 90 mA for 45 minutes. Membranes were washed with Tris Buffered Saline (TBS), unmasked with microwave for 3 minutes in TBS and then blocked with 5% Bovine serum albumin (BSA) in TBS with 0,1% Tween at 37°C for 1 hour, and then incubated with primary antibody (6E10 antibody, diluted 1:500, Covance) at 4°C overnight. After four washes with TBS containing 10% Tween 20, membranes were incubated at room temperature for 1 h with anti-mouse biotinylated secondary antibody (diluted 1:3000, GE Health care). After four washes with TBS, they were incubated at room temperature for 45 minutes with ABC Elite Kit, then blots were developed using ECL Plus (Pierce Biotechnology, USA).

5.6 Genetic analysis

DNA was isolated from frozen brain tissue of 30 animals. A genomic region of ~1.8 Kb, encompassing exon 2 to exon 4 of the bovine apolipoprotein E (APOE), was amplified. The selected region included the entire APOE open reading frame (ORF) (Fig. 4). PCR primers were designed using the Primer3 application and their sequences were: APOE_Bt_Ex2_F (5' CCAATCGCAAGCCAGAAG3') and APOE_Bt_Ex4a_R (5' GAGACTCGGGGTGGGAGTA 3').

PCR reactions were carried out with the following conditions: 5 µl of buffer 10x, 2 µl of genomic DNA, 30 pmol of each primer, 1 µl of dNTPs, 1 U of Taq polymerase (Roche), 10 µl of CG solution (Roche) and 2 µl MgCl2 50 mM, in a final volume of 50 µl (Table 3).

Thermocycling parameters consisted of an initial denaturation step (95 °C, 10 min) followed by 40 cycles of denaturation (94 °C, 1 min), annealing (57 °C, 1 min) and extension (72 °C, 2 min). ApoE sequence was determined by direct DNA sequencing of the PCR products on ABI 3130 Genetic Analyser (Life Technologies) by Big Dye terminator v. 3.1 cycle-sequencing using the amplification primers pairs and two internal primers, APOE_Bt_Ex3_F (F (5' GAGGAGCCCCTGACTACCC3') and APOE_Bt_Ex4b_R (5' ACACCCAGGTCATTCAGGAA 3'). The sequence reactions were prepared as follows: 2 µl Sequencing Buffer 10x, 2 µl of Big Dye Terminator v3.1, 3,2 pmol of the sequencing primer, 50-100 ng of template DNA in a final volume of 20 µl.

All the ApoE sequences were assembled using the program SeqMan II (Lasergene package, DNASTAR Inc.) in order to obtain a consensus sequence for each sample. Polymorphic nucleotides were annotated and the final consensus sequences were assembled into a single data set. Each variable site was enumerated based on the corresponding position in the bovine ApoE sequence. Eventually, allele frequencies of the detected polymorphisms were calculated.

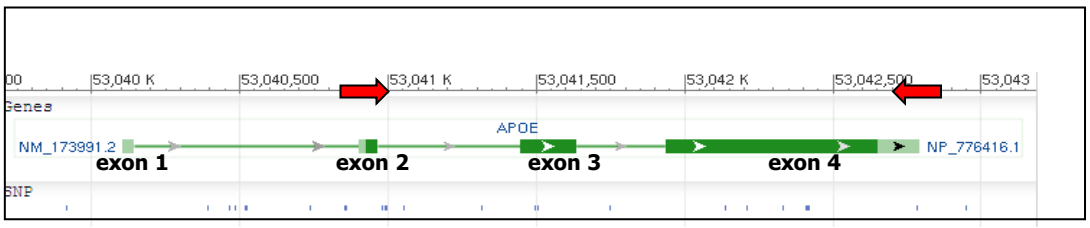

Fig. 4: Genomic regions of the bovine *APOE* gene.
(Light green = mRNA; dark green = ORF; red arrows = PCR primers).

REAGENTS	AMOUNT (µl)
Buffer 10x	5
Primer F	1,5
Primer R	1,5
$MgCl_2$ 50 mM	2
dNTPs	1
Roche Taq polimerase	0,2
H_2O	26,8
CG solution	10
DNA	2

Table 3: PCR mix.

5.7 Statistical analysis

Statistical analysis on IHC and WB results was performed employing Chi-square test, carried out using the SISA online tool (http://www.quantitativeskills.com/sisa/statistics/twoby2.htm), which, in case of small number of data, suggests to apply Fisher test. When more than two classes were tested for association, the Epitools website was used (http://epitools.ausvet.com.au/content.php?page=chi_sq). In genetics, the same websites were used to look for association between each polymorphism detected and WB and IHC profiles. The analysis were performed by categorizing the animals according to the following criteria:

1) Positive or negative cases at the WB analysis;
2) Cases with intracellular or extracellular Aβ accumulation in the cortex or its absence at IHC analysis;
3) Cases with a strong or weak intensity of the signal or its absence in the cortex at IHC analysis;
4) Cases with the intracellular or extracellular Aβ accumulation in the hippocampus or its absence at IHC analysis;
5) Cases with a strong or weak intensity of the signal or its absence in the hippocampus at IHC analysis.

Chapter 6: Results

6.1 Single-labelling immunohistochemistry (IHC) and statistical analysis

In this study the immunoreactivity to 4G8 was detected by IHC analysis in 59 out of the 102 cattle examined. The 4G8 antibody is directed against a central epitope of the APP protein (17-24), and a cross reaction with APP cannot be completely ruled out. However, Aβ immunopositive deposits occurred in two different patterns which were detectable after acid formic pretreatment: the intracellular and the extracellular pattern. The intracellular pattern is characterized by fine and randomly dispersed Aβ-immunoreactive granules in the cytoplasm of neurons, located close to the nucleus (Fig. 5-6) while the extracellular pattern is characterized by aggregates frequently associated to glia or randomly dispersed in each brain area (Fig. 7-8).

In general 16 out of 59 IHC positive cases presented exclusively Aβ extracellular pattern (mean age at death: 84 months); 2 cattle out of 59 IHC positive cases presented exclusively Aβ intracellular pattern (mean age at death: 48 months); 41 presented the coexistence of the extracellular and intracellular patterns (mean age at death: 128,4 months) and the remaining 43 animals were completely negative (mean age at death: 17,1 months). Considering the four neuroanatomical areas, cattle with exclusively extracellular pattern presented marked Aβ deposits (score 3) localized in cerebral cortex (2/16) principally restricted to the gray matter and hippocampus (1/16), where fine and randomly dispersed Aβ immunoreactive granules were frequently present in almost all neurons of the dentate gyrus. Cattle with exclusively intracellular pattern presented slight Aβ deposits principally localized in cerebral cortex (2/2) and cattle with the coexistence of intra and extracellular patterns presented marked Aβ deposits principally localized in cerebral cortex (17/41), hippocampus (4/41) and cerebellum (4/41) with granular deposits localized at the level of Purkinje cells and in the molecular layer. Aβ deposition was never observed in brainstems.

No Aβ immunoreactive plaques and cerebral vessels were found in all samples.

It was then investigated the relationship between age of animals and the presence or absence of Aβ at IHC: out of 59 IHC positive cases, we have found that 33 animals were older than 108,5 months, whereas 38 animals, out of 43 IHC negative cases, were younger than 36,5 months (Table 4). Chi square test disclosed the statistical significance of these data (Table 5).

The association between the intracellular or extracellular Aβ localization related to the same age ranges was also considered: the majority of animals between 36,5-60 months presented a prevalent extracellular Aβ localization (58%), although with aging a coexistence of intracellular and extracellular colocalization was mainly present (52% animals between 108,5-180 months) (Table 6).

We have then tried to correlate 4G8 immunoreactivity to the health status of the animals and interestingly it appeared that in diseased animals Aβ accumulation occurred earlier (from 12,5 months) than in healthy animals (Table 7). Chi square test highlighted the statistical significance of the data regarding healthy and diseased animals (Table 8-9).

All the brain tissue slices tested for Aβ presence at IHC were also processed for APP and the results were similar to those described for 4G8 immunostaining: each section positive to 4G8 immunostaining resulted positive for the presence of APP. Fine granules of APP immunopositivity were mainly localized in the cytoplasm and at the level of the membranes of neurons (Fig. 9).

IHC							
Age (months)	**0-12**	**12,5-36**	**36,5-60**	**60,5-84**	**84,5-108**	**108,5-180**	**>180**
+	**2/59**	**7/59**	**6/59**	**7/59**	**4/59**	**27/59**	**6/59**
-	**32/43**	**6/43**	**1/43**	**2/43**	**0/43**	**2/43**	**0/43**

Table 4 : 4G8 positive or negative cattle (considering all brain areas) subdivided by age (months).

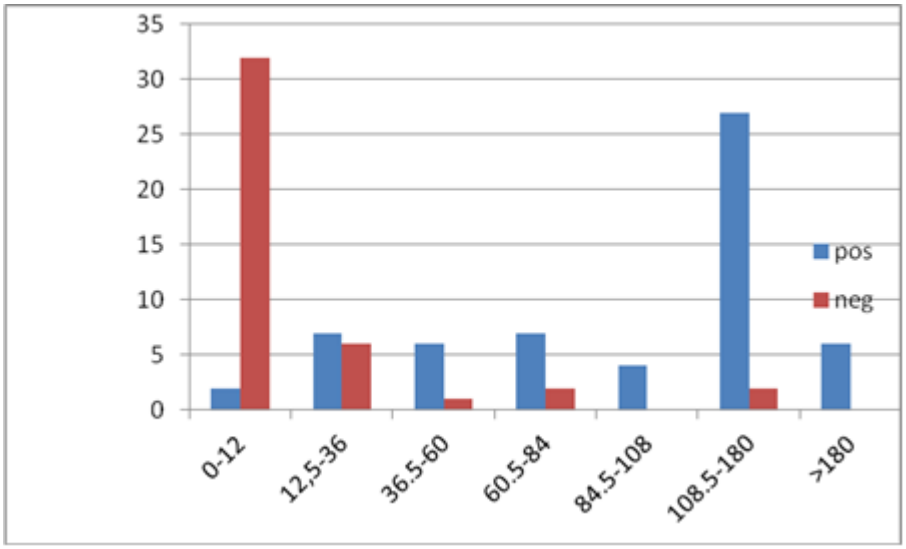

Table 5: Test chi square: 4G8 positive or negative cattle.
Chi-square statistic 63.5011; Degrees of freedom 6; P-value <0.0001.

age (months)	intra	extra	int-ext	neg	cases
0-12	0%	6%	0%	94%	34
12,5-36	8%	38%	8%	46%	13
36.5-60	14%	58%	14%	14%	7
60.5-84	10%	20%	40%	20%	10
84.5-108	0%	25%	75%	0%	4
108.5-180	0%	41%	52%	7%	29
>180	17%	17%	66%	0%	6

Table 6: Frequencies of intracellular and extracellular amyloid localization.

IHC								
Health status	**IHC results**	**Age (months)**						
		0-12	**12,5-36**	**36,5-60**	**60,5-84**	**84,5- 108**	**108,5-180**	**>180**
Healthy	**+**	**0/31**	**1/31**	**0/31**	**0/31**	**1/31**	**25/31**	**4/31**
	-	**17/19**	**1/19**	**0/19**	**0/19**	**0/19**	**1/19**	**0/19**
Diseased	**+**	**2/28**	**6/28**	**6/28**	**7/28**	**3/28**	**4/28**	**0/28**
	-	**15/24**	**5/24**	**1/24**	**2/24**	**0/24**	**1/24**	**0/24**

Table 7: 4G8 positive or negative cattle (considering all brain areas) subdivided by age (months) and health status.

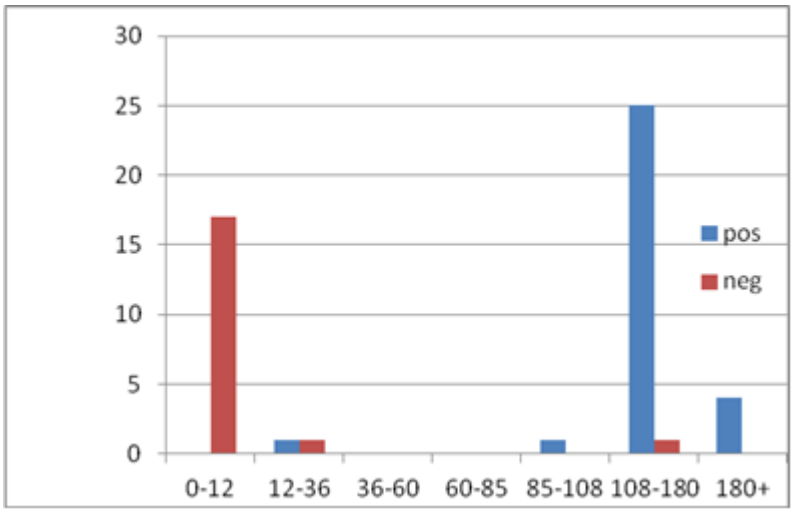

Table 8: Test chi square: healthy animals on 4G8 positive or negative cattle.

Chi-square statistic 43.7965; Degrees of freedom: 4; P-value < 0.0001.

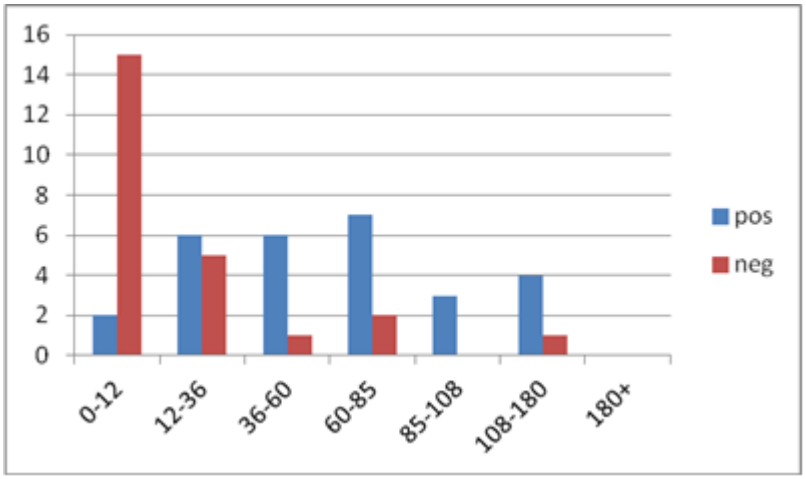

Table 9: Test chi square: diseased animals on 4G8 positive or negative cattle.

Chi-square statistic 20.9978; Degrees of freedom: 5; P-value < 0.0008

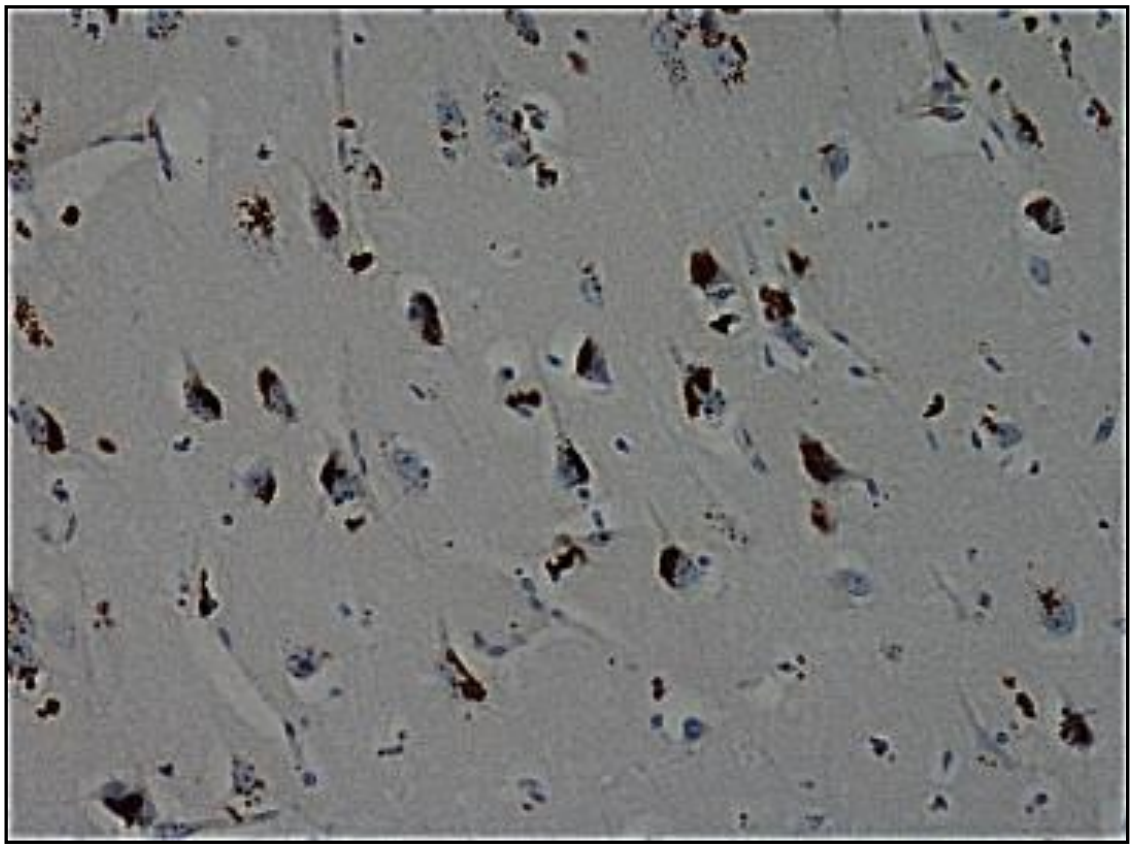

Fig. 5: 4G8 - Intracellular deposits at the level of frontal cortex (20X).

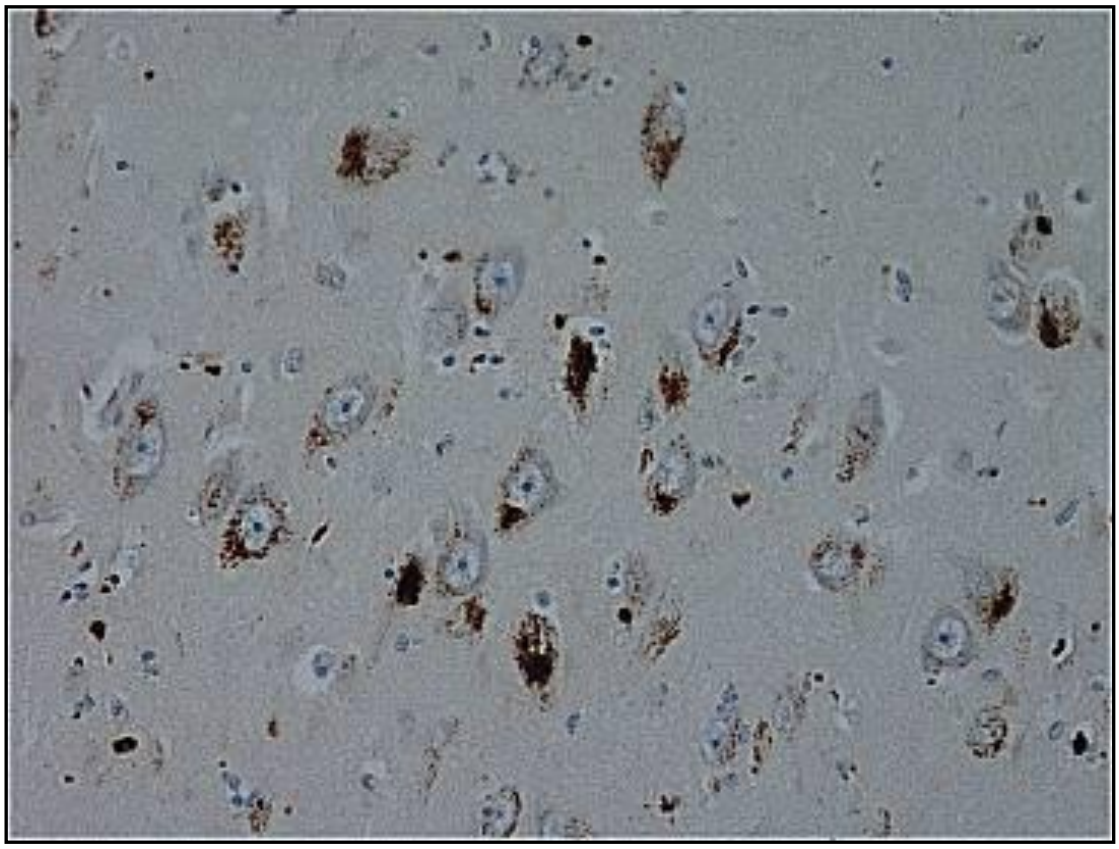

Fig. 6: 4G8 – Intracellular deposits at the level of hippocampus (20X).

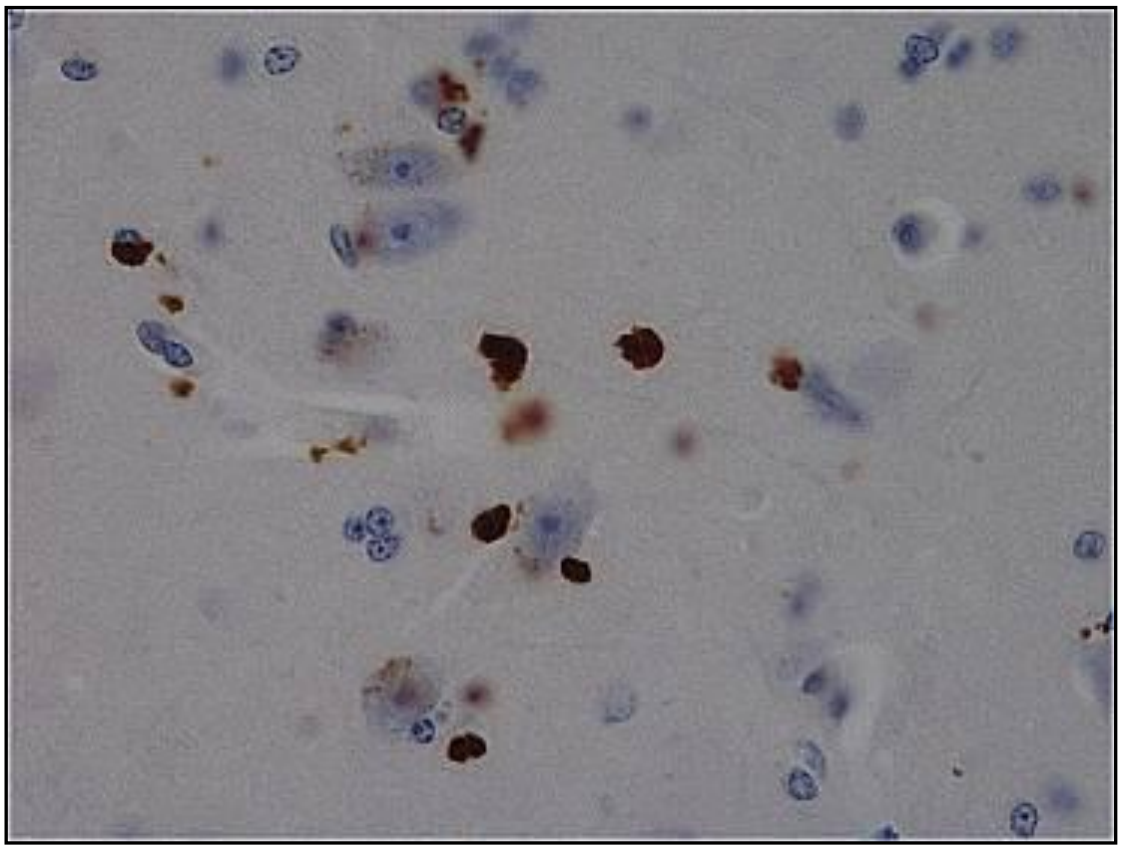

Fig. 7: 4G8 – Extracellular deposits at the level of frontal cortex (40X).

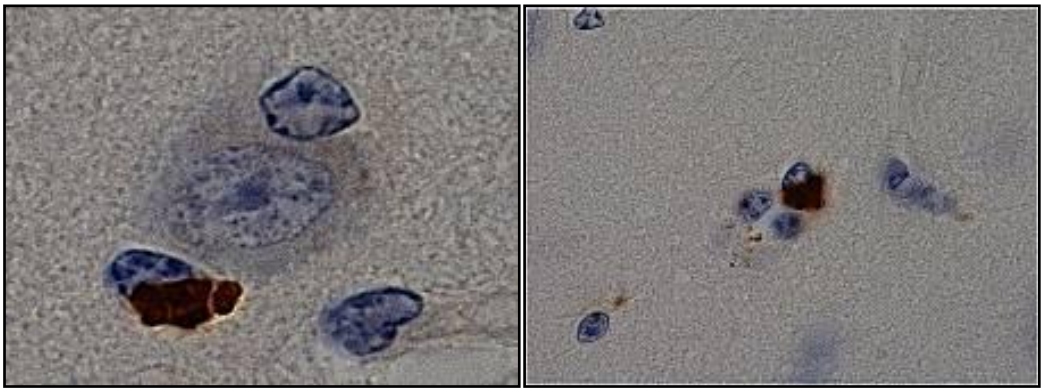

Fig. 8: Glia-associated Aβ deposits in frontal cortex (100 X).

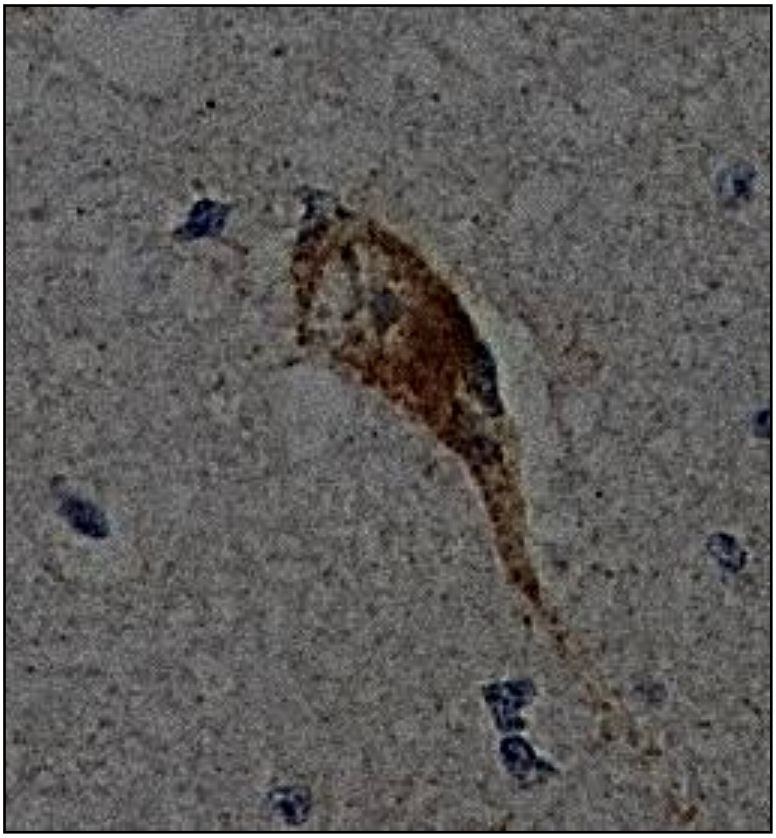

Fig. 9: APP - magnification of an hippocampal neuron (40X).

6.2 Lipofuscin staining

To evaluate intracellular distribution patterns of lipofuscin and 4G8 immunopositive deposits, a dual IHC was performed to simultaneously visualize the two stainings in the same brain section. Lipofuscin was stained in blue and β-amyloid in red. Results showed that lipofuscin is predominantly present in cytoplasmic regions of neurons exhibiting prominent β-amyloid immunolabelling; the labeling patterns of the blue-stained lipofuscin showed that most of this material is not co-localized with red-stained β-amyloid in the neurons: most lipofuscin and β-amyloid occupy distinct cellular compartments in the same neurons. This separate and distinct localization is clearly shown in Fig. 10.

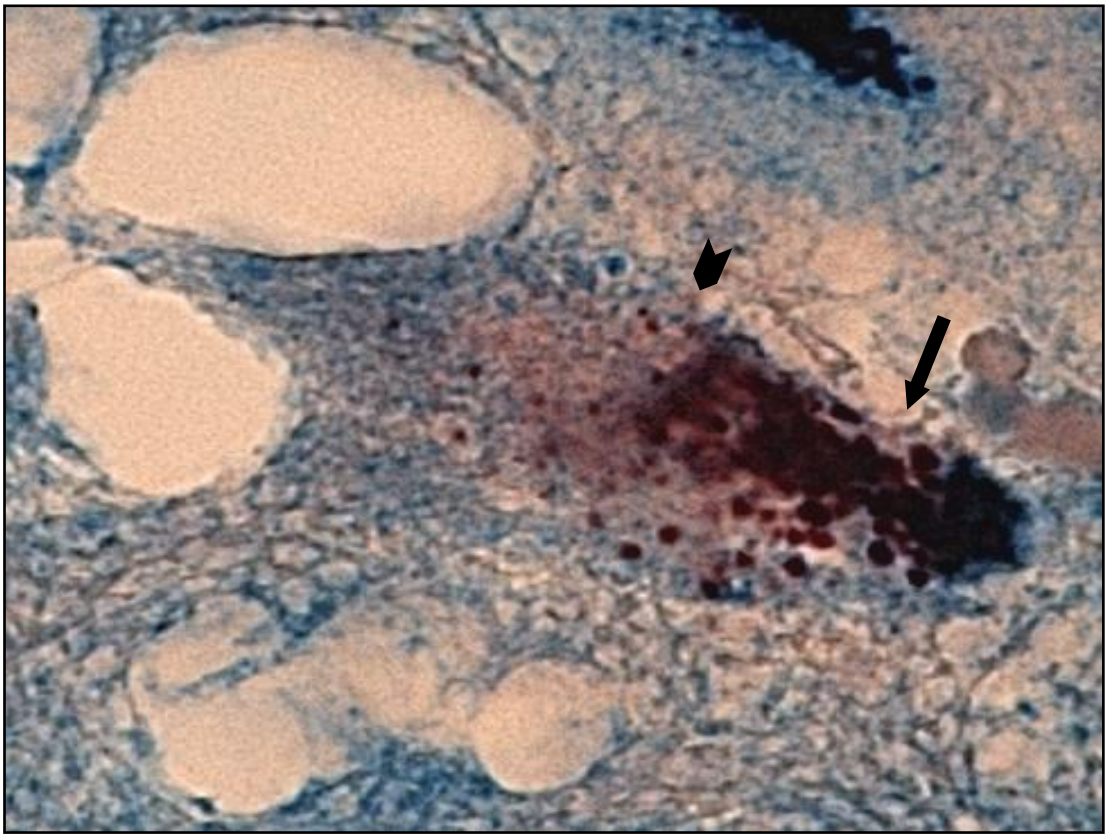

Fig. 10: Dual IHC to detect 4G8 immunoreactive deposits (arrowhead) and lipofuscin (arrow) in cortical brain tissue.

6.3 Immunofluorescence (IF)

- **ß-amyloid, astrocytes and microglia detection.** Glial fibrillary acidic protein (GFAP) immunofluorescence was used for the specific identification of reactive astrocytes. A generalized increase in GFAP signalling and in number of astrocytes was observed in brains with a major accumulation of Aβ deposits, but a different localization of astrocytes and Aβ deposits was often present in these immunopositive sections, in particular at the level of cerebral cortex (Fig. 11-12). In brain areas where coexistence of astrocytes and Aβ deposits was present, cell bodies of reactive astrocytes can be seen located around the Aβ deposits and their fine processes penetrate them (Fig. 13). The majority of compact deposits, with the exception of the small punctuate ones, were accompanied by such peripheral reactive astrocytes. GFAP-positive cells were found also within the white matter in the areas free of amyloid. To understand the exact localization of extracellular amyloid deposits, we performed a double immunofluorescence using 4G8 and Iba-1, an antibody against activated microglia. A strictly relationship between Aβ extracellular deposits and microglia was observed: almost all Aβ extracellular deposits were surrounded and penetrated by immunoreactive microglia cells and the most intense Iba-1 immunoreactivity was distributed principally in the frontal cortex of all the animals tested. (Fig. 14).

- **X34.** No positive staining was observed on sections treated with X34 staining.

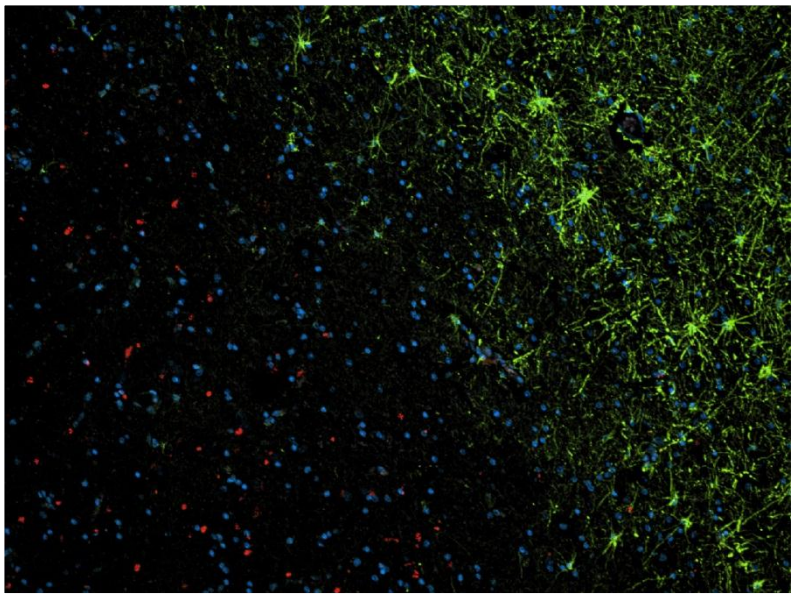

Fig. 11: 4G8 (Alexa fluor 555, red) and GFAP (Alexa fluor 488, green) - frontal cortex. Different localization of astrocytes and Aβ in cattle brain (20X).

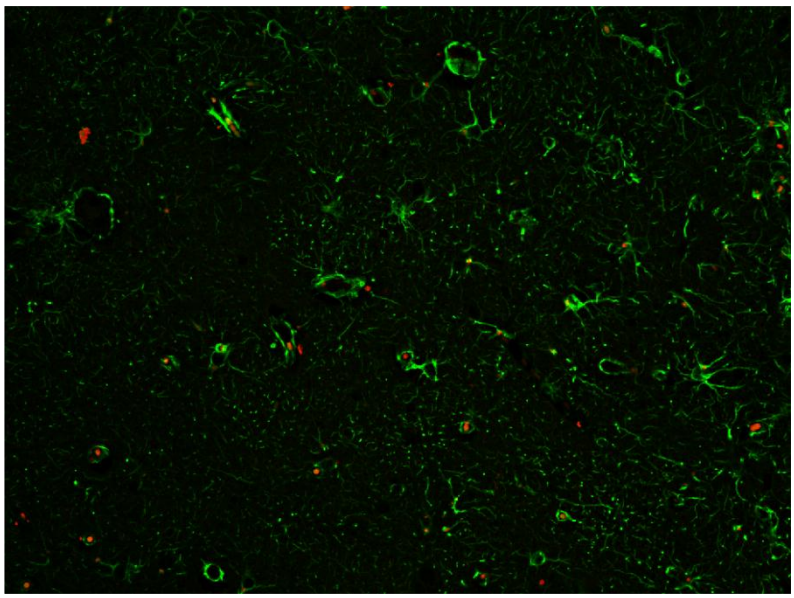

Fig. 12: 4G8 (Alexa fluor 555, red), GFAP (Alexa fluor 488, green) - frontal cortex. Astrocytosis (20X).

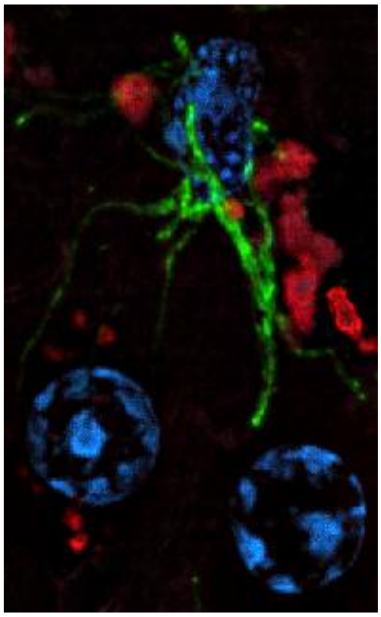

Fig. 13: 4G8 (Alexa fluor 555, red), GFAP (Alexa fluor 488, green) and Dapi for nuclei – frontal cortex. Strictly relationship between astrocytes and Aβ (100X).

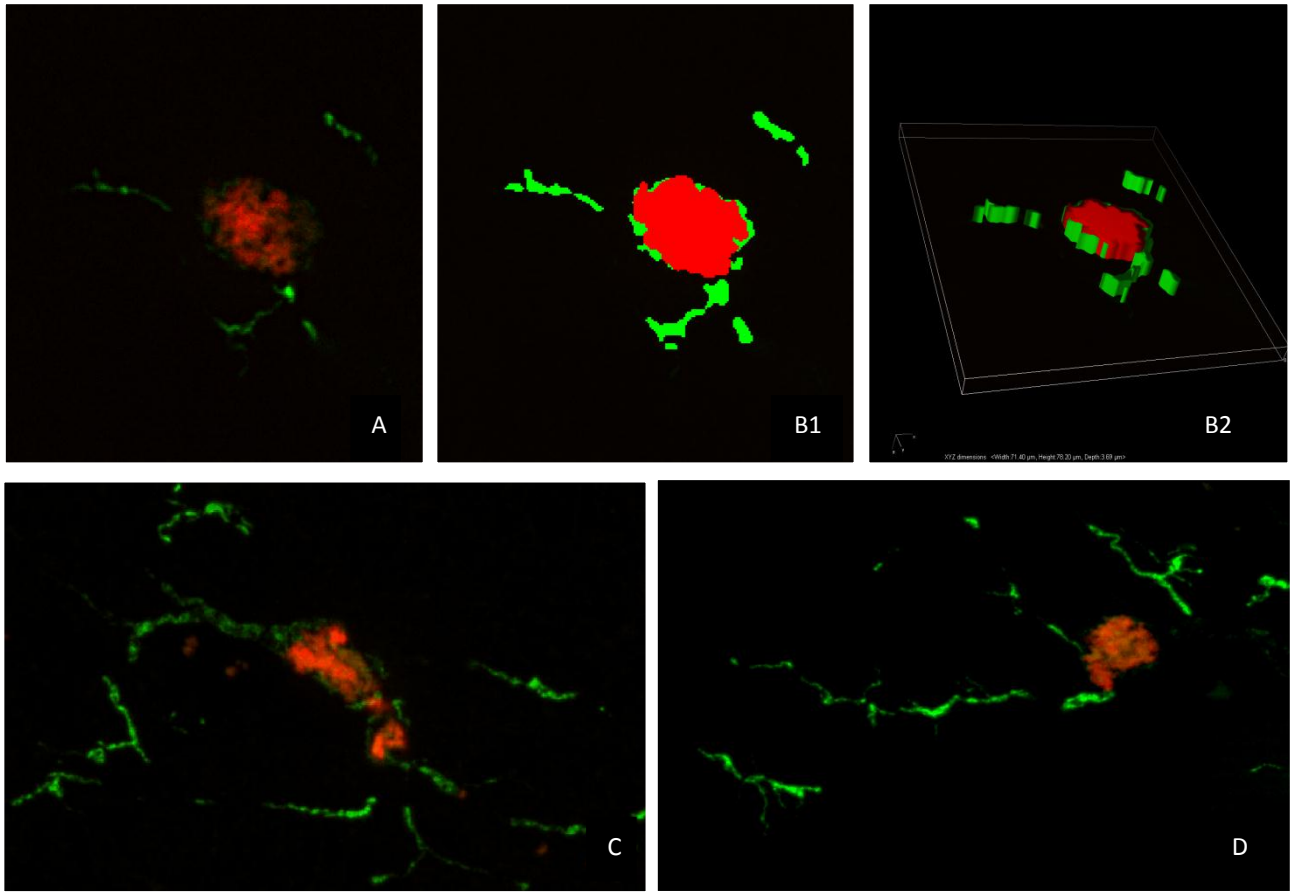

Fig. 14: 4G8 (Alexa fluor 555, red) and Iba-1(Alexa fluor 488, green) – frontal cortex. A) 60X ; B1-B2) 3D reconstruction of image A); C-D) 60X. Extracellular Aβ aggregates are strictly correlated to activated microglia.

6.4 Western Blot (WB) and statistical analysis

A total of 27 cattle brains were examined by WB analysis, including the following areas: 25 cortex, 8 cerebella and 7 brainstems (Fig. 15). Peptides of Aβ (1-38, 1-40, 1-42 and 3-42) was detected in 10 animals out of the 27 examined. Specifically peptide 1-38 was found in 4 cortex out of 10 positive animals; peptide 1-40 was found in 7 cortex out of 10 positive animals; peptide 1-42 was found in 7 cortex out of 10 positive animals and peptide 3-42 was found only in one cortex out of 10 positive animals; 3 cerebella out of 8 examined were positive for amyloid peptides: in particular peptide 1-42 was found in 2 cerebella out of 3 positive cases and peptide 1-40 only in 1 cerebellum out of 3 examined; no Aβ peptides was detected by WB analysis in all 7 brainstems examined.

However, the intensity of the signal in all the cases analized was weaker than the signal detected in humans brain with AD.

Considering the correlation between the age of the animals and their positivity or negativity at WB analysis, although the tested animals were only 27, it appeared that the majority of negative cases belonged to the age range under 12,5 months (Table 10); chi square test performed was not statistically significant ($p<0,8931$) (Table 11).

Relating 27 cases tested to WB analysis with the corresponding IHC positivity or negativity, it appeared that 17 cases have been confirmed in both analysis: 8 animals were positive and 7 were negative to WB and IHC (Table 12).

Fisher test was performed to highlight the statistical significance of the comparison between the two analysis: the obtained p-value ($p=0,08931$), although not properly statistically significant, could indicate a possible trend (Table 13).

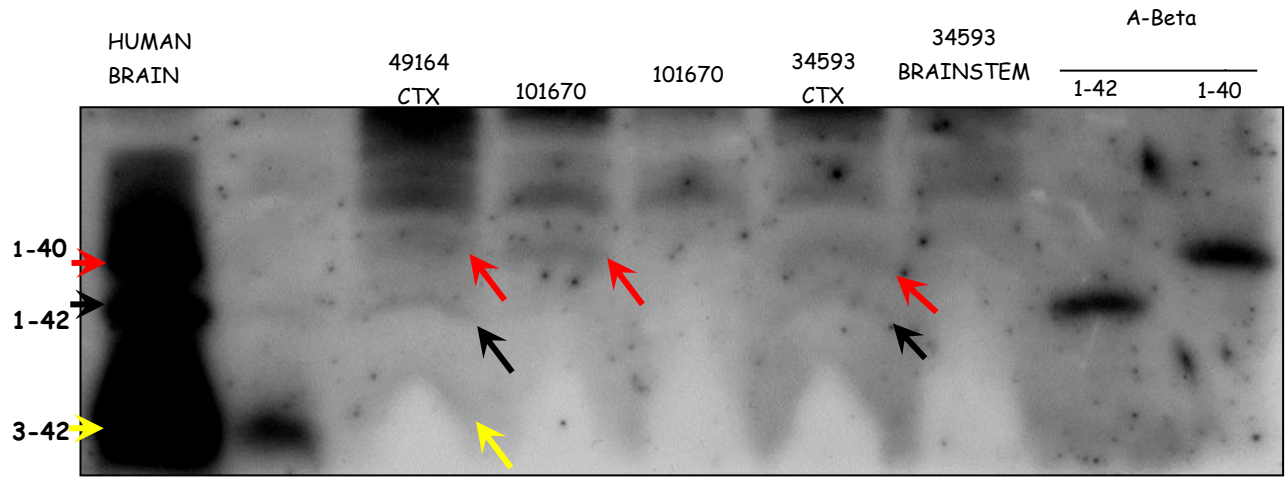

Fig. 15 : Western Blot analysis performed on brain sections of some animals of the study (CTX = cortex)

BLOT							
Age (months)	**0-12**	**12,5-36**	**36,5-60**	**60,5-84**	**84,5-108**	**108,5-180**	**>180**
+	**3/10**	**1/10**	**1/10**	**0/10**	**1/10**	**4/10**	**0/10**
-	**7/17**	**1/17**	**2/17**	**1/17**	**1/17**	**4/17**	**1/17**

Table 10 : Western Blot positive or negative cattle (considering all tested areas) subdivided by age (months)

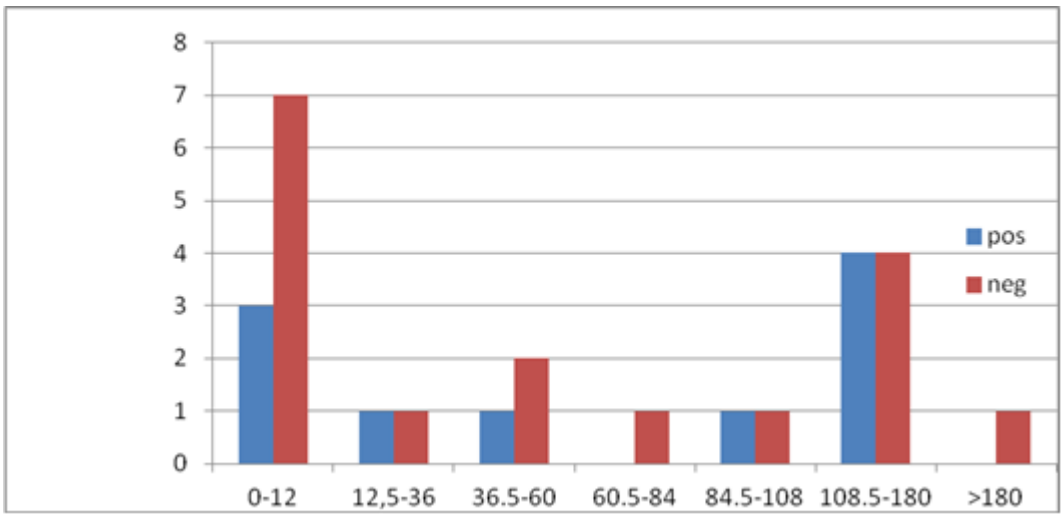

Table 11: Test chi square: Western Blot positive or negative cattle

Chi-square statistic 2.2712; Degrees of freedom 6; P-value < 0.8931 (not statistically significant)

	WB POSITIVE CASES	WB NEGATIVE CASES
IHC POSITIVE CASES	8	8
IHC NEGATIVE CASES	2	9

Table 12: Testing for correlations between IHC and Western Blot analysis

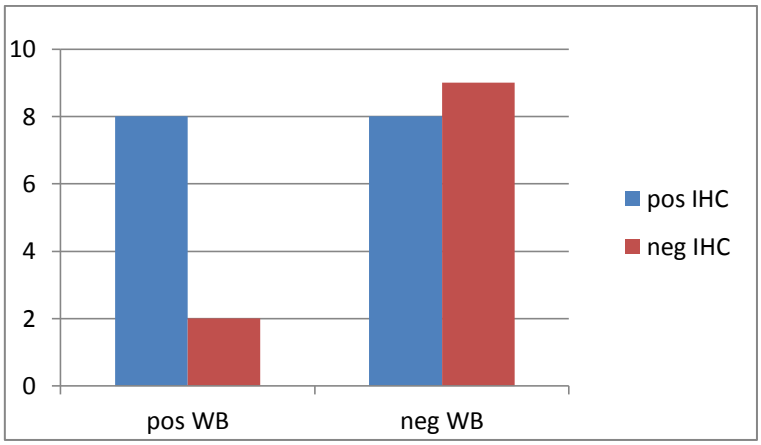

Table 13: Fisher test: comparison between WB and IHC results

P-value = 0,08931.

6.5 Genetic and statistical analysis

PCR products of the expected molecular size (approximately 1750 bp) were obtained using primers and optimized protocols described in the Methods section.

Sequence analysis of the ApoE gene of the 30 cattle resulted in the identification of nine SNPs. A list of the identified polymorphisms with the indication of their position in the sequence and relative allele frequencies is shown in Table 14.

Three polymorphisms were not reported in the GenBank reference database and they included one non-synonymous mutation in exon 2 (841 G/T, codon 8, Val→Leu) and two SNPs in intron 2 (873 G/A) and intron 3 (1544 G/T). The other polymorphisms have all been reported previously.

There is not a reported relationship between these mutations and evident phenotypic changes: the two located in the exon determine two synonymous mutations (exon 3, 1394 C/G, codon 32, Thr ⇒ Thr and in exon 4, 2034 T/C, codon 146, Ser ⇒ Ser) and the function of those already reported in introns is not known. Interestingly, SNPs at positions 1643 (intron 3) and 2034 (exon 4) were in complete linkage disequilibrium (Table 14).

Chi-square tests performed to look for association between each found polymorphism and Western blot and IHC profiles did not detect any statistically significant associations (Table 15; A,B,C,D,E). In Table 15A was performed Fisher test as suggested by SISA online tool. However, there was an interesting results for the position 876 when association was tested for strong or weak intensity of the signal or its absence in the cortex by IHC analysis (Table 16) . This result was obtained excluding the category without available cases (intracellular pattern in cortex). The obtained p-value ($p<0,08931$), although not properly statistically significant, could indicate a possible trend: the variant "G" (guanine) would seem in close relationship with the extracellular Aβ deposits in cerebral cortex, as almost all the "G" were found in cases where the Aβ accumulated mainly extracellular in the cortex (7/8), while only one "G" was present in cases with both intra- and extra-cortex localization. Finally, no "G" was detected in control cattle.

SNP location (Ref.Seq. NC_007316.5)	APOE region	Allele frequency
841 G/T (codon 8, Val→Leu)	exon 2	0.08
873 G/A	intron 2	0.06
876 C/G	intron 2	0.24
894 T/C	intron 2	0.26
952 C/G	intron 2	0.37
1394 C/G (codon 32, Thr)	exon 3	0.61
1544 G/T	intron 3	0.09
1643 A/G	intron 3	0.38
2034 T/C (codon 146, Ser)	exon 4	0.38

Table 14: Identified polymorphisms with their position in the sequence and relative allele frequencies

A. Positive or negative cases at the Western blot analysis

				876				894				952		
	pos/neg blot			C	G			T	C			C	G	
			pos[a]	8	4		pos[a]	8	4		pos[a]	6	6	
			neg[b]	11	5		neg[b]	12	4		neg[b]	9	7	
			p< 0.31304				p<0.28986				p<0.74726			

	1394			1544			1643			2034		
	C	G		G	T		A	G		T	C	
pos[a]	7	5	pos[a]	11	1	pos[a]	8	4	pos[a]	7	5	
neg[b]	8	10	neg[b]	15	1	neg[b]	9	7	neg[b]	9	9	
p<0.45524			p<0.50794			p<0.2637			p<0.26479			

pos[a]: positive cases at WB.

neg[b]: negative cases at WB

B. Cases with intracellular or extracellular Aβ accumulation in the cortex or its absence at IHC analysis

			876			894			952		
	cortex intra extra		C	G		T	C		C	G	
		extra[c]	9	7	extra[c]	11	5	extra[c]	9	7	
		intra[d]	0	0	intra[d]	0	0	intra[d]	0	0	
		int-ext[e]	5	1	int-ext[e]	5	1	int-ext[e]	4	2	
		negative[f]	6	0	negative[f]	4	2	negative[f]	2	4	
		p <0.091			p <0.7635			p<0.4848			

	1394			1544			1643			2034	
	C	G		G	T		A	G		T	C
extra[c]	8	8	extra[c]	15	1	extra[c]	10	6	extra[c]	10	6
intra[d]	2	0	intra[d]	2	0	intra[d]	0	2	intra[d]	0	2
int-ext[e]	2	4	int-ext[e]	5	1	int-ext[e]	4	2	int-ext[e]	4	2
negative[f]	4	2	negative[f]	6	0	negative[f]	2	4	negative[f]	2	4
p<0.3598			p <0.673			p<0,238			p<0,238		

extra[c]: extracellular Aβ accumulation in the cortex at IHC analysis.

intra[d]: intracellular Aβ accumulation in the cortex at IHC analysis.

int-ext[e]: intracellular and extracellular Aβ accumulation in the cortex at IHC analysis.

negative[f]: negative cases at IHC analysis.

C. Cases with a strong or weak intensity of the signal or its absence in the cortex at IHC analysis

				876				894				952		
	IHC cortex signal			C	G			T	C			C	G	
			strong[g]	5	1		strong[g]	5	1		strong[g]	5	1	
			weak[h]	5	3		weak[h]	7	1		weak[h]	5	3	
			no signal[i]	6	0		no signal[i]	4	2		no signal[i]	2	4	
			p<0.2151				p<0.6097				p<0,206			

	1394				1544				1643				2034	
	C	G			G	T			A	G			T	C
strong[g]	4	4		strong[g]	7	1		strong[g]	6	2		strong[g]	6	2
weak[h]	3	5		weak[h]	8	0		weak[h]	5	3		weak[h]	5	3
no signal[i]	4	2		no signal[i]	6	0		no signal[i]	2	4		no signal[i]	2	4
p<0,558				p<0.3998				p<0,3998				p <0,3998		

strong[g]: strong intensity of the signal at IHC analysis.

weak[h]: strong intensity of the signal at IHC analysis.

no signal[i]: absence of the signal at IHC analysis.

D. Cases with the intracellular or extracellular Aβ accumulation in the hippocampus or its absence

				876				894				952		
	Hippocampus			C	G			T	C			C	G	
			ext[j]	3	3		ext[j]	4	2		ext[j]	3	3	
			int[k]	3	1		int[k]	3	1		int[k]	3	1	
			ext- int[l]	2	0		ext- int[l]	2	0		ext- int[l]	2	0	
			negative[m]	10	4		negative[m]	11	3		negative[m]	7	7	
			p<0,5763				p<0,8048				p<0,4858			

	1394				1544				1643				2034	
	C	G			G	T			A	G			T	C
ext[j]	4	2		ext[j]	5	1		ext[j]	3	3		ext[j]	3	3
int[k]	1	3		int[k]	4	0		int[k]	3	1		int[k]	3	1
ext- int[l]	2	0		ext- int[l]	2	0		ext- int[l]	2	0		ext- int[l]	2	0
negative[m]	7	7		negative[m]	13	1		negative[m]	8	6		negative[m]	8	6
p<0,3164				p<0,7562				p<0,5707				p <0,5707		

ext[j]: extracellular Aβ accumulation in the hippocampus.

int[k]: intracellular Aβ accumulation in the hippocampus.

ext-int[l]: extracellular and intracellular Aβ accumulation in the hippocampus.

negative[m]: absence of Aβ accumulation in the hippocampus.

E. Cases with a strong or weak intensity of the signal or its absence in the hippocampus at IHC analysis

				876				894				952		
	Hippocampus signal			C	G			T	C			C	G	
			strong[n]	2	0		strong[n]	2	0		strong[n]	2	0	
			weak[o]	5	3		weak[o]	6	2		weak[o]	5	3	
			no signal[p]	10	4		no signal[p]	11	3		no signal[p]	7	7	
			p<0,5784				p<0,7358				p<0,3895			

	1394				1544				1643				2034	
	C	G			G	T			A	G			T	C
strong[n]	2	2		strong[n]	4	0		strong[n]	2	2		strong[n]	2	2
weak[o]	4	4		weak[o]	7	1		weak[o]	5	3		weak[o]	5	3
no signal[p]	7	7		no signal[p]	13	1		no signal[p]	8	6		no signal[p]	8	6
p<1				p<0,7409				p<0,9165				p<0.9165		

strong[n]: strong signal in the hippocampus at IHC analysis.

weak[o]: weak signal in the hippocampus at IHC analysis.

no signal[p]: absence of the signal in the hippocampus at IHC analysis.

Table 15 (A, B, C, D, E): Chi-square test. A= adenine; C= citosine; G= guanine; T= timine.

	876	
	C	**G**
extra	**9**	**7**
intra	**0**	**0**
int-ext	**5**	**1**
negative	**6**	**0**

Table 16: Chi-square statistics. 4.6229; Degrees of freedom: 2; P-value < 0.091.

Chapter 7: Discussion

Aβ deposition in the brain has been widely investigated to date in many animal species in an attempt to discover a suitable animal model to provide insights into the pathology of human cerebral Aβ amyloidosis. Even if a large amount of data about Aβ deposition in the brain is available for numerous species, both domestic and wild, but mainly belonging to the category of carnivores (i.e. dog and cats), little is still known about the features of Aβ deposition process in the large herbivores. Aim of the present study was at first to characterize the features of Aβ deposition in cattle brain in relation to age and health status, in order to shed light on a possible formation process of Aβ in such species, which had never been investigated earlier. Second aim of our investigations was to compare the obtained findings with the knowledges acquainted to date about the neuropathological and genetic aspects of Aβ deposition process in human brains. Eventually, the third aim was to assess if cattle could represent a suitable animal model to disclose the mechanisms underlying cerebral Aβ accumulation in man.

Regarding immunohistochemical investigations, out of the 102 cattle brains examined in the present study, 59 tested positive for the presence of Aβ whereas 43 resulted negative. About 56% of the cases which tested positive for Aβ presence were older than 109 months, whereas nearly 88% of the cases testing negative for Aβ were younger than 36 months. Another interesting emerging finding was that on the 102 samples tested, 37 out of the 39 cases older than 85,5 months (95%) were positive for Aβ deposition (Table 4).

Chi square test performed confirmed the statistically significance of this result ($p < 0,0001$, Table 5). The aforementioned data would allow the supposition that Aβ deposition in the brain is an age-related process mainly involving cases older than 108 months (9 years). Our findings are similar to what described in humans, where it has been reported that the number of non-demented individuals presenting with cerebral Aβ deposits in the form of senile plaques was higher in older age groups (Hof et al., 1996). In a recent study by Rodrigue et al. (2012) on cognitively normal adults who underwent Aβ PET imaging it was demonstrated that cerebral Aβ deposition increases with age and is particularly elevated in about 20% of adults aged 60 and over.

Several studies regarding other animal species would support the aforementioned hypothesis. In a study on the cat brain (Gunn-Moore et al., 2006), Aβ deposits were immunohistochemically detected inside neurons as well as extracellularly, and the staining intensity appeared to be age-dependent.

Aβ deposition was shown to be closely age-dependent also in the brain of rhesus monkeys (Cork et al., 1990) and of dogs (Cummings et al., 1995); in the latter species such a finding was particularly evident in the cortex area, similarly to what displayed by the cattle examined in the present study.

By examining the classification of the same data according to the health status of the cases considered, it appeared that, in the healthy subjects, β-amyloid deposition was most frequently detected in cases older than 108 months (29/31 = 94%), whereas the 89 % (17/19) of the cases free of β-amyloid deposition belonged to the age range below 12 months. Considering diseased animals, we evidenced that the presence of a neuropathological affection induces an early accumulation of β-amyloid in an uniform way among all the age ranges considered starting from 13 months. In the category of diseased cases, cattle devoid of cerebral β-amyloid accumulations (15/24 = 62%) were mainly younger than 12 months. On the basis of the findings obtained it is conceivable that β-amyloid accumulation process seemingly increases concurrently with age, as reported in humans (Hof et al., 1996), and, in case of concomitant diseases, it could be hypothesized that an underlying neuropathological process may speed up cerebral Aβ accumulation.

By examining the frequencies of Aβ localization (Table 6), it was confirmed that the youngest cases were mainly negative; moreover, Aβ deposition started to increase with age, at first mainly displaying an extracellular pattern (38% between 12,5-36 months, 58% between 36.5-60 months), and then evolving in the older cattle to a prevalent coexistence of intracellular and extracellular deposits (52% between 108.5-180 months, 66% over 180 months).

These findings could be explained the theory reported by LaFerla et al., where it has been proposed that previously secreted Aβ, which forms the extracellular Aβ pool, could be taken up by cells and internalized into intracellular pools. So it is likely that the intracellular Aβ is derived from extracellular Aβ pools and is taken up into the cells through receptors or transporters (LaFerla et al., 2007).

A prevalent extracellular localization of Aβ deposits has been frequently described also in the brain of other animals, particularly cats (Brellou et al., 2005; Head et al., 2005) and dogs (Cummings et al., 1996; Aristotelis et al., 2002): the main extracellular deposition pattern is the diffuse (non-β-sheet) plaque, which can bona fide be assimilated to a form of preamyloid deposit, similarly to our findings in cattle. Actually the extracellular Aβ deposits observed in the cattle brains examined in our study were not in the form of plaques, but appeared more similar to preamyloid lesions, which are also frequently detectable in some form of human dementias (Rostagno et al., 2008) as well as in non-demented elderly (Delaere et al., 1990). In

human AD brain the extracellular localization of Aβ deposits in the form of senile plaques has long been reported, but recently the scientific attention has also been direct to better describe the features and investigate the role of Aβ intraneuronal accumulation in AD pathogenesis (Gouras et al., 2010). A supposition which has been put forward is that intraneuronal Aβ accumulation likely decreases with advancing plaque pathology: such a view would suggest that intraneuronal Aβ accumulation should be seen as an early event in the pathology of AD. In AD patients diffuse plaques characterize the first stages of the disease and may act as the precursor of the neuritic plaques arising afterwards.

In view of such findings, it could therefore be argued that the preamyloid deposits observed in the cattle brains examined could represent an even earlier stage of the Aβ deposition process than the diffuse plaques described in domestic carnivores and man.

The fact that no staining of the tissue sections examined was obtained by employing X34, a highly fluorescent derivative of Congo red, is very likely to be ascribed to a lack of the β-sheet secondary protein structure in the Aβ deposits detected, i.e. a fibrillar organization.

APP staining results appeared alike to those obtained by staining the brain sections under examination with 4G8 antibody, even if, on APP stained tissues, the staining evaluation has been less careful because a quantitative scoring of the signal was not performed. The similarity of APP and 4G8 staining results is likely due to the fact that 4G8 antibody recognizes the same amino acid sequence (17-24 aa) which also APP antibody is directed against to, so a crossreaction between APP and 4G8 can not be rouled out.

A set of the immunohistochemical investigations performed was carried out on the brain sections displaying a more prominent intraneuronal Aβ, in order to exclude an aspecific staining of lipofuscin by 4G8 antibody. Lipofuscin is a lipopigment which physiologically accumulates in certain cell types during aging. Its gradual intracellular accumulation is probably the most characteristic cytological change associated with the process of ageing in the brain. Cytochemically, it is considered to be an inert end product of lysosomal breakdown. It is known that lipofuscin may be aspecifically stained by anti-Aβ antibodies (Bancher et al., 1989). Our attempt to discriminate by double immunohistochemical labelling between Aβ and lipofuscin deposits revealed that most lipofuscin and Aβ occupy distinct cellular compartments in neurons, although there may be some detectable co-localization. Our results seem to correlate with findings previously reported by D'Andrea et al. (2002), which assessed that the bulk of Aβ 42 immunolabelling is present in areas of the cells not occupied by lipofuscin pigments in normal, aged-matched control and AD neurons. This is likely evidence for the possibility that Aβ42 containing endosomes may not make a major contribution to

lipofuscin formation in neurons. However, since lipofuscin progressively accumulates throughout the life of neurons, it is conceivable that the bulk of existing lipofuscin may have been deposited within these neurons prior to the onset of Aβ42 accumulation.

The relationship of glial cells to deposits of Aβ protein has long been studied in the brain of both humans and animals.

Regarding microglia, a study reported in literature on AD brains revealed that about 50% of diffuse plaque deposits appeared to be associated with reactive microglia, compared to nearly 100% of senile plaques (Itagaki et al., 1989). The finding that the intensity of the microglial reaction increased with the density of the surrounding amyloid deposits has been disclosed also by other studies (D'Andrea et al., 2004), leading to the supposition that the main association of microglia with dense core plaques is due to the presence of injured or dying neurons as plaque constituents. Such neurons would release lysosomal enzymes, cellular DNA and other factors capable of inducing the microglial activation.

In the cattle brains examined in our study almost all the extracellular β-amyloid deposits detected, even if they were not well consolidated in the form of plaques but just displaying as preamyloid aggregates, appeared surrounded by activated microglial cells. The involvement of microglia cells in the process of preamyloid deposition has not been much investigated to date; however, keeping in mind such limitations, we have found an association of preamyloid deposits with microglial cells in the brain. Conversely, a previous investigation in the AD human brain by El Hachimi and Foncin (1994) reported a lack of involvement of microglial cells with preamyloid deposits. Our findings would let us suppose that in preamyloid aggregates the amyloid substance itself or some other yet unknown co-factors, probably unrelated to neurons, might act as potent microglial-activating materials.

Regarding astrocytes involvement in the Aβ deposition process it is known from literature that these cells appear associated with both neuritic and diffuse plaques. Some studies have indicated that astrocytes activate subsequently to microglia suggesting a cause and effect relationship (Gatan and Overmier, 1999). Microglia activation can activate astrocytes, which in turn act to temper and regulate, sometimes even to inactivate, the phagocytic microglial activity.

In the majority of brain areas analyzed in our study, there was not a real colocalization between astrocytes and Aβ; however, when present, we have detected the involvement of astrocytes in the process of preamyloid deposition. The finding we obtained confirmed what previously described by other authors about astrocyte morphology in AD brains (Itagaki et al.,

1989): cell bodies of reactive astrocytes labelled with GFAP were located around preamyloid deposits, with some of them sending fine processes penetrating the aggregates.

Regarding immunobiochemical analysis for the detection of the different Aβ isoforms in cattle brains, a limitation of the present study was that frozen samples were available for a fewer cases (n = 27) than all those tested by IHC. In fact, the Chi square test was not statistically significant ($p < 0,8931$, Table 11).

Although at WB analysis a small amount of data was obtained, it appeared that the largest part (7/17 =41%) of the cases testing negative was younger than 12 months of age, whereas no overt association can be drawn between age-range and detection of positivity. Regarding the neuroanatomical distribution, the immunobiochemical analysis disclosed the presence of Aβ peptides mainly at the level of the cerebral cortex, with 76% of the cortices examined testing positive (19/25), whereas 37,5 % (3/8) of the examined cerebella displayed Aβ peptides. Aβ was never detected immunobiochemically at the brainstem level (0/7), thus confirming the related immunohistochemical results. Regarding the types of Aβ peptides detected, the same Aβ isoforms characterizing AD brains were disclosed in the cattle brain, i.e. Aβ 1-38, 1-40, 1-42 and 3-42, but, given the paucity of the samples tested, no correlations can be drawn among Aβ types and age ranges.

WB results were then compared with IHC analysis to disclose possible correlations.

Fisher test analysis, although the p-value was not completely statistically significant ($p = 0,08931$), likely suggests a trend in the comparison of IHC and WB results. Eight out of the 10 cases positive at WB analysis were also positive at IHC, while 2 out of 10 resulted negative. This is most likely due to the fact that WB is much more sensitive than IHC and could have unmasked IHC false negative cases.

Among the cases detected positive at IHC, 8 out of 16 were also positive at WB analysis, while the remaining amount resulted WB negative. Further analysis by mass spectrometry (SELDI-TOF), that is going to be performed in our study, could clarify if some of the cases testing negative at WB but positive at IHC were false positive because of an aspecific staining of the ageing pigment lipofuscin. Moreover, the fewer amount of positive cases detected at WB analysis in comparison to IHC could be ascribed to the antibody used to immunohistochemically detect Aβ, i.e. 4G8, which can cross react also with APP (Aho et al., 2010). Therefore it can not be excluded that 4G8 antibody immunoprecipitated not only Aβ but also the full-length APP, thus producing some false positive cases at IHC.

Interestingly, in 6 out of the 8 cases positive at both IHC and WB analysis, the presence of Aβ-42 isoform was detected at the immunobiochemical analysis. In other animal species such

as dogs (Aristotelis et al., 2002), cats (Head et al., 2005) and monkeys (Kimura et al., 2001), the diffuse plaques detected were mainly Aβ-42 positive. It would seem that also in cattle amyloid deposits are mainly characterized by the presence of the Aβ-42 peptide and an extension of the present study by investigating at WB analysis a larger number of the animals considered could confirm this hypothesis.

The genetic analysis was focused on the ApoE gene because it is known that APOE ε4 is a major risk factor for AD in humans (Bekris et al., 2010); specifically two SNPs in exon 4 (at codons 112 and 158) of the APOE gene that define three polymorphic alleles (ε2, ε3 and ε4) are known. The results from our study show that codons 112 and 158 of the bovine gene are not polymorphic. However we found 9 SNPs, among which 3 are novel.

An interesting result was detected for the position 876 for which statistical analysis suggested an association between a SNP at this position and cases with a strong or weak intensity of the signal or its absence in the cortex at IHC analysis. Actually, the frequence of the allelic variant "G" seemed to be in relationship with the extracellular cortical accumulation of Aβ.

The position of the polymorphism, located in one of the first introns near the 5' UTR, could lead to the hypothesis that the 876 polymorphism has a regulatory role in gene expression, as reported in many studies (Chorev et al., 2012).

There are some studies in humans focused on the role of ApoE genotype in the Aβ intracellular or extracellular depositions in the brain. In a study analizing 20 patients with AD and 10 controls (Christensen et al., 2010), the analysis revealed a strong association between the ApoE4 genotype and the presence of intraneuronal Aβ.

Given that ApoE is involved in neuronal cholesterol transport, it could not be excluded that perturbed intracellular trafficking might influence the intraneuronal or extraneuronal Aβ aggregation, in human as well as in cattle brains.

Further studies with the involvement of a larger number of animals could shed light on the role of this polymorphism. The next aim of our research will be the study of the role of other genes involved in the Aβ deposition process in humans, starting from presenilin 1 (PSEN-1) and presenilin 2 (PSEN-2).

Ringraziamenti

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