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Immune-Inflammatory Responses in Alzheimer's Disease: Therapeutic Implications

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Alla mia famiglia

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A Claudia, per la sua saggezza e il suo affetto…

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Senza dimenticare le colleghe di lavoro e di studio che in questi anni mi sono state vicine.

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Chapter 1

GENERAL INTRODUCTION

1.1 Aging and Brain

Aging is a natural process that is defined as a progressive deterioration of biological functions after the organism has attained its maximal reproductive competence. The most prominent characteristics of aging are a progressive decrease in physiological capacity, a reduced ability to respond adaptively to environmental stimuli, an increased susceptibility to diseases, and an increased mortality. Many theories have been advanced to explain aging, but the biological mechanism(s) that underlies aging is(are) still unknown.

Aging produces several changes in human brain. The human brain shrinks with aging. It is known that decreasing in weight and volume in aging brain is due to a loss of neurons and myelinated axons (Peters, 2002). Changes in brain white matter are prominent features of the aging brain. During aging an increase in microglial activation also occurs in several brain regions, including the hippocampus (Finch and Cohen, 1997). Possible mechanisms may include microglial reaction to advanced glycation end products (AGEPs) (Morgan et al., 1999), which

activate nuclear transcription factor kappaB (NFkB) and induce the transcription of pro-inflammatory cytokines during aging (May and Ghosh, 1998). Thus, age dependent alterations in gene expression cause a disruption of metabolic homeostasis (Mattson, 2002; Mocchegiani et al., 2006).

Normal brain aging is associated with chronic low-grade inflammation. A growing amount of literature is related to the development of dementia and in particularly Alzheimer's Disease (AD), which is a heterogeneous and progressive neurodegenerative disease that in Western societies accounts for the majority of clinical dementia.

Many inflammatory mediators have been detected in regions of the brain of patients with AD and astrocyte and microglial cells activation has a fundamental role in the inflammatory pathogenesis, as stated by the amyloid cascade/neuroinflammation hypothesis (Eikelenboom P, et al 2006).

Astrocytes are responsible for the production of the neurotoxic substances, such as reactive oxygen and nitrogen species, pro-inflammatory cytokines, complement proteins and other inflammatory mediators that bring important neurodegenerative changes (Vasto S, et al 2006).

The microglia activation can be due to local or systemic inflammation. In fact, a strong local inflammatory stimulus such as a previous head trauma is a risk factor for AD and several epidemiological studies clearly show that blood elevations of acute phase proteins, markers of systemic inflammatory stimuli, may be risk important factors for cognitive decline and dementia (Balistreri CR, et al 2008; Vasto S, et al 2007).

It has been seen that several genetic and environmental factors are involved in AD onset as recently reviewed (Listì F, et al 2006), thus confirming that the inflammatory status cannot be *per se* the only "hit" or condition able to force the development of the pathogenesis (Franceschi F , et al 2000).

1.2 Alzheimer's Disease

Clinically and pathologically Alzheimer's disease (AD) represents a sequential progressive neurodegenerative disorder. AD is etiologically heterogeneous and accounts for a majority of dementia in Western societies. It was identified for the first time by Alois Alzheimer, a Bavarian psychiatrist, who defined the pathological syndrome in a woman named Auguste D., showing several cardinal features of the disorder that are currently observed in most patients (Maurer et al., 2007). Symptoms like progressive memory impairment, disordered cognitive function and altered behaviour including paranoia, delusions, loss of social appropriateness, and a progressive decline in language function are common in many patients affected by AD. The first phase of this gradual, relentless process, is characterized by well conserved patient's awareness, and intact mechanical and sensory functions (McKhann et al., 1984). However, as individuals keep on losing ground cognitively, occupations such as walking and movement synchronization often resemble extra-pyramidal motor disorders similar to Parkinsonism (Allan et al., 2005).

The most important risk factor for AD is age. AD prevalence is approximately 1% between 65 and 69 years and it is higher than 50% in individuals above 95 years. Although the mean age of AD onset is

around 80 years, early-onset disease, defined arbitrarily as the illness occurring before the age of 60, can happen - though it is rare. Thus, early-onset cases make up about 6–7% of all AD evaluation (Aronson et al., 1991; Campion et al., 1999).

Besides aging, which is the most obvious risk factor for the disease, epidemiological studies have suggested several tentative associations. Some can be linked to a decreased reserve capacity of the brain, including reduced brain size, low educational and occupational attainment, low mental ability in early life, and reduced mental and physical activity during late life.

The brain reserve capacity is determined by the number of neurons and their synaptic and dendritic arborisation together with lifestyle-related cognitive strategies. A low reserve capacity has been linked with early presentation of some pathological changes of the disease (Mayeux R. 2003; Mortimer JA et al 2003).

Whether a brain trauma initiates the pathogenic cascade leading to plaque and tangle formation or whether it simply reduces the brain reserve capacity is unclear. Other risk factors are associated with vascular disease, including hypercholesterolemia, hypertension, atherosclerosis, coronary heart disease, smoking, obesity, and diabetes. Whether these are true causal risk factors for Alzheimer's disease,

driving the pathogenic processes resulting in plaque and tangle formation, or whether they induce cerebrovascular pathology, which adds to clinically silent disease pathology thus exceeding the threshold for dementia, needs to be established.

Although environmental factors might increase the risk of sporadic Alzheimer's disease, this form of the disease has been shown to have a significant genetic background. A large population based twin study showed that the extent of heritability for the sporadic disease is almost 80% (Gatz M, et al 2006).

1.3 Pathophysiology of AD

Neuro-pathological hallmarks are senile plaques and neurofibrillary tangles in the medial temporal lobe structures and cortical areas of the brain (Fig. 1), together with a degeneration of the neurons and synapses (Nussbaum and Ellis 2003; Findeis M.A 2007).

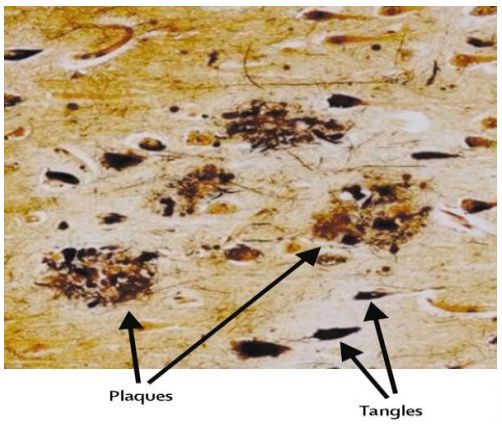

Fig. 1. Plaques and tangles in the cerebral cortex in Alzheimer's disease
Plaques are extracellular deposits of Aβ surrounded by dystrophic neurites, reactive astrocytes, and microglia, whereas tangles are intracellular aggregates composed of a hyperphosphorylated form of the microtubule-associated protein tau.

Extracellular senile plaques result from the accumulation of several proteins and an inflammatory reaction around deposits of amyloid. It is a fibrillar protein (Aβ), produced by the cleavage of a much larger protein, the β-amyloid precursor protein (APP), by a series of proteases (Akiyama et al., 2000; Findeis, 2007). The senile plaques are believed to evolve over a long period of time and the fibrillary nature is due to 42 amino acid long Aβ peptide accumulation. Besides, Aβ plaques

contain dystrophic neurites, activated microglia and reactive astrocytes (Rogers et al., 1988; Dickson et al., 1988; Akiyama et al., 2000).

Aggregated amyloid fibrils and inflammatory mediators secreted by microglial and astrocytic cells equally contribute to neuronal dystrophy (Nussbaum and Ellis, 2003; Findeis, 2007).

Neurofibrillary tangles are intracellular deposition of hyperphosphorylated degenerate filaments, which result from aggregations of the microtubular protein tau (Selkoe, 2001).

The abnormal hyperphosphorylation of tau makes it resistant to proteolysis and this might lead to several-fold increase in the levels of tau in AD. The hyperphosphorylated tau causes sequestration of normal tau and other microtubule-associated proteins, leading to inhibition and disruption of microtubules and impaired axonal transport (Iqbal K et al 2005).

Tau also becomes prone to aggregation leading to formation of intracellular neurofibrillary tangles, compromising neuronal and synaptic function.

Neurofibrillary tangles are not an exclusive marker of AD: they also feature in other neurodegenerative diseases named tauopathies. Among these pathologies, Fronto-Temporal Dementia with Parkinsonism (FTDP-17), an hereditary disease associated to numerous tau

mutations, is characterized at cellular level by deposition of paired helical filaments (PHF, Kidd, 1963) of hyperphosphorylated tau (Hutton et al., 1998).

Under physiological conditions, APP is processed by the non-amyloidogenic pathway, where cleavage by α-secretase releases a soluble fragment. In AD, this process is significantly altered, where increased amount of APP is cleaved by other endo-proteases such as β- and γ-secretase, generating highly amyloidogenic protein molecules of 40-42 amino acid residues.

APP is the Aβ peptide precursor and it is a transmembrane glycoprotein widely expressed (it is also present on platelets), produced by the endoplasmatic reticulum and mainly involved in the neuronal and dendritic growth, and synapses formation (Priller et al., 2006).

The catabolitic feature of APP is influenced by different enzymes as α-β-γ secretases (PS1 and PS2 are co-factors in the processing of APP by secretases); as regards the different enzymes, different amounts of Aβ can be produced. APP is processed by two competing pathways: the amyloidogenic and the non-amyloidogenic .

The amyloidogenic pathway is due to the consecutive action of two proteases, β- and γ-secretases, catalysing the release of the N- and C-terminal of the protein, respectively producing Aβ40 and Aβ42

isoforms. In the non-amyloidogenic pathway, α-secretase protects the protein from amyloidogenic changes by cleaving within the Aβ sequence (Fig. 2).

The peptide Aβ40, which constitutes most of the 90% secreted Aβ protein, is more soluble and less amyloidogenic compared to the Aβ42 isoform. The great part of amyloid tissue is constituted by Aβ42 isoform which is highly amyloidogenic (Selkoe, 2001; Findeis, 2007).

This peptide is highly neurotoxic especially due to the self aggregation and its capacity to form insoluble plaques either in vitro or in vivo (Nussbaum and Ellis, 2003; Findeis, 2007; Newman et al., 2007).

The amyloid cascade hypothesis is the central hypothesis for the cause of AD, which states that the initiating event in AD is an imbalance between the production and clearance of Aβ in the brain. Support for this hypothesis includes the finding that the mutations implicated in the familial disease are present in the genes for both the substrate (APP) and the key enzyme (presenilin) for Aβ generation. Most *APP* mutations also cluster around the secretase sites, and both the *APP* and presenilin mutations increase Aβ42 production (Blennow K et al 2006).

Furthermore, the knowledge that people with Down's syndrome, who possess an extra *APP* gene, develop Aβ plaques early in life, and the recent finding of a duplication of the *APP* locus in families with familial Alzheimer's disease, support the notion that life-long APP over-expression triggers Aβ deposition (Rovelet-Lecrux A et al 2006).

Another neuropathological hallmark of AD is the appearance of neurofibrillary tangles that consist of a hyperphosphorylated form of the microtubule-stabilizing protein tau, often conjugated with ubiquitin. The abnormal hyperphosphorylation of tau makes it resistant to proteolysis and this might lead to several-fold increase in the levels of tau in AD.

The hyperphosphorylated tau causes sequestration of normal tau and other microtubule-associated proteins, leading to inhibition and disruption of microtubules and impaired axonal transport. Tau also becomes prone to aggregation leading to formation of intracellular neurofibrillary tangles, compromising neuronal and synaptic function. Tau pathology starts early in the disease process in neurons in the transentorhinal region, spreads to the hippocampus and amygdala, and later to the neocortical association areas. Whether tau hyperphosphorylation and tangle formation are a cause or a consequence of Alzheimer's disease is unknown.

Recently, a number of researchers showed that the levels of transcripts for a number of pro-inflammatory markers were elevated in AD, specifically in response to tau (Yoshiyama et al., 2007; Klein et al., 2009; Wang et al., 2010). Although a relationship between neurofibrillary degeneration and β-amyloidosis is debatable, it is certain that hyperphosphorylation of tau is related to neuroinflammation and could be responsible for the progression of AD.

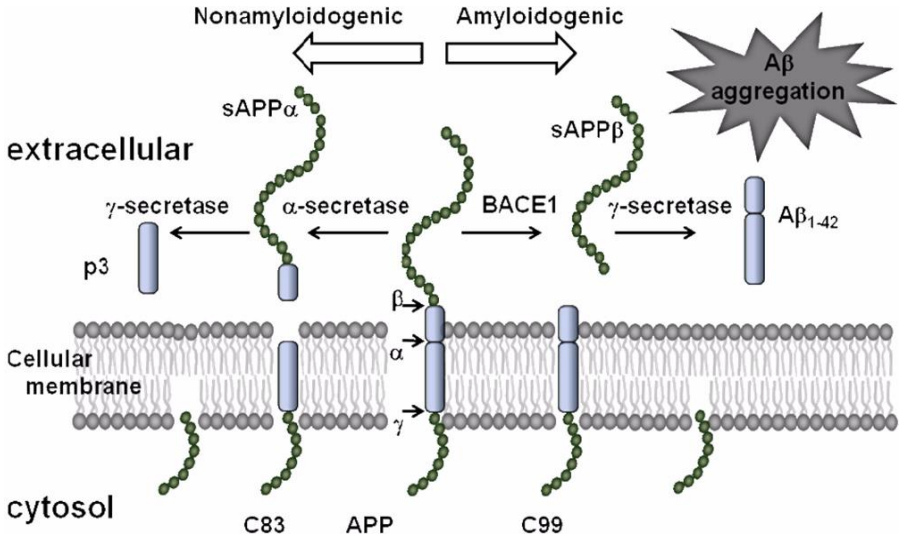

Fig. 2. Schematic representation of APP processing and Aβ accumulation. Mature APP is metabolized by 2 competing pathways, the α-secretase pathway that generates sAPPα and C83, and the β-secretase pathway that generates sAPP β and C99. C99 is a substrate for γ-secretase, generating Aβ. Aβ aggregates into small multimers (dimers, trimers, etc.) known as oligomers.

1.4 Genetics

Although the complete etiopathogenesis of AD still remains unclear, genetic studies have provided valuable insights into this complex and heterogeneous disorder. Twin and family studies have shown that certain genes contribute to the development of AD, especially with respect to the age at which the disease manifests, and more recently, the development of non-cognitive symptomatology. Early onset familial AD is a very rare autosomal dominant disorder caused by highly penetrant mutations in APP and presenilin genes, both linked to Aβ metabolism. Around twelve different mutations have been identified in APP gene at the level of α-, β-, or γ-secretase cleavage sites, which can lead to alteration in the normal proteolysis of amyloid precursor protein. Similarly, more than fifty missense mutations of the presenilin-1 gene (PS1) are associated with familial AD; several mutations of presinilin-2 gene (PS2) are associated with rare cases of early onset familial AD (Crentsil V. 2004; Cacabelos R 2002).

These mutations of APP, PS1 and PS2 may share a common pathogenetic mechanism leading to accumulation of β-amyloid protein as a result of abnormal amyloid precursor protein metabolism.

In contrast, sporadic AD is a very common disorder. Many studies have reported association of the APOE-4 allele with late-onset AD, and

APOE-2 has shown a protective effect. Moreover, APOE-4 may influence AD pathology by interacting with APP metabolism and Aβ protein accumulation, enhancing hyperphosphorylation of tau protein and neurofibrillary tangle formation, reducing choline acetyltransferase activity, increasing oxidative processes, modifying inflammation-related neuroimmunotrophic activity and glial activation, altering lipid metabolism, lipid transport and membrane biosynthesis in sprouting and synaptic remodelling, and inducing neuronal apoptosis (Vasto S et al 2008; Scapagnini G et al 2010).

1.5 Inflammation in AD

Inflammation is a complex cellular and molecular response to insults (stress, injury or infection), an attempt to defend against these insults. Inflammation is also a process that has been closely related to the onset of various neurodegenerative diseases, including AD.

This inflammatory response in neurons (neuroinflammation) includes activation of microglia, astrocytes, macrophages and lymphocytes, resulting in the release of inflammatory mediators such as cytokines, chemokines, neurotransmitters and ROS (Tansey et al, 2007).

The release of mediators leads to recruitment of monocytes and lymphocytes through the blood brain barrier (BBB) (Lossinsky and Shivers, 2004; Taupin, 2008) as well as activation of additional microglia, promoting their proliferation, and resulting in further release of more inflammatory factors (Das and Basu, 2008). The emerging evidence suggests that inflammatory responses contribute to the progress of AD, accelerating the course of the disease.

The major players involved in the inflammatory process in AD are thought to be the microglia and the astrocytes and possibly to a less extent the neurons, all of which are cellular components of the brain who have many critical roles in the homeostasis and function of the

brain (Akiyama et al., 2000a,b). The microglia are cells that support and protect the neurons and their functions in the CNS and act as immunocompetent defense cells that orchestrate the endogenous immune response of the CNS.

The microglia are composed mostly of mesodermally derived macrophages (Streit&Kincaid-Colton, 1995) and they are able to express major histocompatibility complex type II (MHC II), pro-inflammatory cytokines, chemokines, reactive oxygen species, and complement proteins (Tuppo and Arias 2005). Microglia also express scavenger receptors that mediate adhesion of the microglia to Aβ fibril-coated surfaces, leading to ROS secretion and cell immobilization (El Khoury et al., 1996). Secretion of ROS can result in further neuronal damage.

Microglia activated by Aβ show an increase in cell surface expression of MHC II protein along with increased secretion of proinflammatory cytokines IL- 1β, IL-6, and TNF-α, the chemokines IL-8, macrophage inflammatory protein-1α (MIP-1α), and monocyte chemoattractant protein-1. Although microglia have neuroprotective functions, neurotoxic mechanisms involving continuous activation of microglia and toxic factors released by microglia, may lead to

neuroinflammation. Subsequently, the continuous activation of microglia may act as trigger for the progression of AD pathology.

The role of astrocytes in the inflammatory process associated with AD is more difficult to ascertain. Senile plaques in the AD are known to be associated with reactive astrocytes (Dickson, 1997) and astrocytes cluster at sites of Aβ deposits. Similar to microglia, they also secrete various proinflammatory molecules such as ILs, prostaglandins, leukotrienes, thromboxanes, coagulation factors, complement factors, proteases, and protease inhibitors.

The aggregation of astrocytes around Aβ deposits in AD suggests that these lesions produce chemotactic molecules that induce astrocyte recruitment.

Astrocytes activated by Aβ produce chemokines, cytokines, and ROS that may result in neuronal damage. Chemokines released by astrocytes attract microglia, which further express proinflammatory products, thus increasing neuronal damage in the pathogenesis of AD (Fig. 3).

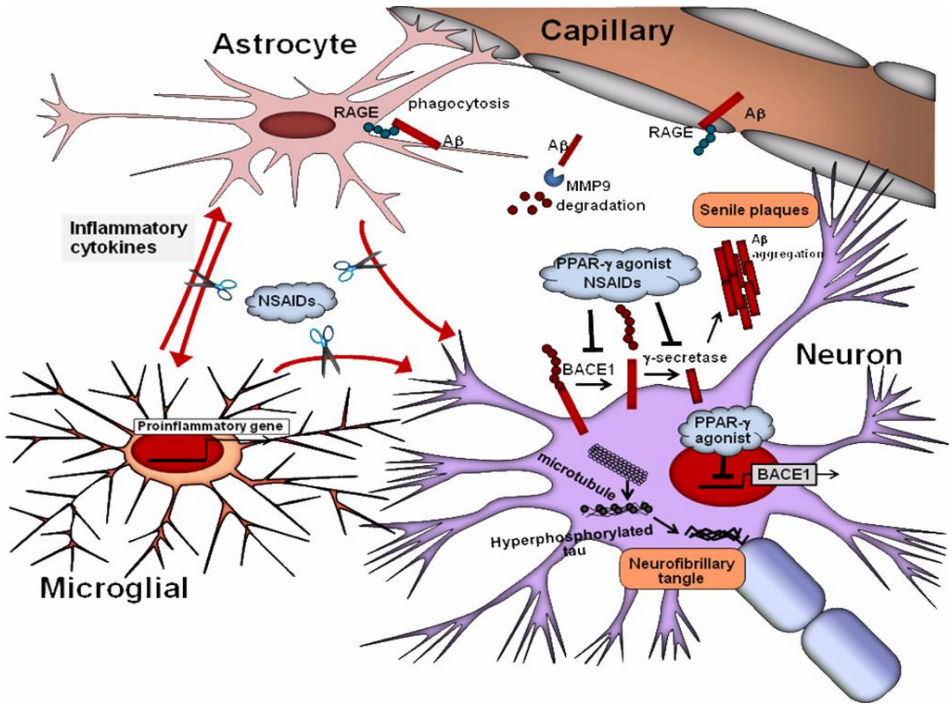

Fig. 3. Schematic representation of inflammation-mediated neuronal loss in Alzheimer's disease and possible preventive effects of NSAIDs and PPAR-γ agonists.

In addition to the observation that inflammatory mediators are present in AD lesions, there is also epidemiological and genetic evidence showing that inflammation contributes to AD pathology. Prospective case–control studies show that higher serum levels of some acute-phase proteins and cytokines, as C-reactive protein, alpha1-antichymotrypsin, interleukin (IL)-6 are a risk factor for the development of cognitive decline and AD (Dik et al., 2005; Perry, 2004; Schmidt et al., 2002; Licastro and Chiappelli, 2003; Yaffe et al., 2003).

The polymorphisms of cytokines and other inflammatory genes seem to be a genetic risk factor for AD. Moreover, the inflammatory hypothesis is further supported by the epidemiologic finding of reduced incidence of AD in patients who use anti-inflammatory drugs compared to controls (Hoozemans and O'Banion, 2005; int'Veld et al., 2001; McGeer and McGeer, 1998).

Other studies suggested that non-steroidal anti-inflammatory drugs, could be the key of the protective effect, actually anti-inflammatory drugs do not only inhibit ciclo-oxygenase, but also activate the receptor PPARγ which is a transcription factor that works shutting down the expression of pro-inflammatory genes in mononucleate phagocyte (Bernardo A et al 2005).

Further studies on the association between inflammation and AD are revealed in the association between dementia and the inflammatory marker high-sensitivity C-reactive protein (CRP) which has been studied in a cohort of Japanese American in the Honolulu-Asia Aging Study. The C-reactive protein concentrations were measured and associated with dementia which was assessed in a clinical examination that included neuroimaging and neuropsychological testing. The results showed that, compared with men in the lowest quartile (<0.34mg/L) of high-sensitivity C-reactive protein, men in the upper three quartiles had

a 3-fold significantly increased risk for all dementias combined, Alzheimer's disease, and vascular dementia. These data support the view that inflammatory markers may reflect not only peripheral disease, but also cerebral disease mechanisms related to dementia, and that these processes are measurable long before clinical symptoms appear (Schmidt R et al 2002).

The role of an individual genetic background and predisposition for the extent of an inflammatory response is determined by the variability of genes encoding endogenous mediators that constitute the pathways of inflammation. Several studies report a significant different distribution, in patients and controls, of pro-inflammatory genes, the alles of which are underrepresented in control subjects and overrepresented in patients affected by AD (Licastro and Chiappelli, 2003; Licastro et al., 2005, 2006; Lio et al., 2006a; McGeer and McGeer, 2001a).

1.6 Immuno-inflammatory Genes

As reported above, inflammatory molecules might play a central role in AD lesion development and a number of family and case control studies have been directed to investigate inflammatory molecule variants as susceptibility candidate genes for the disease.

Actually, the genes involved in the inflammation process are numerous and the role of an individual genetic background might show a predisposition to inflammation and its healthy or chronic resolution.

Primary responses are mediated by pathogen recognition receptors such as Toll-like receptor (TLR), pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6, anti-inflammatory cytokines such as IL-10 and eicosanoids (Imahara and O'Keefe 2004; Vasto et al., 2007c).

As such, we reviewed several data clearly showing that inflammatory genetic variation may contribute to AD susceptibility, strengthening the role of inflammation in AD. In fact, proinflammatory genotypes were significantly overrepresented in AD patients, whereas anti-inflammatory genotypes were significantly underrepresented (Candore et al., 2006; Candore et al., 2007; Vasto et al., 2007c).

IL-1 overexpression seems to occur early in plaque evolution. For instance, it is already evident in diffuse, non-neuritic Aβ deposits, and

can be observed in autopsied histochemical brain samples from fetuses and young children with Down's syndrome (Griffin WS et al 1994).

The early overexpression of IL-1 in AD suggests that it could play a key role in plaque evolution, in particular, IL-1 promotes the synthesis (Mackenzie IR. 2000) and processing of APP and may therefore promote further amyloid production and deposition in plaques (Buxbaum JD, 1998).

Also IL-6 can be measured in vivo and it reveals that elevated levels of IL-6 cause significant CNS damage and behavioural deficits. Transgenic mice that chronically overexpress IL-6 exhibit dose- and age-related deficits in avoidance learning that closely correlate with specific neuropathological changes (Heyser CJ et al 1997).

Moreover, IL-6 has been shown to induce cellular neuropathological changes indicative of a chronic inflammatory response in another transgenic mouse model that develops motor impairment and seizures. Hence, pro-inflammatory cytokines seem to be involved in APP metabolism and in the Aβ peptide production.

Besides, increasing evidences suggest the involvement of innate immunity receptors in the activation of microglial cells.

The CD14 antigen/Toll like receptor-4 (CD14/TLR4) receptor complex seems to be overexpressed on microglial cells inducing

activation through the binding of Aβ peptides. This receptor complex is known to be involved in cellular activation by micro-organisms components, as lipopolysaccharide (LPS) or other highly hydrophobic and aggregate structures, or endogenous molecules produced by cell and DNA damage. So, in the AD brain all these molecules may contribute to development and progression of neurodegeneration through the TLR4/NF-kB pathway (Walter S et al 2005; Fassbender K et al 2004; Fassbender K et al 2004; Lehnardt S et al 2004).

These studies will permit the detection of a risk profile which will potentially allow both the early identification of individuals susceptible to disease and the possible design or utilize of drug at the right dose for a desired effect, i.e., a pharmacogenomic approach for this disease.

1.7 Oxidative Stress and Therapeutic Perspectives

Oxidative stress has been implicated in a variety of pathophysiological conditions, including neurodegenerative disorders, and oxidative stress–mediated neuroinflammation which has been demonstrated to actively participate to AD ethiopathology.

In particular, cortical and hippocampal oxidative stress is a very early event in the pathogenesis of sporadic AD and correlates with the development of specific cognitive deficits in this condition. Inflammation is strictly related to ROS production, which act as signals to activate inflammatory genes. Actually, besides the pathological hallmarks of the disease, AD brains exhibit evidence of reactive oxygen species (ROS)–mediated injury, and free radical oxidative damage to key intracellular targets such as DNA or proteins has been shown to be a major cause of the neuronal cell death related to AD (Butterfield DA et al 1997).

Oxidative stress is the result of an unbalance between oxidant production and antioxidant defenses. The brain, compared to other organs, is more susceptible to oxidative stress for the following reasons: (1) High content of peroxidizable unsaturated fatty acids; (2) high oxygen consumption per unit weight; (3) high content of lipid

peroxidation key ingredients (iron and ascorbate); and (4) the scarcity of antioxidant defenses systems.

Within the cell, ROS are physiologically present at minimal concentration as byproducts of aerobic metabolism as well as second messengers in many signal transduction pathways and, in normal conditions, there is a steady-state balance between pro-oxidants and antioxidants

In AD, an overproduction of free radicals seems to be mostly related to mitochondrial dysfunctions, to the Aβ peptides themselves, and to the presence of unbound trace metal ions.

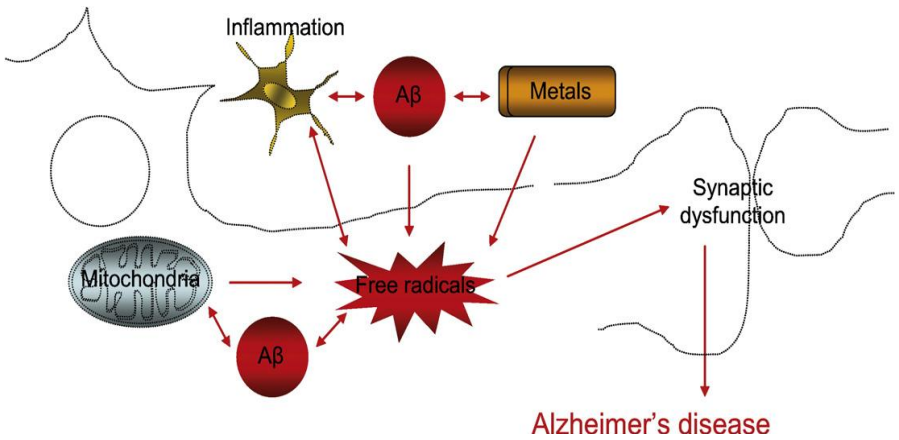

Fig. 4 Scheme of the generation and role of free radicals in AD. In cells, free radicals can be generated by two major sources: mitochondria and NADPH oxidase. Several key players,such as metals and Aβ, can exacerbate their production. Once accumulated inside the cell, free radicals can cause lipid, protein, DNA, and RNA damage that can exacerbate AD.

Today, it is evident that the three sources are not independent from each other, and recent hypotheses predict that, in the early stages of the disease, Aβ peptide enters the mitochondria where it induces ROS generation and subsequent oxidative stress (de la Monte SM, Wands JR 2006).

Aβ soluble oligomers, which have been shown to be cytotoxic via the formation of ROS, represent another source of oxidative stress, as well as the activation of microglial oxidase and the inflammatory response (Calabrese V et al 2006; Vasto S, 2007). These conditions well explain why the biomarkers of lipid peroxidation, such as free 4-hydroxynonenal (HNE), are abundantly present in several areas of the AD brain. Furthermore the presence of carbonylated-, HNE- and nitrated-proteins demonstrates the occurrence of protein modification by oxidation.

Signs of oxidative damage in AD patients have also been found in the cerebrospinal fluid, in urine, and in serum and, although the results of some studies are contradictory, it seems that the levels of oxidative damage parallels the progression of the disease (Butterfield DA, Sultana R.2007; Scapagnini G, et al 2010).

All together these findings suggest that increased oxidative damage is not the terminal sequelae of the disease but instead it plays an initial

role. They also suggest that damage does not mark further destruction by reactive species and is instead marked by a broad array of increased cellular defenses (Perry et al., 2002).

Aging, the major risk factor for AD, leads to loss of free radical scavenging ability by endogenous mechanisms and it is widely known that free radical-induced oxidative stress increases in brain aging. Oxidative damage to key intracellular targets such as DNA or proteins by free radicals has been shown to be a major cause of the neuronal cell death related to AD.

Since oxidative stress may underlie some - if not all - aspects of AD neurodegeneration and since to date most of the available treatments are merely symptomatic, a considerable research has been aimed at reducing the effects of oxidative stress in order to prevent AD progress, by using free radical scavengers. Thus, one therapeutic strategy is to delay the onset of AD dementia sufficiently long as to slow the neuronal damage associated with Aβ-induced oxidative stress, particularly Aβ-induced lipid peroxidation (Finkel T, Holbrook NJ.2000; Hensley K et al 1995, Sultana R et al 2006).

Brain-accessible antioxidants potentially may provide the means of implementing this therapeutic strategy of delaying the onset of AD, acting as neuroprotective agents.

By definition, neuroprotection is an effect that may result in salvage, recovery or regeneration of the nervous system, its cells, structure and function. Although there are several lines of evidence supporting the hypothesis that neuroprotection may be a practical and achievable pharmacological target, few effective compounds have been developed for clinical application.

One of the more important system devoted to the antioxidant defense in brain is represented by the "heat shock proteins" (Hsps) and among the Hsps family, an important role has been attributed to heme oxygenase-1 (HO-1 or Hsp-32).

HO-1 catalyzes the conversion of heme to biliverdin and iron. Biliverdin, in turn, is reduced to bilirubin, an antioxidant. Since HO-1 is induced in proportion to the level of heme (Keyse and Tyrrell, 1989), the induction of HO-1 suggests that there may be abnormal turnover of heme in AD.

In the CNS, the HO system has been reported to be very active and its modulation seems to play a crucial role in the pathogenesis of neurodegenerative disorders. Deregulation of the HO system has been associated with the pathogenesis of Alzheimer's disease, multiple sclerosis and brain aging (Takeda A et al 2000; Schipper HM. 2000).

Many studies clearly demonstrate that activation of HO-1 in neurons is strongly protective against oxidative damage and cell death. In fact, the activation of HO-1 seems to represent an important defensive mechanism for neurons exposed to oxidative stress (Chen K et al 2000; Panahian N et al 1999).

Thus, modulation of HO-1 should represent a potential pharmaceutical strategy for the treatment of neurodegenerative disorders. Because oxidative stress may be responsible for some aspects of AD neurodegeneration, extensive research has been aimed at reducing the effects of oxidative stress to prevent AD progression by using free radical scavengers. In recent years there has been a growing interest, supported by a large number of experimental and epidemiological studies, in the beneficial effects of some commonly used food-derived products in preventing various age-related pathologic conditions, included brain aging (Racchi M et al 2008).

1.8 Goal of the Thesis

The overall aim of this thesis was to study some hot topics concerning AD pathophysiology that have an important impact on therapeutic perspectives. The manuscripts included in this thesis have addressed different aspects of the most common form of senile dementia, hence we have focused our attention on inflammation, cytokines, immune response, oxidative stress, as well as on exploring the related therapeutic possibilities.

In fact, taking into account the future epidemic of AD, prevention and treatment are important goals of ongoing research. As discussed in this thesis, events contributing to the onset and development of AD are numerous and complex. I believe that the investigation of AD pathophysiology, particularly disentangling inflammation, is likely to provide important clues about how to develop drugs that can slow or delay AD.

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Chapter 2

Biomarkers of aging

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Biomarkes of aging

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1. ABSTRACT

Aging is a complex process that negatively impacts the development of the different systems and its ability to function. Moreover, the Aging rate in humans is not the same, principally due to genetic heterogeneity and environmental factors. The aging rate is measured as the decline of functional capacity and stress resistance. Therefore, several attempts have been made to analyse the individual age, (so-called biological age) compared to chronological age. The biomarkers of aging are age-related body function or composition, these markers aim to assess the biological age and predict the onset of age-related diseases and/or residual lifetime. Such biomarkers should help in one hand to characterise the biological age and on the other hand to identify individuals at high risk of developing age-associated diseases or disabilities. Unfortunately, most of the markers under discussion are related to age-related diseases rather than to age, so none of these markers discussed in literature is a true biomarker of aging. Hence, we discuss some disease-related biomarkers useful for a better understanding of aging and the development of new strategies to counteract it, essential for improving the quality of life of the elderly population.

2. INTRODUCTION

Aging results from a breakdown of self organizing system and reduced ability to adapt to environment. In addition, it has been suggested that normal human Aging is associated with a loss of complexity in a variety of fractal-like anatomic structures and physiological processes (1). Furthermore, using a variety of measures that employ fractal analysis, Aging has been shown to be associated with a loss of complexity in blood pressure (2), respiratory cycle (3), stride interval (4), and postural sway dynamics (5).

However, there is a judge difference among people that age: there are people at the age of 90 years old still in good mental and physical condition and other that at 60 years old have extensive cognitive difficulties and chronic diseases. Overall, understanding Aging means being able to quantify physical inability, mental functional capacity, organs and apparatus deregulation (6).

Aging is considered a process that changes the performances of most physiological systems and increases susceptibility to diseases and death. The Aging phenotype

is a complex interaction of stochastic, environmental, genetic and epigenetic variables. However, these variables do not create the Aging phenotype but generate the lost of molecular fidelity and therefore as the random accumulation of damage in the human organism's cells, tissues, or whole organism during life increases, the probability of disease and death also augments in proportion (7).

In the society, the public perception of advanced Aging involves the inability to survive due to chronic diseases and the combined loss of mobility, sensory functions, and cognition (8) with an exponential growth of health costs linked to increased size of elderly in the Western World. So, biomarkers of human Aging are urgently needed to assess health state of elderly and the possible therapeutic interventions.

What a biomarker for Aging should be or predict is quite broadly defined. At the minimum, a biomarker should not only (i) reflect some basic property of Aging, but also (ii) be reproducible in cross-species comparison, (iii) change independently of the passage of chronological time (so that the biomarker indicates biological rather than chronological age), (iv) be obtainable by non invasive means, and (v) be measurable during a short interval of life span. A biomarker should reflect the underlying Aging process rather than disease (9). It should vary as an individual ages, but not strictly chronologically; instead, its quantity should correlate with remaining life span and with the likelihood of acquiring multiple age-related conditions. Furthermore, scientists will likely need a set of numerous biomarkers, perhaps some that provide a window on particular tissues and others that give a glimpse of an entire organism, to replace life span as the best measure of Aging.

Another problem, which is even more challenging, is that unless we understand how Aging "works," we might not be able to define ideal biomarkers at all. A biomarker would have only limited utility without understanding of the biological complexity of the system and above all how we can influence the complexity of structure. Moreover, which tissues or organs are preferable to evaluate as a predictable marker? Maybe it can be taken from blood, urinary tract or central nervous system; biochemical markers are better suitable than histology markers that are still to find out.

Unfortunately, most of the markers under discussion are related not only to age, but also to diseases, and thus none of the markers discussed in literature is a pure biomarker of Aging. A recent report has stated that biomarkers collected in physical exams, such as markers of cardiovascular diseases (CVD) and diabetes are useful predictors of healthy Aging (10). In the present review, we will discuss markers based on immunosenescence, inflammatory responses and oxidative stress. The review is based on data from author laboratories rather than on an extensive review of the literature.

3. BIOMARKERS OF IMMUNOSENESCENCE

The modifications of the immune system in the elderly are evaluated as a deterioration of the immune system, the so-called immunosenescence, which is thought to be mostly the result of the declining effectiveness of T cells. It contributes to higher morbidity and mortality caused by the increased susceptibility to infectious diseases or their reactivation as well as to autoimmune phenomena and cancer (11-13).

It is well established that the percentage and the number of naïve T cells is lower in the elderly than in the young. Age-associated thymic involution is thought to materially contribute to this phenomenon. Reciprocally, the percentage and numbers of memory and effector-memory cells are higher in the elderly. In fact, lifelong and chronic antigenic load results the major driving force of immunosenescence, which impacts on lifespan by reducing the number of virgin antigen-non experienced T cells, and filling the immunological space with expanded clones of memory and effector, antigen-experienced T cells. Thus, the repertoire of cells available to respond to antigenic challenge from previously unencountered pathogens is shrinking (11,14,15).

Several studies have underlined the importance of ubiquitous viruses causing chronic latent infections, such as Herpes Viruses, in determining characteristic aspects of T-cell branch senescence such as the progressive exhaustion of naïve lymphocytes, the increase in memory cells and the T repertoire shrinkage. Particularly, the Herpes virus Cytomegalovirus (CMV) seropositivity has been associated with many of the same phenotypic and functional alterations of T-cell immunity previously considered as Aging biomarkers. CMV-specific lymphocytes represent, even in immunocompetent subjects, a sizable proportion of both the CD8+ and the CD4+ memory compartment and they increase with age, with a significant increase in the proportions of highly differentiated effector memory and effector $CD45RA^{+}$ CD8 T cells in comparison to younger subjects. Furthermore, the increase of these cells is due to the expansion of terminally differentiated exhausted lymphocytes and expanded clones restricted towards specific epitopes, with the accumulation of large oligoclonal expansions of $CD8^{+}$ T cells (16-20).

Thus, the analysis of T cells in longitudinal studies in octogenarians and nonagenarians has defined an "immune risk phenotype" (IRP), originally characterized by an inverted CD4/CD8 ratio and low lymphoproliferative response, that constitutes a major predictor of no survival. In addition, very old subjects with a health status impaired by the most common age-related diseases exhibit an increase in CMV-specific effectors T cells, mostly CD4+, with a parallel increase in anti-CMV IgG antibodies. As previously stated, persistent CMV infection induces chronic stimulation of specific T cells that leads to terminal differentiation to senescent cells with an altered functional capability. Thus, elderly have high expansions of CMV specific CD8 T cells that display an effector memory phenotype characterized by the low expression of CD28 and increased expression of NK-associated receptors. Therefore, a critical indicator of incipient mortality is T cell repertoire attrition. Furthermore, several studies have suggested a positive association between *in vitro* T cell function and individual longevity (17, 21-29).

Also in the B compartment age-dependent changes indicate that advanced age is characterized by lack of B instructive immune response to new extracellular pathogens. B cell number is decreased and the B-cell repertoire is influenced by Aging through the quality of the antibody response. Changes in B cell repertoire have been described, and decreased B cell diversity in old age is correlated with poor health status. In addition, decreased IgM and IgD levels in elderly suggest a shift from the naïve (CD27-) compartment of the B cell branch towards the memory (CD27+) compartment. However, data are controversial since not all studies have shown, in elderly, a significant decrease of naïve CD27- B cells and an increase of CD27+ memory B cells (30-35).

Circulating B cells can be divided on the basis of the expression of IgD and CD27 into different functional subsets. In aged, a double-negative (DN) IgD-CD27- B cell subset is significantly increased. The origin of DN cells is not well understood as they might derive from activated CD27+ memory cells that have lost CD27 expression (32, 36-38). Hence, they might be similar to the effector T exhausted T cells, previously stated.

Of interest, B naïve lymphocytes are increased in offspring of healthy centenarians. It is well known that centenarian offspring, who are in their 70s and 80s, have a survival advantage when compared with control subject of the same age range whose parents died at an average life expectancy (39). The main lymphocyte differences observed between the two groups concern B cells. Indeed naïve B cells are more abundant as well as double negative B cells are less abundant in centenarian offspring. These data are similar to that found in previously experiment on young subjects. So, B cell compartment of the offspring of centenarians seems to be more similar to that of young respect to the old one (40).

However, the age-dependent B cell changes here briefly discussed indicate that the loss of naive B cells could represent a hallmark of immunosenescence and could provide a biomarker possibly related to the life span of humans (32).

The importance of a well-preserved NK cell function in elderly is underscored by data showing that low NK cell activity is associated with development of infections and death due to infection in immunologically normal elderly subjects with an impaired performance status. The relative risk for the development of infection increased in accordance with the decrease in the NK cell activity and a low NK cell activity was associated with short survival due to infection. Furthermore, people aged > 85 year with low numbers of NK cells were reported to have three times the mortality risk in the first 2 years of follow-up than those with high NK cell numbers. Other aspects of NK cell function, such as the secretion of chemokines or interferon-γ in response to interleukin (IL)-2 are also decreased in the aged. Hence, high NK cytotoxicity associates with healthy Aging and longevity, whereas low NK cytotoxicity associates with increased morbidity and mortality due to infections, atherosclerosis, and poor response to influenza vaccination. Together, these results support the notion that preserved NK cytotoxicity should be considered a biomarker of healthy Aging and longevity, whereas low NK cytotoxicity is a predictor of morbidity and mortality due to infections (29, 41-43).

4. BIOMARKERS OF INFLAMMATION

Aging is accompanied by chronic low-grade inflammation state, showed by a 2 to 4-fold increase in serum levels of inflammatory mediators which acts as predictors of mortality independent on pre-existing morbidity. This pro-inflammatory status of the elderly underlies biological mechanisms responsible for physical function decline, and inflammatory age-related diseases are initiated or worsened by systemic inflammation (44-46). In fact, an inflammatory response appears to be the prevalent triggering mechanism driving tissue damage associated with different age-related diseases and the term "inflamm-Aging" has been coined to explain the underlining inflammatory changes common to most age-associated diseases (44,45,47-49). It is mostly the consequence of the body ability to counteract and modulate the effects of a variety of stressors, which cause the accumulation of molecular and cellular scars. However, a wide range of different aetiological factors contributes to increased low-grade inflammatory activity in elderly including a decreased production of sex steroids, smoking, subclinical disorders such as atherosclerosis, asymptomatic bacteruria, a higher amount of fat as well as cellular senescence (49,50).

However, there is a link among an individual exposure to past infection, levels of chronic inflammation and increased risk of heart attack, stroke, Alzheimer's disease (AD), Parkinson's disease, cancer, type-2 diabetes, sarcopenia, functional disability and high mortality risk. In addition, within individuals, C-reactive protein (CRP) levels are also correlated with the number of seropositivities to common pathogens, suggestive of infection history (44, 51-62).

Tumor necrosis factor(TNF)-alfa is an independent prognostic marker for mortality in persons aged 100 years and in elderly nursing home patients detectable serum level of TNF- alfa were associated with death within 13 months. Plasma levels of TNF-α are correlated linearly with Interleukin (IL)-6 and CRP reactive protein in centenarians, indicating an interrelated activation of the entire inflammatory cascade in the oldest old. High circulating levels of TNF- alfa and IL-6 as well as CRP have been related to CVD and frailty. Moreover, TNF- alfa has been linked to AD and type 2 diabetes, and IL-6 has been demonstrated to be a strong predictor of mortality itself. Increased levels of CRP, IL-6 and TNF- alfa are associated with insulin resistance in elderly non-diabetic subjects and highly sensitive CRP levels are significant predictors of subsequent diabetes and metabolic syndrome as well as AD. The prospective InCHIANTI study demonstrated that high levels of IL-6, CRP and IL-1, are significantly associated with poor physical performance and muscle strength (60,63-78).

On the other hand, white blood cell count (WBC) is an important predictor of all-causes mortality in aged, mostly CVD and a high leukocyte count may identify high-risk individuals who are not currently identified by traditional CVD factors (79,80).

During normal Aging, the gradual loss of telomeric DNA in dividing somatic cells can contribute to replicative senescence, apoptosis, or neoplastic transformation. Hence, an association between telomere length and mortality in 143 normal unrelated individuals over the age of 60 years. Those with shorter telomeres in blood DNA had poorer survival, attributable in part to a higher mortality rate from heart disease and a mortality rate from infectious disease. Telomere shortening of blood cells, likely due to increased rounds of replication depending on life-long immune-inflammatory stimuli, contributes to mortality in many age-related diseases. Hence, these results suggest their possible role as biomarkers. However, unfortunately they have not been yet extensively confirmed (81).

The majority of above described immune-inflammatory aspects, that characterize the immunosenescence, are also detectable in extreme longevity, where a higher frequency of genetic markers associated with a reduced pro-inflammatory ability seems to counteract the onset of the main age-related disorders. Centenarians are quite capable of mounting effective inflammatory responses; however, inflammatory status, correlated to increasing risk of developing frailty and diseases, is compensated by the concomitant development of strong and effective anti-inflammatory responses (39, 82,83).

4.1.Oxidative stress

The "free radical theory of Aging" has captivated the scientific attention as a possible biological explanation of the entire Aging process (84, 85). Oxidative stress has been linked to a variety of medical problems related to Aging, such as CVD, cancer, diabetes and AD (86, 87). Many *in vitro* markers of oxidative stress are available, but most are of limited value *in vivo* because they lack sensitivity and/or specificity or require invasive methods. Thus the results of studies investigating oxidative stress in human Aging are still controversial, and there are still limited and conflicting results available in the literature (88-91).

There is considerable literature supporting a key role of oxidative stress in the clinical phenotype, with direct evidence of significant increases in oxidative DNA damage, protein oxidation and lipid peroxidation (86,87,92). Moreover, numerous studies have demonstrated that oxidative stress is increased in frail, institutionalised elderly people, and may lead to an accelerated Aging, while in free living elderly this increase is not always significant.

An example of biomarkers of oxidative stress, *in vivo*, is the measure of lipid peroxidation, although producing contradictory results. Lipid oxidation not only causes membrane disruption, it also produces aldehydic species, such as malondialdehyde (MDA), able to perpetrate further damage by binding to and modifying proteins (93). MDA, though certainly not perfect, has widely performed as a biomarker to ascertain whether lipoperoxidation has taken place (93, 94) and it has been often utilized to evaluate human Aging. *In vivo* results in elderly are quite ambiguous. In numerous studies plasma MDA, evaluated by means of the thiobarbituric acid test, was significantly higher in healthy elderly, confirming the presence of increased lipoperoxidation in old age. Nevertheless, other studies in healthy older subjects, reported a biological antioxidant status similar to those of younger elderly subjects (95).

Recently, isoprostanes (IsoPs), compounds that are produced *in vivo* by free radical-induced peroxidation of arachidonic acid, have been also proposed to assess oxidative stress status *in vivo*, but there are still few evidences of their consistency (96). The data from literature suggest that lipid peroxidation is a less sensitive marker of oxidative stress than protein oxidation (90). The most widely studied oxidative stress-induced modification to proteins is the formation of carbonyl derivatives. Carbonyl formation can occur through a variety of mechanisms including direct oxidation of certain amino-acid side chains and oxidation-induced peptide cleavage. Protein carbonyl level is a stable and generic signal of protein oxidative damage (97, 98). The importance of protein oxidation in Aging is supported by the observation that levels of oxidized proteins increase with animal age, but again in humans *in vivo* studies results are poor and uncertain. Although all organs and all proteins can potentially be modified by oxidative stress, certain tissues and specific protein targets may be especially sensitive. For example, recent studies have applied redox proteomic, a branch of proteomics that identifies oxidatively-modified proteins, to characterize specific proteins in brain Aging, and a number of proteins that are oxidatively modified in AD have been identified (99).

Oxygen free radicals can induce a variety of damage to DNA, including DNA single and double strand breaks, base modifications and abasic sites (100). 8-hydroxy-2-deoxyguanosine (8-OHdG) is by far the most studied oxidative DNA lesion and has gained much attention because of its mutagenic potential (101). The formation of 8-OHdG in leukocyte DNA and the excretion of 8- OHdG into urine have been frequently measured by high performance liquid chromatography or mass spectrometry to assess oxidative stress in humans. Compared with the determination of 8-OHdG in leukocyte DNA, the measurement of urinary 8-OHdG offers some advantages, because it is non-invasive, there is less production of artifacts during sample procedure, it better represent a marker of oxidative DNA damage and repair from all cells in the organism (102). Although some studies have identified an age-related increase of 8-OHdG in healthy volunteers (103), prospective study of oxidatively damaged DNA as a predictor of risk for age related pathologies is extremely difficult and few interesting results have been obtained to date. In addition, the accumulation of oxidation products several studies have

associated Aging with a progressive loss of antioxidant defence (104, 105).

Reactive oxygen species (ROS) production is largely counteracted by an intricate antioxidant defence system that includes the enzymatic scavenger superoxidodismutase (SOD), catalase and glutathione peroxidise (GSH-Px). SOD speeds the conversion of superoxide to hydrogen peroxide, whereas catalase and glutathione peroxidise convert hydrogen peroxide to water. The most recently discovered SOD isoenzyme is the extracellular SOD (EC-SOD) that plays a primary role as main enzymatic scavenger of superoxide in the extracellular space (106). Numerous researches evaluated the impact of Aging process on EC-SOD activity, but the results are yet disparate. There are conflicting evidences about the effect of Aging on GSH-Px activity. In the French PAQUID study, there were no changes in GSH-Px with age (107). BELFAST study has shown a decline in GSH-Px in well free-living nonagenarians (95) and other studies have demonstrated the same in institutionalized old subjects (109). In a recent population-based study, cognitive decline was associated with lower activity of the protective selenium-dependent GSH-Px and a higher activity of Cu/Zn-SOD (110).

In addition to these well characterized antioxidant enzymes a variety of other non-enzymatic, low molecular mass molecules are important in scavenging ROS. These include ascorbate, pyruvate, flavonoids, carotenoids, uric acid and perhaps most importantly, glutathione (GSH), an ubiquitous antioxidant which is present in millimolar concentrations within cells. GSH depletion can enhance oxidative stress, GSH level and the ratio between GSH and oxidized glutathione (GSSG) is decreased in models of Aging and correction of low tissue glutathione increased longevity (109,110). Thus it has been speculated that glutathione status could be an indicator of health and functional age. In the BELFAST study GSH plasma levels were increased in nonagenarians compared to septo/octogenarians (95). It has been recently shown in 204 volunteers with a broad age spectrum that blood GSH concentration declines with age (111). Another study, measuring cysteine/cystine and GSH/GSSG redox in plasma of 122 healthy individuals aged 19-85 years, showed a steady, linear increase in oxidative events throughout adult life and in particular that the capacity of the GSH antioxidant system is maintained until 45 years in healthy subjects and then declines rapidly (112).

Recently, total plasma carotenoid levels have been suggested as a possible health indicator in elderly populations. The 'Epidemiology of Vascular Aging' (EVA) study have determined the association between baseline total plasma carotenoids and mortality. Low total plasma carotenoid levels were significantly associated with all-cause mortality in men but not in women (113).

In the context of stress response, eukaryotic cells are able to induce an evolutionarily highly conserved class of proteins known as heat shock proteins (HSPs) or stress proteins. A large body of evidence support a critical role for HSPs in cellular protection against ROS and a variety of other insults, including heat, hypoxia, ischemia, excitotoxicity, glucose deprivation, cancer, and Aging (114). The cellular protection of HSPs is attributed to their molecular chaperone function by facilitating nascent protein folding and refolding or degradation of abnormally folded proteins. Recently, the role of extracellular Hsp70 (also referred to as serum Hsp70) begun to be addressed and it has been proposed as a potential biomarker of healthy Aging (115,116), easily measurable in the blood by the classical sandwich enzyme-linked immunosorbent assay (ELISA) (117). Rea *et al* (118) examined serum Hsp70 in 60 individuals with ages ranging from 20 to 96 years. They demonstrated a progressive decline in serum Hsp70 levels in older age groups. Similarly, Jin *et al* (119), in their study of 327 healthy male donors aged between 15 and 50 years, demonstrated a decline in serum Hsp70 at older ages (between 30 and 50) although at younger ages, they noted a positive correlation with age. Terry *et al.*, (120) in their cross-sectional study, have assessed serum Hsp70 levels from participants enrolled in either the New England Centenarian Study (93 centenarian offspring plus 43 controls) or the Longevity Genes Project (87 centenarians plus 83 controls), showing that serum Hsp70 levels are lower in those individuals that reach an advanced age. In addition, they have suggested that low serum Hsp70 levels are associated with longevity independently on other covariates such as age, gender, race, income, alcohol, CVD, and a variety of other age-related diseases. It should be noted, however, that none of these studies, examined the changes in serum Hsp70 in the same individuals over time.

5. CONCLUSIONS

Aging is a complex process that negatively impacts the development of the different systems and their ability to function. A long life in a healthy, vigorous, youthful body has always been one of humanity greatest dreams. During the last years, an increasing number of scientific meetings, articles, and books have been devoted to anti-Aging therapies. This subject, full of misleading, simplistic, or wrong ideas, is very popular among the general public, whose imagery has been fascinated by all possible tools to delay Aging and to get immortality. Hence, the search for Aging biomarkers continues because such biomarkers would have tremendous relevance to the current push to identify drugs that ameliorate the Aging processes.

As discussed by Johnson (121), The American Federation for Aging Research has proposed the following criteria for a Biomarker of Aging:

1. It must predict the rate of Aging, exactly telling where a person is in his/her total life span.
2. It must monitor a basic process that underlies the Aging process rather than the effects of disease.
3. It must be able to be tested repeatedly without harming the person, as a blood test.
4. It must be something that works in humans and in laboratory animals. Hence, it should be tested in laboratory animals before being validated in humans.

Table 1. Biomarkers of unsuccessful aging

Unsuccessful Aging		Ref
Memory T cells	↓	14-16
Effector T cells	↑	
T cell repertoire	↓	
IgG anti CMV	↑	17,21-29
DN B cells	↑	40
CRP	↑	44,51-62
TNF	↑	60,63-78
IL-6	↑	
IL-1	↑	
WBC	↑	79,80
Telomere length	Short	81
MDA	↑	93-95
8-OHdG	↓	101-103
Plasma carotenoids	↓	113

For the explanation see the text

Table 2. Biomarkers of successful aging

Successful Aging		Ref
Naive T cells	↑	16-20
T cells function	↑	
NK function and number	↑	29,41-43
IgM	↑	31-33,40
Naive B cell	↑	
GSH-Px	↑	106,110
GSH	↑	
Serum HSP70	↓	115,118

For the explanation see the text

For over 15 years, the National Institute on Aging has supported research on biomarkers of Aging. While many important findings have developed, no biomarker has yet been identified" (http://www.infoaging.org). The general feeling of the Aging community is that biomarkers fulfilling all of the above criteria are unlikely to exist. Hence, we discussed some disease-related biomarkers (summarized in Table 1) as well as markers found in successful Aging (summarized in Table 2) useful for a better understanding of Aging and for the development of new strategies to counteract it essential for improving the quality of life of the elderly population. In fact, this kind of knowledge is useful to anti-Aging strategies aimed to slow Aging and to postpone death by preventing infectious diseases and delaying the onset of age-related diseases (55, 11, 122,123).

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Chapter 3

Inflammation, Cytokines, Immune Response, Apolipoprotein E, Cholesterol, and Oxidative Stress in Alzheimer's Disease: Therapeutic Implications

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Inflammation, Cytokines, Immune Response, Apolipoprotein E, Cholesterol, and Oxidative Stress in Alzheimer Disease: Therapeutic Implications

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Abstract

Alzheimer disease (AD) is a heterogeneous and progressive neurodegenerative disease, which in Western society mainly accounts for senile dementia. Today many countries have rising aging populations and are facing an increased prevalence of age-related diseases, such as AD, with increasing health-care costs. Understanding the pathophysiology process of AD plays a prominent role in new strategies for extending the health of the elderly population. Considering the future epidemic of AD, prevention and treatment are important goals of ongoing research. However, a better understanding of AD pathophysiology must be accomplished to make this objective feasible. In this paper, we review some hot topics concerning AD pathophysiology that have an important impact on therapeutic perspectives. Hence, we have focused our attention on inflammation, cytokines, immune response, apolipoprotein E (APOE), cholesterol, oxidative stress, as welll as exploring the related therapeutic possibilities, i.e., nonsteroidal antiinflammatory drugs, cytokine blocking antibodies, immunotherapy, diet, and curcumin.

Introduction

The most common form of dementia, Alzheimer disease (AD) is a progressive neurodegenerative illness that affects nearly 0.6% of those persons aged 65–69, 1.0% of ages 70–74, 2.0% of ages 75–79, 3.3% of ages 80–84, and 8.4% of persons 85 and older. Thus, it represents a growing public health problem as life expectancy increases. AD is characterized by progressive memory deficits, cognitive impairment, and personality changes with behavioral and emotional disturbances that may eventually interfere with the patient's ability to perform the basic activities of daily living. These symptoms are a result of neuronal death, especially in the limbic and association cortices, which have roles in memory and navigation.[1–3] Neuronal death follows a period of progressive synaptic dysfunction caused by the senile plaques, characterized by the accumulation of proteins in the form of β-pleated sheet fibrils, which are composed mainly of a 42-amino-acid peptide known as β-amyloid (Aβ), and neurofibrillary tangles, mainly composed by a cytoskeletal hyperphosphorylated protein tau. The relative contributions of Aβ and tau to the process of neuronal death is the subject of intense debate in the field. However, the "amyloid cascade hypothesis" supports the idea that Aβ is the main pathogenetic factor of AD due to the aberrant metabolism of the amyloid precursor protein. The subsequent massive production and deposition of the peptide in extracellular sites are responsible for a concatenate series of events that result in neurotoxicity and consequent neuronal death. Many inflammatory mediators have been detected in several regions of the brain of AD patients and in astrocyte and microglial cells activation, which have a fundamental role in the inflammatory pathogenesis, as stated by the amyloid cascade/neuroinflammation hypothesis.[4–6]

Events contributing to AD development are numerous and complex. The pathogenesis involves a multitude of variables, many of which remain to be quantified. Recent work highlighting the importance of immune-inflammatory responses in AD has led to the prospect of exciting future therapeutic options. A better understanding of the interplay

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among inflammatory mediators, oxidative stress, and acquired immunity is necessary. Several inflammatory factors influencing AD development, i.e., environmental factors (proinflammatory phenotype) and/or genetic factors (proinflammatory genotype), have been described.[4–11]

Taking into account the future epidemic of AD, prevention and treatment are important goals of ongoing research. Furthermore, a better understanding of AD pathophysiology must be accomplished to make this objective feasible. In the present paper, we will review some hot topics concerning AD pathophysiology and their subsequent relationship to therapeutic perspectives.

Inflammation and Antiinflammatory Drugs

Inflammation clearly occurs in pathologically vulnerable regions of the AD brain with the same characteristics of peripheral inflammatory responses. In the periphery, the presence of degenerating tissue and the deposition of highly insoluble abnormal materials are classical stimulants of inflammation. Likewise, in the AD brain, the occurrence of damaged neurons, neuritis, the deposition of highly insoluble Aβ42 peptide and neurofibrillary tangles provide obvious stimuli for inflammation. Moreover, senile plaques in AD brains are associated with reactive astrocytes and activated microglial cells, which overexpress cytokines and acute-phase proteins.[4,12,13]

The microglial activation can be due to local or systemic inflammation. In fact, a strong local inflammatory stimulus such as a previous head trauma is a risk factor for AD, and several epidemiological studies clearly show that blood elevations of acute-phase proteins, which are markers of systemic inflammatory stimuli, may be risk factors for cognitive decline and dementia. Furthermore, in experimental animals, chronic systemic inflammatory response induced by lipopolysaccharide administration also induces glial activation.[14–16] A systemic inflammatory challenge in an animal with a chronic neurodegenerative disease leads to a significant increase in acute neurodegeneration, because microglia in the diseased or aged brain are "primed" and they switch their phenotype to produce neurotoxic molecules when they respond to systemic inflammatory signals. It has been suggested that in the diseased or aging brain, signals from systemic inflammation do not evoke a protective homeostatic response in the host, but evoke an exaggerated response that contributes to disease progression. In a retrospective general practitioner database study, the presence of two or more infections over a 4-year follow-up period was shown to increase the odds of developing AD by around two-fold. Direct evidence in humans that systemic inflammatory events might affect AD was shown in a 2-month study of AD patients, in which systemic infections and raised plasma levels of interleukin-1β (IL-1β) are both associated with an increased rate of cognitive decline.[17,18]

Periodontal disease (PD) can be used to establish further evidence between inflammation and AD. PD is a model of chronic inflammatory disease able to influence the general status of health and the respective quality of aging. PDs are a heterogeneous group of diseases that affect the supporting structures of the teeth (gingiva, root cement, alveolar bone, and periodontal ligament). In a generic way, it is possible distinguish gingivitis as the early stage not involving tooth attachment and showing an irritation of the gums from the periodontitis that affects all of the tissues around the tooth up to the final solution of the unhappy dental loss. The etiology is complex, clinical manifestations differ, and several classifications have been proposed. The chronic stimulation of inflammation sustained by Gram-negative anaerobic bacteria of dental plaque has been correlated with various systemic diseases, such as preterm and low birth weight, atherosclerosis and cardiovascular diseases, worsening diabetes control, poor wound healing, aspiration pneumonia, and osteoporosis.[19–21]

At present, PD is accredited as a complex model, in which direct and indirect dynamics as well as innate, endogenous, and exogenous factors are involved. In fact, local acquired immunity reacts, producing inflammatory mediators that are able to alter vessel permeability and allowing monocytes to penetrate in the inflamed tissue. The chronic stimulation of inflammation by bacterial plaque involves several cell populations and several networks of cytokines, allowing the loss of attachment and the bone defects formation, because they amplify the inflammatory reaction and activate the effectors mechanism responsible for tissue destruction. At the same time, bacteria directly or by means of their lipopolysaccharides could reach the blood circle.[21,22]

New links have been hypothesized, on the basis of the same model, for renal diseases, obesity dismetabolic syndrome, and pancreatic cancer; the most interesting link suggested is that with AD.[21] It is important to emphasize that among periodontal bacteria, some species are capable of invading the brain, changing the cytokine milieu and possibly contributing to existing pathological mechanisms.[23] However, it is likely that the relationship depends on the systemic inflammation.

This mechanism implies that PD-derived inflammatory molecules increase brain inflammation. The interaction between periodontal bacteria and host response results in locally increased production of inflammatory molecules. The host response to subgingival periodontal pathogens engages both innate and instructive immune responses, resulting in the alteration of local vasculature, generation of an inflammatory response, immune cell priming, and the secretion of inflammatory mediators. In periodontal health, bacteria and host response are in balance. In gingivitis, the bacterial challenge elicits an innate immune response in the adjacent gingival tissue that is able to limit bacterial-induced pathology. In periodontitis, the balance between bacteria and host response is disrupted, resulting in increased inflammatory infiltrate and the production of inflammatory mediators. Tissue destruction occurs mainly by activation of osteoclasts, matrix metalloproteinases, and other proteinases by the host inflammatory response. In severe PD, these proinflammatory molecules may induce systemic inflammation and therefore may access the brain via the systemic circulation. Proinflammatory molecules derived locally from periodontal tissue may stimulate trigeminal nerve fibers, leading to increased brain cytokines. These cytokines may act on the already primed glial cells, resulting in an amplified reaction and possible progression of AD. A test of this hypothesis would entail examining whether PD affects the progression of AD manifested clinically as earlier onset or as more severe disease.[21,24]

In this context, both cross-sectional and longitudinal studies have shown that patients with dementia are more likely to have poor oral health. For example, the Nun Study, a longitudinal study of aging and AD, has offered an opportunity for studying oral health and cognitive function. Study authors found that a low number of teeth increases the risk of a higher prevalence and incidence of dementia, even in patients without the apolipoprotein-E4 (APOE4) allele (see below), although without conclusive evidence around the casual role played by each other.[21,25–27] In conclusion, evidence-based data regarding the association of PD with neurodegenerative disorders are still lacking; however, it seems plausible that via contribution to systemic/brain inflammation, brain cytokines increase and activate the neurodegenerative pathway.

The role of local and systemic inflammation raises the possibility that some proinflammatory cytokine-blocking antibodies or soluble receptors could also be beneficial for preventing or treating AD (see below). Moreover, there are other less potent well-known inflammation-modulating drugs, such as statins and nonsteroidal antiinflammatory drugs (NSAIDs) that have few side effects and can be used with safety, even in very old subjects. On this basis, it is reasonable to assume that antiinflammatory treatments could be useful to counteract and to reduce both the age-dependent and the AD-dependent inflammatory status, either preventing or treating the development of AD. In particular, several epidemiological studies have suggested that long-term treatment with NSAIDs may reduce the risk of developing AD[28–31](for statins, see below). On the other hand, a recent report based on a longitudinal study of men and women aged 70 years and older with a family history of AD (ADAPT Study) showed that the use of naproxen or celecoxib did not improve cognitive function.[32] However, several critical issues have been raised concerning these study results; i.e., it has been claimed that the ADAPT Study does not indicate that NSAIDs, if taken during adulthood and for an extended period, cannot prevent or delay the onset of dementia.[33] In addition, examining the effects on AD risk of NSAID use for >5 years in a large health-care database including 49,349 cases and 196,850 controls, it has been clearly demonstrated that long-term NSAID use was protective against AD. They found that long-term users of NSAIDs were at lower-than-expected risk of AD. They found that the protective effect did not seem to be identical for each NSAID: Some showed clear protective effects, others did not, and in others the effect on AD risk was unclear. Findings were clearest for ibuprofen. Aβ42-suppressing NSAIDs did not differ from others, suggesting a key role for cyclooxygenase inhibition.[34]

Cytokines and Anticytokines Therapy

As reviewed recently, cytokines are critical in the pathophysiology of AD. Although evidence for cellular and humoral immunity is not present in the AD brains of individuals, cytokines including tumor necrosis factor-α (TNF-α and IL-1, as well as other components of the immune response, such as members of the complement cascade, are present.[35,36] Accordingly, cytokine gene polymorphisms have been claimed to play a key role in the pathophysiology of AD. Many genetic studies have reported significant associations between different polymorphisms of pro- and antiinflammatory cytokines and AD. However, there are controversial findings from other studies that have not replicated the initial results.[4] Meta-analysis provides a means to quantitatively synthesize association data across studies of the same genetic variant. The use of the meta-analyses has recently become an important part of genetic research, mainly to reconcile previously conducted studies that gave inconsistent results. Hence, two recent meta-analyses performed by our group on IL-1β and TNF-α clearly demonstrate the association between some functional single-nucleotide polymorphisms (SNPs) and AD.[37,38]

In this paper, we present data on a meta-analysis focused on transforming growth factor-β (TGF-β) expressed in the brain and implicated in the pathophysiology of AD. TGF-β might have dual proinflammatory and antiinflammatory roles, although the precise effects of TGF-β in AD are not well understood. [3,35] However, characterization of the roles of TGF-β in AD has been addressed in experimental models of the disease. In transgenic AD-prone mice, overexpression of TGF-β reduced plaque burden. Activation of TGF-β in microglial cells might lead therefore to increased degradation of Aβ.[39] Polymorphisms in the genes regulating the expression of TGF-β1 have been hypothesized to enhance the risk of developing AD. In particular, the most studied polymorphism of TGF-β, the C/T SNP at the position 509 in the 5′-flanking region of TGF-β1 gene, was shown to be associated with increased expression and also with increased plasma level of TGF-β.[40,41] However, genetic association studies investigating the association with this SNP and the risk of AD gave very contrasting results. The main causes explaining the lack of replicability of the results between different studies seems to be the heterogeneity of the enrolled study populations and the small sample size of most studies, leading to a loss of statistical power. To overcome some of the limitations mentioned above and to increase the relevance of statistical analysis, a meta-analysis of all available case–control studies on the association between TGF-β1 SNPs and the risk of AD has been performed (Di Bona et al., unpublished observations).

The primary sources of the studies were the AlzGene database (www.alzgene.org), updated September, 2009, and the PubMed database. The medical subject headings were "TGF-β1," "polymorphisms," and "Alzheimer's disease" for the PubMed search, and the selection of the specific polymorphism (TGF-β −509; TGF-β −800; TGF-β +10; TGF-β +25) for the search on the AlzGene database. Data for TGF-β +25 SNPs were not extensively analyzed by meta-analysis, because less than four homogeneous samples were available.[42–44] Criteria for the inclusion in the analysis were: Diagnosis of AD according to the *Diagnostic and Statistical Manual of Mental Disorders* (DSM), and the National Institute of Neurological Disorders and Stroke–Alzheimer Diseases and Related Disorders (NINDS-ADRDA) working group criteria,[45] case–control studies, and available genotype. Analysis was performed as previously described,[37,38,46] and the 95% confidence interval (95% CI) of the odds ratio (OR) was also calculated. The putative risk genotype in homozygosity was compared to the other two aggregated genotypes data, condensing the results into one statistic.[37,38]

TGF-β −509 SNP

Six case–control studies on the association between TGF-β −509 SNP and AD were identified.[41,42,47–49] The effect of the TGF-β −509 TT genotype on the risk of AD is shown in the Table 1. The effect of the TT genotype seems to increase the AD risk in two[41,48] out of six studies, but a statistically significant difference was observed only in one.[41] The study-pooled summary OR was 1.00 (TT vs. CT + CC, OR = 1.00; 95% CI, 0.78–1.28), suggesting that TT subjects as high TGF-β producers do not have a higher risk of developing AD. There was evidence of heterogeneity between the results of individual studies ($\chi^2 = 9.42$, degrees of freedom [df] = 5, p = 0.09, $I^2 = 46.9\%$).

TGF-β +10 SNP

Four case–control studies on the association between TGF-β +10 SNP and AD were identified.[43,44,48,50] The effect of the TGF-β +10 CC genotype on the risk of AD is shown in the Table 1. The effect of the CC genotype seems to increase the AD risk in two out of four studies, but a statistically significant difference was not observed in any study.[43,48] The pooled summary OR was 0.98 (CC vs. CT + TT, OR = 0.98; 95% CI, 0.61–1.57), suggesting that subjects with the TGF-β +10 CC genotype do not have a higher risk of developing AD. Nor did we observe any difference in disease risk when the TT genotype was compared with the other two aggregated genotypes (CT + CC) (data not shown).

TGF-β −800 SNP

Four case–control studies on the association between TGF-β −800 SNP and AD were identified.[41,42,44,49] The effect of the TGF-β −800 genotype on the risk of AD is shown in the Table 1. The effect of the AA genotype was to increase the AD risk in all the studies, but none of them showed a statistically significant difference. The pooled summary OR was 1.52 (AA vs. AG + GG, OR = 1.52; 95% CI, 0.86–2.96), suggesting that subjects with the TGF-β genotype −800 AA could have an higher risk of developing AD, although it does not reach any statistical significance. Nor did we observe a statistically significant difference in disease risk when the GG genotype was compared with the other two aggregated genotypes (AA + AG) (data not shown). There was no evidence of heterogeneity between the results of individual studies ($\chi^2 = 0.81$, df = 3, $p = 0.85$, $I^2 = 0\%$).

This study summarizes the evidence to date regarding the association between common polymorphisms that comprehensively capture the variability of the TGF-β gene and the risk of AD. The analysis of pooled data ruled out the role of TGF-β SNPs in modifying AD risk that was hypothesized by some investigators. However, a remarkable heterogeneity was observed between the results of individual studies, suggesting differences among the enrolled populations and a possible population-specific genetic effect of the TGF-β SNPs. Alternatively, genetic or environmental factors may also play a contributing role and may explain differences between the result of individual studies. We could not explain heterogeneity by subgroup analysis, taking into account study and patients characteristics, because of the low number of available studies.

So these data do not seemingly support a role for TGF-β SNPs in the pathophysiology of AD. However, inflammatory mediators do not act alone; they act within a complex network in which they are interacting reciprocally.[51] Accordingly, we found combinations of alleles in eight inflammatory genes and APOE that distinguish AD risk groups.[52]

These studies are relevant to AD treatment. The occurrence of a high-risk genetic profile linked to the presence of high-responder alleles of proinflammatory cytokines or of low-responder alleles of antiinflammatory cytokines might suggest the treatment with biologics, such as monoclonal antibodies directed versus the proinflammatory cytokines. Decreasing the level of systemic inflammation in AD patients might be a suitable chemopreventive treatment because microglia may be activated by systemic stimuli.[5,53]

In fact, a recently published small, open-label pilot study suggested that inhibition of the inflammatory cytokine TNF-α employing the perispinal administration of etanercept produced sustained clinical improvement in a 6-month, open-label pilot study in patients with AD ranging from mild to severe. This approach uses therapeutic delivery of etanercept across the dura via the cerebrospinal venous system, a confluence of the venous plexuses of the spine and the brain, in which flow is bidirectional owing to the absence of venous valves. Continued open-label clinical experience with this new treatment modality, now for more than 2 years, suggests that weekly maintenance treatment with perispinal

Table 1. Random-Effects Meta-Analyses Using Genotypic Contrasts for −509, +10, −800 Transforming Growth Factor-β Single-Nucleotide Polymorphisms

SNP	*Studies* (n)	*Ethnicity*	*Participants* (n)	*Genotypic summary OR (95% CI)*, p *value*	*Heterogeneity* p *value*, I^2
−509	6	3 Europe (France, The Netherlands, Spain) 2 United States 1 Asia (Japan)	10,452	TT vs. CT + CC: 1.00 (0.78–1.28), $p = 0.98$	$p = 0.09$; 46.9%
+10	4	2 Europe (Italy, The Netherlands) 1 United States 1 Asia (Japan)	7663	CC vs. CT + TT: 0.98 (0.61–1.57), $p = 0.92$	$p = 0.02$; 69.9%
−800	4	Europe (Italy, The Netherlands, Spain) 1 United States	9898	AA vs. AG + GG: 1.52 (0.86–1.20), $p = 0.15$	$p = 0.85$; 0%

Di Bona et al., unpublished observations.
SNP, Single-nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

etanercept may have a sustained positive effect. In addition, rapid clinical improvement within minutes of dosing has been observed on a repeated basis in multiple patients. It is hypothesized that perispinal administration of etanercept may enable rapid delivery to the central nervous system (CNS) via the cerebrospinal venous system, resulting in improvement in synaptic mechanisms that have been dysregulated by excess TNF-α. Although some benefit was observed, firm conclusions cannot be drawn at present from these small trials. Perispinal etanercept for AD merits further study in randomized clinical trials.[54–56]

Immune Response and Immunotherapy

Some evidence suggests the involvement of a systemic immune response in AD, although so far it is poorly characterized. However, changes in the distribution and reactivity of immune cells in the blood have been observed. Neuroinflammation induces the efflux of CNS proteins, such as Aβ, or inflammatory mediators across the blood–brain barrier. This may cause systemic immune reaction and recruitment of myeloid or lymphocytic cells into the CNS. Thus, communication between the CNS and immune system in AD could influence both the lymphocyte distribution in the blood and the production of immune mediators.[11] Actually peripheral blood mononuclear cells (PBMCs) from AD patients produce higher levels of some cytokines, such as IL-1β and IL-6, compared to levels of PBMCs from control subjects.[57] Moreover, an immune disregulation was recently documented as dramatic alterations on $CD4^+$ subsets in patients with mild AD. In particular, decreased percentages of naïve cells and an increase of memory cells, an increased number of $CD4^+$ lymphocytes that lack the co-stimulatory molecule CD28, and a reduction of $CD4^+CD25^{high}$ T regulatory cells have been observed.[58]

To investigate the systemic signs of immune processes in AD, Pellicanò et al. (unpublished observations) have examined the distribution of lymphocyte blood subsets in AD patients, comparing the results to data obtained in healthy controls. A decrease of B lymphocytes in AD patients due to a decrease of exhausted memory cells has been observed. In addition, the expression of activation markers on PBMCs from AD patients activated *in vitro* by recombinant (r)Aβ42, was increased respect to controls. Stimulation by rAβ42 also induced the production of the proinflammatory and anti-inflammatory cytokines, chemokines, and growth factors (Table 2). This kind of study, which supportsthe involvement of systemic immunity in AD patients, can provide the basis for the search of immune biomarkers in AD for monitoring the effectiveness of therapeutic interventions.[10]

In fact, immune-based therapies targeting Aβ have generated considerable interest as a possible mechanism for reducing Aβ in the brain. Current views see immunization with the Aβ peptide or the infusion of preformed antibody specific for human Aβ as possible therapeutic approaches to improve the cognitive status in AD patients. Animal models of AD have provided positive results from both approaches. Thus, an initial clinical trial using immunization with human Aβ in AD patients was started, but then was halted because of a high incidence of meningoencephalitis. Passive immunotherapy in animal models of AD has provided similar benefits comparable to those seen with active immunotherapy, and in human beings it has the potential of being effective without inducing T cell–mediated encephalitis.[59,60] Thus, intravenous immunoglobulin (IVIg) has been proposed as a potential agent for AD immunotherapy because it contains antibodies against Aβ. In a recent study, administration of IVIg to patients with mild AD led to transient, reproducible, and dose-dependent increases in serum anti-Aβ antibody titers and parallel increases in plasma Aβ levels. After 6 months of IVIg therapy, the cerebrospinal fluid (CSF) Aβ levels decreased, whereas after 3 months without IVIg, a washout period, CSF Aβ levels increased to their pretreatment baseline levels. When IVIg therapy was restarted, a decrease in CSF Aβ levels was again observed. In the meantime, a mean improvement of 2.5 points in Mini-Mental State Examination (MMSE) scores after 6 months was observed, with a decline of these scores toward baseline during the washout period.[61]

Furthermore, a retrospective case–control analysis demonstrated that previous treatment with IVIg is associated with a reduced risk of developing AD. Compared with untreated controls, patients who received previous IVIg for other indications had a 42% lower risk of developing AD over a period of approximately 4 years. This study provides epidemiologic evidence that previous IVIg may have a protective effect on the development of AD.[62] It is well known that IVIg contains natural anti-Aβ antibodies, and these natural antibodies have the capacity to prevent Aβ oligomer-induced neurotoxicity in N2A neuroblastoma cells. This neuroprotective effect may reflect the therapeutic potential of the natural anti-Aβ antibodies found in IVIg for the treatment of patients with AD.[63] It is also possible that other activities of IVIg, unrelated to its content of anti-Aβ antibodies, such as the modulation of inflammatory and immune reactions, may complement the effects of anti-Aβ antibodies on cognitive function in AD patients. The mechanisms of action of IVIg are complex, involving modulation of expression and function of Fc receptors, interference with

Table 2. Cytokines, Chemokines, and Growth Factors Whose Secretion by Alzheimer Disease Peripheral Blood Mononuclear Cells Is Significantly Increased When Stimulated with rAβ42 with Respect to Control Cultures

Cytokines	*Chemokines*	*Growth factors*
IL-1β	Eotaxin	GM-CSF
IL-1ra	MIP-1β	G-CSF
IL-6	RANTES	
IFN-γ		
TNF-α		
IL-10		
IL-8		

Cells were cultured in 24-well flat-bottomed plates at 1.5×10^6 cells per well in RPMI medium. The PBMCs were either unstimulated or stimulated by oligomeric rAβ42 (10 μg/mL). The supernatants of the cultured PBMCs were collected and the following cytokines and chemokines were evaluated using Luminex 100 (BioRad) according to manufacturer's instructions (Pellicanò et al., unpublished observations).

IL-1, Interleukin-1; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; MIP-1β, macrophage inflammatory protein-1β; GM-CSF, granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor.

activation of complement and the cytokine network and of idiotype network, regulation of cell growth, and effects on the activation, differentiation, and effector functions of dendritic cells and T and B cells.[64] These promising preliminary findings encourage additional research involving both adequate, well-controlled, randomized clinical trials to evaluate the effects of IVIg more thoroughly in AD and well-designed studies that gain insight to immune response in AD patients.

APOE, Cholesterol, and Diet

The APOE4 allele is the only known genetic variant that has been clearly associated with increased risk of late-onset AD. In the human population, three variants of the APOE gene have been found—ε2, ε3, and ε4—that are able to influence the concentration of the lipoprotein in the bloodstream. The APOE proteins are associated with 10% of the difference in cholesterol concentration in the bloodstream whereas the allele ε4 is tightly associated with the highest concentration. It has been demonstrated that subjects carrying APOE4 allele have a nearly double chance of developing AD whereas individuals not carrying the APOE4 allele have a decreased risk of AD by 40%. Yet fewer than half of all AD patients possess the ε4 allele and not all ε4 carriers develop the disease. It has also been suggested that the main effect of the allele is to anticipate the onset of the disease.[4,65,66]

Although there have been numerous studies attempting to elucidate the underlying mechanism accounting for increased risk, the influence of APOE4 on AD onset and progression needs more convincing and critical evidence. However, prevailing indications suggest that the differential effects of APOE isoforms on Aβ aggregation and clearance play the major role in AD pathogenesis. Actually, the ε4 allele accelerates amyloid deposition and promotes Aβ aggregation in cholesterol-rich lipid rafts, enhancing aggregation into senile plaques. In addition to its role in Aβ aggregation, it has been well argued that APOE4 promotes inflammatory responses. Other potential mechanisms, such as the differential modulation of neurotoxicity and tau phosphorylation by APOE4 isoforms, as well as its role in synaptic plasticity, have not been ruled out.[4,67–69]

On the other hand, the association between APOE, cholesterol, and AD has been the subject of intense scrutiny by numerous groups. High cholesterol levels at midlife are a considerable risk factor for dementia/AD in most of long-term follow-up studies. Moreover, the power of dietary fats on AD development was observed in APOE4 subjects only. To get a glimpse of cholesterol expression on AD, we have to consider that the brain is very rich in cholesterol that is actively turned over among neurons and glial cells via apolipoproteins and their receptors play an essential role in synaptic plasticity. In addition, different cholesterol synaptic distribution depends on different APOE alleles. The best-studied role of cholesterol in AD is in amyloid precursor protein (APP) processing and Aβ generation. Increased cholesterol leads to increased cleavage of APP and increased Aβ production, whereas reduction of cellular cholesterol decreases the γ-secretase activity, which is responsible for Aβ generation.[4,70,71]

Epidemiological studies have suggested a possible protective effect for the cholesterol-lowering statin drugs that inhibit 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, the enzyme that catalyzes the rate-limiting step in cholesterol, in AD patients. Numerous studies have examined the role of statins in the prevention of dementia and treatment of established AD. As matter of fact, as a biological point of view, it seems feasible that statins could prevent dementia due to their role in cholesterol reduction and in antiinflammatory activities that are not directly dependent on lowering blood cholesterol. However, while evidence from retrospective case–control studies suggests a beneficial role of statins in the prevention of AD, a similar benefit has not been established in prospective cohort studies or randomized clinical trials.[4,72,73]

On the other hand, cognitive impairment can be influenced by a number of other factors. The potential effect of nutrition has become a topic of increasing scientific and public interest. In particular, there are arguments that nutrients (food and/or supplements), such as vitamins, trace minerals, and lipids, can affect the risk of cognitive decline and dementia, especially in frail elderly people at risk of deficiencies. Unmistakably, aging is associated with cognitive dementia and AD; concomitantly, aging is also associated with malnutrition and reduced intake of micro- and macronutrients.[74] The role of diet in cognitive decline has not been looked into broadly, but because several dietary factors affect the risk of cardiovascular disease and some studies have suggested a link between cognitive decline and cardiovascular disease,[75] diabetes mellitus,[76] and hypertension,[77] it might be assumed that dietary intake might also influence the risk of dementia.

High energy intake and increase in body mass index during middle age have been associated with defeat of cognitive function in old age,[78] whereas low calorie intake was protective against AD.[79] Nonetheless, the diet of AD patients seems to have deficiencies in omega-3 fatty acids, especially docosahexaenoic acid/eicosapentaenoic acid long-chain omega-3 fatty acids, and too few antioxidants. Unfortunately, there are only a few studies comparing diets of AD patients with age-matched controls, and they tend to show AD patients with lower amounts of omega-3 fatty acids, excess omega-6 fatty acids (which would also cause inflammation), too much sugar (insulin resistance and metabolic syndrome), and fewer antioxidants.[80] Unfortunately, in the Rotterdam study, no correspondence was found between increased risk of dementia and dietary intake rich in saturated fatty acids, trans fatty acids, and cholesterol.[81]

On the other hand, there are studies showing a positive association between high intake of saturated and trans-unsaturated (hydrogenated) fats and increased risk of AD, because intake of polyunsaturated and monounsaturated fats seems to be protective against cognitive decline in the elderly.[82,83] Overall the role of lipid in AD is difficult to pinpoint because the presence of the APOE4 allele seems to respond more efficiently to diet modulation, whereas the presence of APOE3 and APOE2 is less influenced by diet.[84,85]

Also homocysteine-related vitamins and antioxidant nutrients (vitamins E and C, carotenoids, flavonoids, enzymatic cofactors) seem to have a role in AD risk, and epidemiologic data suggest a protective role of the B vitamins, especially vitamins B9 and B12, regarding cognitive decline and dementia.[86] Nonetheless other studies have shown conflicting results.[87]

Ambiguous results are evident also on the dietary fruit and vegetables supplementation. The Chicago Health and

Aging Project has shown an association between cognitive decline with older age and high vegetable but not fruit consumption,[88] whereas intervention studies on rats given a dietary supplement of fruit and vegetables extracts have shown a slowed age-related decline in neuronal and cognitive functions.[89] However, the results on antioxidant nutrients may suggest the importance of having a balanced combination of several antioxidant nutrients to exert a significant effect on the prevention of cognitive decline and dementia, while taking into account the potential adverse effects of these nutrients.[84]

Higher adherence to a Mediterranean diet seems to be associated with borderline reduction in risk for developing mild cognitive impairment (MCI) and a reduction risk for conversion from MCI to AD,[26] whereas a Japanese diet seems to be less protective.[90] The substantial difference consists in the intake of animal fat and complex carbohydrates, whereas a Japanese diet is poor in animal fat and rich in complex carbohydrates.

Overall, these studies take in account several among the known confounding factors and consider the impact of food habits, such as the regional cultures, social status, and educational level, together with the genetic effect. In conclusion, there are a great amount of large prospective studies demonstrating association with lower all-cause and cause-specific mortality; nevertheless, there is a need of further analysis to verify if those association can be a useful tools for preventing diseases.

Oxidative Stress and Therapeutic Perspectives

Oxidative stress is considered one of the most critical among the factors linked to AD pathophysiology.[91] Actually, besides the pathological hallmarks of the disease, AD brains exhibit evidence of reactive oxygen species (ROS)–mediated injury, and free radical oxidative damage to key intracellular targets such as DNA or proteins has been shown to be a major cause of the neuronal cell death related to AD.[92] Oxidative stress is the result of an unbalance between oxidant production and antioxidant defenses. The brain, compared to other organs, is more susceptible to oxidative stress for the following reasons: (1) High content of peroxidizable unsaturated fatty acids; (2) high oxygen consumption per unit weight; (3) high content of lipid peroxidation key ingredients (iron and ascorbate); and (4) the scarcity of antioxidant defenses systems. Under normal conditions, free radicals are produced from a number of sources, among which are enzymatic, mitochondrial, and redox metal ion–derived sources as well as inflammatory response.[93] In AD, an overproduction of free radicals seems to be mostly related to mitochondrial dysfunctions, to the Aβ peptides themselves, and to the presence of unbound trace metal ions. Today, it is evident that the three sources are not independent from each other, and recent hypotheses predict that, in the early stages of the disease, Aβ peptide enters the mitochondria where it induces ROS generation and subsequent oxidative stress.[94]

The mechanisms associated with Aβ-mediated neurotoxicity are still partially unknown, but there is evidence suggesting that oxidative stress plays a key role.[94,95] Several groups showed that among the 42 amino acids forming Aβ(1–42), the methionine located at position 35 is critical for Aβ-associated ROS production and neurotoxicity.[96,97] Furthermore, activation of microglial nicotinamide adenine dinucleotide phosphate (NADPH) oxidase by Aβ oligomers, Aβ fibrils, and senile plaque is responsible for the production of both extracellular and intracellular ROS, with direct toxicity to neurons and sustained production of several proinflammatory and neurotoxic cytokines.[98,99]

On the other hand, irrespective of the source and mechanisms that lead to the generation of intracellular toxic oxidants, mammalian cells have developed highly refined inducible systems to counteract stressful conditions and to fight off oxidation.[100] Activation of antioxidant pathways is particularly important for tissue with relatively weak antioxidant defenses, such as the brain. Increasing evidence points out that reduced cellular expression and activity of antioxidant proteins are fundamental triggers for AD.[101] Aging, the major risk factor for AD, leads to loss of the free radical scavenging ability by endogenous mechanisms.[102]

Cortical and hippocampal oxidative stress is a very early event in the pathophysiology of sporadic AD and correlates with the development of specific cognitive deficits. This regional distribution has been strictly related to the amyloid load, because the cerebellum showed low levels of Aβ, no oxidative stress relative to controls, and essentially no neuronal loss.[103,104]

Among cellular antioxidant defenses, heat shock proteins have been regarded as cytoprotectants protecting the brain cells from oxidative damages encountered during the neurodegenerative diseases progression. Heme oxygenase-1 (HO-1) is a 32-kD stress protein that catalyzes the degradation of heme to biliverdin.[105] The HO-1 gene is redox regulated, and its activation represents a protective system potentially active against brain oxidative injury.[106] Its expression in AD patients brain is significantly increased,[107] and the spatial distribution of HO-1 expression in diseased brain is essentially identical to that of pathological expression of tau.[108] HO-1 immunoreactivity is greatly increased in neurons and astrocytes of the hippocampus and cerebral cortex of individuals with AD and colocalizes in senile plaques and neurofibrillary tangles. HO-1 is thought to downregulate the production of tau protein, and recently HO-1 polymorphisms have been considered as a possible responsible for increased AD susceptibility.[109] Deregulation of the HO system has been associated with the pathogenesis of AD[110], multiple sclerosis,[111] and brain aging.[112] Many studies clearly demonstrate that activation of HO-1 in neurons is strongly protective against oxidative damage and cell death.[113,114] In fact, the activation of HO-1 seems to represent an important defensive mechanism for neurons exposed to oxidative stress. Thus, modulation of HO-1 should represent a potential pharmaceutical strategy for the treatment of neurodegenerative disorders.[8]

Because oxidative stress may be responsible for some aspects of AD neurodegeneration, and because to date most of the available treatments are merely symptomatic, extensive research has been aimed at reducing the effects of oxidative stress to prevent AD progression by using free radical scavengers. Thus, one therapeutic strategy is to delay AD onset long enough so as to slow the neuronal damage associated with Aβ-induced oxidative stress, particularly Aβ-induced lipid peroxidation. Brain-accessible antioxidants potentially may provide the means of implementing this

therapeutic strategy of delaying the onset of AD, acting as neuroprotective agents. Spices and herbs often contain active phenolic substances endowed with potent antioxidative and chemopreventive properties,[115] and recently a series of papers have focused on specific neuroprotective effects of some of those polyphenols derived from nutritional sources.[116] Curcumin (1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione), a coloring agent and food additive commonly used in Indian culinary and traditional medical preparations from time immemorial, is extracted from the rhizome of *Curcuma longa*.[117] It is a polyphenolic substance that has the potential to inhibit lipid peroxidation and to effectively intercept and neutralize ROS (superoxide, peroxyl, hydroxyl radicals)[118,119] and nitric oxide (NO)-based free radicals (nitric oxide and peroxynitrite).[120] It is generally assumed that the phenol moiety is responsible for antioxidant properties of any plant phenolic compound. Consequently, the free radical chemistry of curcumin (an *o*-methoxyphenol derivative) has focused on its phenol ring. The possible involvement of the β-diketone moiety in the antioxidant action of curcumin has been considered,[121] and the H-atom donation from the β-diketone moiety to a lipid alkyl or a lipid peroxyl radical has been reported as the potentially more important mechanism underlying its antioxidant action.[122] Of particular interest is the ability of curcumin to inhibit cyclooxygenase enzymes[123] and to reduce the activation of nuclear transcription factor NF-κB.[124] Its antiinflammatory properties and cancer-preventive activities have been consistently reported using *in vitro* and *in vivo* models of tumor initiation and promotion.[125]

In addition to its ability to scavenge carcinogenic free radicals,[126] curcumin also interferes with cell growth through inhibition of protein kinases. Although the exact mechanisms by which curcumin promotes these effects remains to be elucidated, the electrophilic properties of this yellow pigment appear to be an essential component underlying its pleiotropic biological activities. Curcumin contains two electrophilic α,β-unsaturated carbonyl groups, which can react with nucleophiles such as glutathione.[127] In addition, curcumin can increase the activity of γ-glutamyl-cysteinyl synthetase and other glutathione (GSH)-linked detoxifying enzymes.[128] Low concentrations of curcumin potently induce HO-1 expression and activity in vascular endothelial cells, in rat astrocytes, and in cultured hippocampal neurons. Preincubation (12 h) of cultured neurons with a low concentration of curcumin resulted in an enhanced cellular resistance to glucose oxidase–mediated oxidative damage. This cytoprotective effect was considerably attenuated by zinc protoporphyrin IX, a specific inhibitor of HO activity.[129–131] In other experiments, it has been demonstrated the efficacy of curcumin to protect cortical neurons against apoptotic cell death induced by Aβ.[132] The ability of curcumin to induce HO-1 can explain, at least in part, the strong antioxidant and antiinflammatory properties of curcumin, which depend more by its action as cellular signals than by its radical scavenger effect.[133]

The involvement of curcumin in restoring cellular homeostasis and rebalancing redox equilibrium by the activation of defensive genes suggests that it might also be a useful adjunct in AD treatment. Neuroprotective effects of curcumin have been demonstrated by Rajakrishnan[134] in ethanol-induced brain damage; oral administration of curcumin in rats caused a significant reversal in lipid peroxidation, brain lipid modifications, as well as increase in glutathione levels. Epidemiological studies have suggested that curcumin, as one of the most prevalent nutritional and medicinal compounds used by the Indian population, is responsible for the significantly reduced (4.4-fold) prevalence of AD in India compared to United States.[135] Furthermore, elderly Singaporeans who ate curry with turmeric had higher MMSE scores than those who did not.[136] However, the relatively short duration of follow up, cultural factors, and other potential confounders suggest caution in interpreting these findings. Consistent with these data, convincing evidence has been provided that dietary curcumin given to an AD transgenic mouse model (Tg2576) for 6 months resulted in a suppression of indices of inflammation and oxidative damage in the brain of these mice and reversed Aβ-induced cognitive deficits.[137,138] The same group demonstrated in a successive study that curcumin was a better Aβ40 aggregation inhibitor than ibuprofen and naproxen and prevented Aβ42 oligomer formation and toxicity at very low concentrations (between 0.1 and 1.0 μM).[139] They also showed that curcumin readily entered the brain to label plaques *in vivo*, inhibiting the formation of Aβ oligomers and their toxicity. Among the several mechanisms by which curcumin is able to clear amyloid is the induction of heat shock proteins (HSPs), which function as molecular chaperones to block protein aggregate formation.[140]

Recently, curcumin has been evaluated, with preliminary encouraging results, in a pilot clinical trial in AD patients.[141] Curcumin is highly lipophilic and might cross the blood–brain barrier and reach the brain, and, although its bioavailability is very low, because the drug is rapidly metabolized by conjugation, curcumin may reach brain concentrations sufficient to activate signal transduction events and to decrease Aβ aggregation.[142] Other plant-derived phenolic agents with analogous chemical structures to curcumin have been demonstrated to strongly activate HO-1 expression and to defend cells against oxidative stress—in particular carnosol,[143] zerumbone,[144] resveratrol,[145] rosolic acid,[146] and sulforaphanes.[147] Furthermore it has been demonstrated that other phenolics, such as caffeic acid phenethyl ester (CAPE),[130] ethyl ferulate (EFE),[148] and epigallocatechin-3-gallate (EGCG),[149] are able to protect neurons via HO-1 induction.

Conclusions

Today, many countries have rising aging populations and are facing an increased prevalence of age-related diseases, such as AD, with growing health-care costs. Understanding the AD pathophysiology process plays a prominent role in developing new strategies that can extend the health of the elderly population. In fact, taking into account the future epidemic of AD, prevention and treatment are important goals of ongoing research. As discussed in this review, events contributing to the onset and development of AD are numerous and complex. We have focused our attention on many hot topics involved in AD pathophysiology, such as inflammation, cytokines, immune response, APOE, cholesterol, and oxidative stress, and have explored the related therapeutic possibilities, i.e., NSAIDs, cytokine blockade, immunotherapy, diet, and curcumin. We believe that the investigation of AD pathophysiology, particularly

disentangling inflammation, is likely to provide important clues about how to develop drugs that can slow or delay AD.

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Chapter 4

Immune-Inflammatory Responses and Oxidative Stress in Alzheimer's Disease: Therapeutic Implications

Immune-Inflammatory Responses and Oxidative Stress in Alzheimer's Disease: Therapeutic Implications

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Abstract: Alzheimer's disease (AD) is a heterogeneous and progressive neurodegenerative disease which in Western society mainly accounts for clinical dementia. AD has been linked to inflammation and oxidative stress. Neuro-pathological hallmarks are senile plaques, resulting from the accumulation of several proteins and an inflammatory reaction around deposits of amyloid, a fibrillar protein, Aβ, product of cleavage of a much larger protein, the beta-amyloid precursor protein (APP) and neurofibrillary tangles. Inflammation clearly occurs in pathologically vulnerable regions of AD and several inflammatory factors influencing AD development, i.e. environmental factors (pro-inflammatory phenotype) and/or genetic factors (pro-inflammatory genotype) have been described. Irrespective of the source and mechanisms that lead to the generation of reactive oxygen species, mammalian cells have developed highly regulated inducible defence systems, whose cytoprotective functions are essential in terms of cell survival. When appropriately activated, each one of these systems has the possibility to restore cellular homeostasis and rebalance redox equilibrium. Increasing evidence support the notion that reduction of cellular expression and activity of antioxidant proteins and consequent augment of oxidative stress are fundamental causes for ageing processes and neurodegenerative diseases, including AD. The better understanding of different molecular and cellular inflammatory mechanisms is crucial for complete knowledge of AD pathophysiology, hence for its prevention and drug therapy. Accordingly, two lines of preventive therapeutics can be outlined, the first based on anti-inflammatory drugs, the second one on anti-oxidative properties.

Keywords: Alzheimer's disease, curcumin, IL-6, inflammation, oxidative stress.

INTRODUCTION

Alzheimer's disease (AD) is a heterogeneous and progressive neurodegenerative disease which in Western society mainly accounts for clinical dementia. AD has been linked to inflammation and oxidative stress. Neuro-pathological hallmarks are senile plaques, resulting from the accumulation of several proteins and an inflammatory reaction around deposits of amyloid, a fibrillar protein, beta-amyloid (Aβ), product of cleavage of a much larger protein, the Aβ precursor protein (APP) and neurofibrillary tangles. Amyloid deposition, due to the accumulation of Aβ, is the main pathogenetic mechanism. Inflammation clearly occurs in pathologically vulnerable regions of AD and several inflammatory factors influencing AD development, i.e. environmental factors (pro-inflammatory phenotype) and/or genetic factors (pro-inflammatory genotype) have been described [1-3]. A growing number of studies in AD have also reported alterations in systemic immune responses including changes in lymphocyte distribution and activation. Studies in animal models for AD support the notion that immune cells infiltrate the brain and may modulate the disease [4].

On the other hand, irrespective of the source and mechanisms that lead to the generation of reactive oxygen species, mammalian cells have developed highly regulated inducible defensive systems, whose cytoprotective functions are essential in terms of cell survival. When appropriately activated, each one of these systems has the possibility to restore cellular homeostasis and rebalance redox equilibrium. Activation of antioxidant pathways is particularly important for tissue with relatively weak endogenous antioxidant defences, such as the brain. Increasing evidence, in fact, support the notion that reduction of cellular expression and activity of antioxidant proteins and consequent augment of oxidative stress play a central role in ageing processes and neurodegenerative diseases, including AD [3,5].

The better understanding of different molecular and cellular immune-inflammatory mechanisms as well as oxidative stress is crucial for complete knowledge of AD pathophysiology, and for its prevention and drug therapy.

PATHOPHYSIOLOGY OF AD

Amongst the existing entities of dementia spectrums, AD and dementia with vascular component are the most prevalent forms of dementia. These disorders have common and unique molecular pathological characteristics that result in serious reductions in nervous-system functionality [6]. AD, the most common cause of dementia, accounts for 50 to 70 percent of dementia cases. It is a severe neurodegenerative disorder characterized by progressive memory and cognitive impairment [7,8].

Under physiological conditions, APP is processed by the non-amyloidogenic pathway, where cleavage by α-secretase releases a soluble fragment. In AD, this process is significantly altered, where increased amount of APP is cleaved by other endo-proteases such as β- and γ-secretase, generating highly amyloidogenic protein molecules of 40-42 amino acid residues. Soluble Aβ rapidly aggregates into fibrils triggering the misfolding of other Aβ species. *In vitro* studies have shown that extracellular fibrillar Aβ peptides induce apoptosis in cultured neurons [9]. The amyloid cascade hypothesis is the central hypothesis for the cause of AD, which states that the initiating event in AD is an imbalance between the production and clearance of Aβ in the brain [10]. Another neuropathological hallmark of AD is the appearance of neurofibrillary tangles that consist of a hyperphosphorylated form of the microtubule-stabilizing protein tau, often conjugated with ubiquitin. The abnormal hyperphosphorylation of tau makes it resistant to proteolysis and this might lead to several-fold increase in the levels of tau in AD. The hyperphosphorylated tau causes sequestration of normal tau and other microtubule-associated proteins, leading to

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inhibition and disruption of microtubules and impaired axonal transport [11]. Tau also becomes prone to aggregation leading to formation of intracellular neurofibrillary tangles, compromising neuronal and synaptic function.

Although the complete etiopathogenesis of AD still remains unclear, genetic studies over the past two decades have provided valuable insights into this complex and heterogeneous disorder. Twin and family studies have shown that certain genes contribute to the development of AD, especially with respect to the age at which the disease manifests, and more recently, the development of non-cognitive symptomatology [12]. Early onset familial AD is a very rare autosomal dominant disorder caused by highly penetrant mutations in APP and presenilin genes, both linked to Aβ metabolism. Around twelve different mutations have been identified in APP gene at the level of α-, β-, or γ-secretase cleavage sites, which can lead to alteration in the normal proteolysis of amyloid precursor protein. Similarly, more than fifty missense mutations of the presenilin-1 gene (PS1) are associated with familial AD; several mutations of presinilin-2 gene (PS2) are associated with rare cases of early onset familial AD [13]. These mutations of APP, PS1 and PS2 may share a common pathogenetic mechanism leading to accumulation of β-amyloid protein as a result of abnormal amyloid precursor protein metabolism. In contrast, sporadic AD is a very common disorder.

Many studies have reported association of the APOE-4 allele with late-onset AD, and APOE-2 has shown a protective effect. Moreover, APOE-4 may influence AD pathology by interacting with APP metabolism and Aβ protein accumulation, enhancing hyperphosphorylation of tau protein and neurofibrillary tangle formation, reducing choline acetyltransferase activity, increasing oxidative processes, modifying inflammation-related neuroimmunotrophic activity and glial activation, altering lipid metabolism, lipid transport and membrane biosynthesis in sprouting and synaptic remodelling, and inducing neuronal apoptosis [2].

Several studies have reported susceptibility loci on chromosome 1, 2, 5, 9, 10, 12, 14, 18, 19 (close to APOE), and 21 (close to the APP gene) [14]. Other polymorphisms that may also be associated with AD are linked to the angiotensin-converting enzyme, Cystatin C, tau genes, estrogen receptor [15-18]. Genes involved in the neurodevelopmental process have also been considered good candidates to confer susceptibility to AD. All these genetic factors may interact in unknown genetic networks leading to a cascade of pathogenic events characterized by abnormal protein folding, with subsequent accumulation of abnormal proteins, ubiquitin-proteosome system dysfunction, exicitotoxic reactions, oxidative stress, mitochondrial injury, synaptic failure, altered metal homeostasis, axonal and dendritic transport dysfunction and chaperone misoperation [19,20].

IMMUNOLOGY OF AD

Aβ deposition plays a key role in AD pathogenesis, indeed it causes a chronic inflammatory response which contributes to neurodegeneration. The immune response that occurs secondary to amyloid deposition in the brain, results in the activation of complement cascade and microglial cells, and the recruitment of astrocytes [21]. Complement activation contributes to the local inflammatory response by the production of inflammatory mediators that leads to the migration of activated glial cells towards amyloid plaques causing neuronal cell dysfunction and finally degeneration [22].

Aggregated amyloid fibrils and inflammatory mediators secreted by microglial and astrocytic cells equally contribute to neuronal dystrophy. The microglia activation can be due to local or systemic inflammation. In fact a strong local inflammatory stimulus such as a previous head trauma is a risk factor for AD and several epidemiological studies clearly show that blood elevations of acute phase proteins, markers of systemic inflammatory stimuli, may be risk factors for cognitive decline and dementia. Furthermore, in experimental animals, chronic systemic inflammatory response induced by lipopolysaccharide administration also induces glial activation [23].

Microglial cells represent 10% of the cells of the adult central nervous system (CNS), but following activation they undergo morphological and phenotypic changes and differentiate into macrophage-like cells. In response to amyloid beta deposition in AD, microglial cells express different cells surface receptor and they can gain phagocytic or neurotoxic properties with an increased production of reactive oxygen species (ROS) [22,24]. The basic function of astrocytes is to protect neurons. In the early phase of AD there is an astrogliosis that represent a response to the accumulation of the amyloid beta in the brain parenchyma and in the cerebral microvasculature [25]. Migration of astrocytes to amyloid beta plaques is promoted by the chemochines CCL2 and CCL3 released by activated microglial cells that surround the plaques [26]. There is evidence that indicate an involvement of the immune system, other than neuroinflammatory processes in CNS, accompanied by changes or defects in immune responses in the blood of AD subjects [27]. It is possible that injury signals or peptide derived from the AD brain stimulate peripheral immune responses. This phenomenon seems to be due to the communication between central and systemic immune responses in neurodegeneration. There are changes of efflux of CNS proteins, like Aβ, or inflammatory mediators, like cytokines and chemokines, across the blood-brain-barrier (BBB) that may induce systemic immune reaction and the recruitment of lymphocytic or myeloid cells into the CNS [4].

Concerning T cells, many studies find an increased number of these cells in the brain of subjects affected by AD when compared with other neurological disease and age-matched controls [28]. The BBB consists of endothelial cells with tight junctions, but T cells could migrate from blood to brain in AD because peripheral T cells of AD patients overexpress MIP-1α, which is the ligand of CCR5 on brain endothelial cells. This interaction promotes T cells migration to the endothelial tight junctions [29]. Studies on peripheral blood mononuclear cells of AD patients have shown that there is an increased expression of the chemokines receptor CCR2 and CCR5, the chemokine RANTES (CCL5) and the Th1 cytokine interferon (IFN)-γ while MCP-1 chemokine and the Th2 cytokine interleukin(IL)-4 are decreased. So chemokines, chemokine receptors and cytokines production are altered not only in the CNS but also in the blood cells [27].

The molecular and cellular components that mediate the communication between peripheral inflammation and the brain have been studied in experimental models, and major routes of communication are known, all of which lead to the synthesis of cytokines and inflammatory mediators in the brain parenchyma, which are typically associated with tissue injury [30].

Other changes in immune function that occur in AD include an altered lymphocyte subpopulation distribution. Richartz-Salzburger and collaborators have shown a significant decrease of CD3+ T cells and CD19+ B cells, indicating a general decline of immune activity in AD. A slight increase of CD4+ Th cells and a decrease of the CD8+ CTL without significant change of the CD4+/CD8+ ratio. On the contrary CD16+CD56+ NK cells are not altered [31]. Recent studies on circulating CD4+ and CD8+ cells demonstrate dramatic alteration in naïve and memory subsets of CD4+ lymphocytes in AD patients, with an enhanced decrease on percentage of naïve CD4+ cells, elevated memory cells and increased proportions of CD4+ but not CD8+ cells lacking the costimolatory molecule CD28. These data evidence that the immune system of AD undergoing persistent antigenic challenge and it could lead to a premature immunosenescence [32].

Recently, we reported data on immune-inflammatory parameters evaluated in PBMC obtained from AD patients. We showed no changes in lymphocytes subsets with the exclusion of B cells that are reduced in AD subjects. The study of B cell naïve/memory compartment shows a reduction of IgD-CD27- B cells in AD patients compared with age-matched healthy controls. Inflammatory cytokines IL-1β, IL-6, IFN-γ, tumor necrosis factor(TNF)-α, chemokines MIP-1β and RANTES as well as chemokines receptors CCR2 and CCR5, are up-regulated in AD patients after *in vitro* stimulation with recombinant Aβ peptide. Also CD36, a scavenger receptor, is over-expressed in monocytes of AD patients.

All together these data confirm the involvement of systemic immunity in AD and suggest to continue these kind of study to obtain biomarkers useful in the monitoring the effectiveness of therapeutics [33].

IMMUNE-INFLAMMATORY GENES

Variations in immune-inflammatory genes such as cytokine, cyclo-oxygenase, lipo-oxygenease, toll-like receptor have also been considered to be important in the risk for AD [1,2]. In particular, cytokine gene polymorphisms have been claimed to play a key role in pathophysiology of AD, as demonstrated by two recent meta-analysis performed by our group on IL-1β and TNF-α [34,35]. Therefore, in the present paper, we present data on a meta-analysis focused on IL-6.

IL-6 is a pleiotropic cytokine involved in the regulation of the acute inflammatory response. The expression of IL-6 mRNA resulted to be increased in brain areas where amyloid deposition and astroglia activation are prominent in AD patients and increased IL-6 levels in the brain have been implicated in early stages of plaque formation [36].

Polymorphisms in the genes regulating the expression of IL-6 have been hypothesized to enhance the risk of developing AD. In particular, the most studied polymorphism of IL-6, the G/C polymorphism at the position -174 in the 5' flanking region of IL-6 gene, was shown the be associated with a decreased expression and also with a reduced plasma level of IL-6 [37]. However, genetic association studies, investigating the association with this polymorphism and the risk of AD gave very contrasting results. The main causes explaining the lack of replicability of the results between different studies seems to be the heterogeneity of the enrolled study populations, and the small sample size of most studies, leading to a loss of statistical power. To overcome some of the limitation mentioned above and to increase the relevance of statistical analysis a meta-analysis of all available case-control studies on the association between IL-6 -174 single nucleotide polymorphism (SNP) and the risk of AD have been performed. The studies were selected and analysed according to criteria and methods discussed in the previous meta-analysis [34,35]. In particular, the putative risk genotype in homozygosity (GG) was compared to the other two aggregated genotypes data (CG+CC), condensing the results into one statistic. This allowed us to highlight the effect of the putative risk genotype, as well as to maintain the statistical power compared to analyze separately all possible transmission models which would have lead to a loss of observation and, thus, of statistical power.

Sixteen case-control studies on the association between IL-6 -174 SNP and AD were identified [38-53]. The studies selected included a total of 3811 patients and 10716 healthy controls (6303 of which in the only study of van Oijen *et al.* [51]). The percentage of AD females patients ranged from 58% to 73%, but it was missing in the study from van Oien *et al.* [51,52]. The mean age of AD patients ranged from 63,9±10.4 to 80.6±7.8. The sample size of the studies varied greatly ranging from 51 to 753, as well as the number of controls, ranging from 36 to 6303. Neuropathological examination was assessed only in one study [39], the imaging examination of the brain to support the clinical diagnosis only in 3 out of 16 [33,44,50]. Only in three studies [39,44,53] controls were selected by mini-mental state examination (MMSE). All studies were performed on Caucasian population, except 3, which were conducted on Asian population [41]. The effect of the IL-6 -174 GG genotype on the risk of AD is shown in the Fig. (**1**).

The effect of the TT genotype was to increase the AD risk in 9 [38-40,42,47-50,52] out of 16 studies, but a statistical significant difference was observed in only three [40,48,50]. The pooled summary OR was 1.04 (GG vs CG+CC: OR=1.00; 95% C.I.: 0.84-1.22) and did not achieve statistical significance suggesting that subjects with the high producer IL-6 genotype (GG) do not have a higher risk of developing AD. There was evidence of heterogeneity between the results of individual studies ($\chi 2$=59.20, d.f.=15, p=0.00001, I2=73.4%). To reduce heterogeneity and to evaluate whether there was a different genotype effect in predefined subgroups of studies we performed subgroup analysis in relation to patient (age, gender) and study characteristics (type of AD diagnosis, MMSE in controls, sample size and statistical power of individual studies), but we did not find any difference in the overall effect (data not shown).

The current meta-analysis summarizes the evidence regarding the association between IL-6 -174 SNP and AD, representing a pooled total of 3811 cases and 10716 controls. The analysis of pooled data ruled out the role of IL-6 -174 SNP in modifying AD risk that was hypothesized by some investigators. However, a remarkable heterogeneity was observed between the results of individual studies, suggesting differences among the enrolled populations, and a possible population specific genetic effect of the -174 IL-6 SNP. Alternatively, also genetic or environmental factors may play a contributing role and may explain differences between the result of individual studies. We tried to explain heterogeneity by subgroup analysis, taking into account study and patients characteristics, but we fail in identifying variables explaining the observed heterogeneity. However, we must consider that we analyzed summary results which describe only variation between-study, not between patients because they reflect group averages rather than individual patient data. Only an individual patient data meta-analysis could address this issue, but no study reported such detailed information, which call for development of publicly available databases aimed at the collection and analysis of biologic information on a single-patient basis.

So, these data do not seemingly support a role for this IL-6 SNP in pathophysiology of AD. However, it is to take into account that inflammatory mediators do not act alone but they act in a complex network reciprocally interacting [54]. Accordingly, we found combinations of alleles in eight inflammatory genes (including this SNP) and APOE that distinguish AD risk groups [55].

OXIDATIVE STRESS

Oxidative stress has been implicated in a variety of pathophysiological conditions, including neurodegenerative disorders, and oxidative stress–mediated neuroinflammation has been demonstrated to actively concur to AD ethiopathology (extensively reviewed [56]). In particular, cortical and hippocampal oxidative stress is a very early event in the pathogenesis of sporadic AD and correlates with the development of specific cognitive deficits in this condition. Inflammation is strictly related to ROS production, which act as signals to activate inflammatory genes. Within the cell, ROS are physiologically present at minimal concentration as by-products of aerobic metabolism as well as second messengers in many signal transduction pathways and, in normal conditions, there is a steady-state balance between pro-oxidants and antioxidants. However, when the rate of free radical generation exceeds the capacity of antioxidant defences, oxidative stress ensues with consequential severe damage to DNA, protein and lipid. One of the major sources of ROS production and oxidative stress in AD brain is believed to be the impaired mitochondrial electron transport

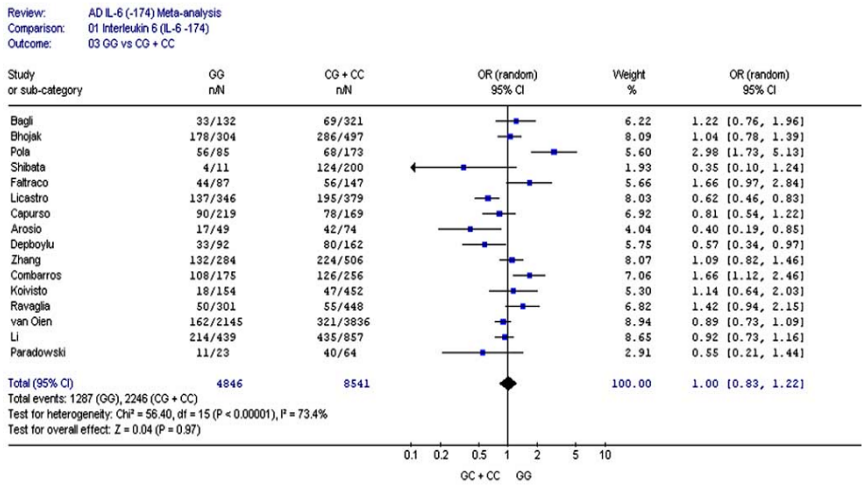

Fig. (1). Meta-analysis of 16 case-control studies of the IL-6 -174 polymorphism and the risk for AD using the random-effects model. Odds Ratio (OD) for each study and the pooled estimate of the OD for the risk of AD with its Confidence Interval (CI) are plotted on the graph. Studies are arranged chronologically based on the year of publication.

chain. Aβ soluble oligomers, which have been shown to be cytotoxic via the formation of ROS, represent another source of oxidative stress, as well as the activation of microglial oxidase [57] and the inflammatory response [2]. These conditions well explain why the biomarkers of lipid peroxidation, such as free 4-hydroxynonenal (HNE), are abundantly present in several areas of the AD brain [58]. Furthermore the presence of carbonylated-, HNE- and nitrated-proteins demonstrates the occurrence of protein modification by oxidation [59]. Signs of oxidative damage in AD patients have also been found in the cerebrospinal fluid, in urine, and in serum, and, although the results of some studies are contradictory, it seems that the levels of oxidative damage parallels the progression of the disease [3].

Oxidative stress is counteracted in biological systems by a large set of endogenous antioxidants, including enzymes such as superoxide dismutases, catalase, and glutathione peroxidase, as well as low molecular weight compounds, such as glutathione, generally found at levels sufficient enough to defend cells from oxidative insult. The brain is particularly low in the antioxidant enzymes, particularly those scavenging H2O2, namely glutathione peroxidase or catalase. On the other hand mammalian cells have developed highly regulated inducible defensive systems that have the possibility to restore cellular homeostasis and rebalance redox equilibrium [3]. One of the more important system devoted to the antioxidant defense in brain is represented by the so called "heat shock response" sustained by the "heat shock proteins" (Hsps). Among the Hsps family, an emerging role has been attributed to heme oxygenase-1 (HO-1 or Hsp-32) which is responsible of the transformation of the heme moieties into carbon monoxide and biliverdin. All the byproducts of HO-1 activity play a significant role in physiological cell functions [60]. In the CNS, the HO system has been reported to be very active [61,62] and its modulation seems to play a crucial role in the pathogenesis of neuro-degenerative disorders. Deregulation of the HO system has been associated with the pathogenesis of AD, multiple sclerosis and brain ageing [63,64]. Many studies clearly demonstrate that activation of HO-1 in neurons is strongly protective against oxidative damage and cell death [65]. In a very elegant study, Panahian *et al*. using transgenic mice over-expressing HO-1 in neurons, demonstrated the neuroprotective effect of this enzyme in an experimental model of ischemic brain damage [66]. The neuroprotective effects of over-expressed HO-1 can be attributed to: (*i*) increase in cGMP and bcl-2 levels in neurons; (*ii*) inactivation of p53, a protein involved in promoting cell death; (*iii*) increase in antioxidant sources and (*iv*) increase in the iron sequestering protein, ferritin [66]. Particularly interesting is the role played by HO-1 in AD. Significant increases in the levels of HO-1 have been observed in AD brains in association with neurofibrillary tangles and also HO-1 mRNA was found increased in AD neocortex and cerebral vessels [63,67]. HO-1 increase was not only in association with neurofibrillary tangles, but also co-localized with senile plaques and glial fibrillary acidic protein-positive astrocytes in AD brains [68]. In addition Takeda *et al*. explored the relationship between HO-1 and tau protein, this latter being the major component of neurofibrillary tangles, the intraneuronal AD lesion. In transfected neuroblastoma cells overexpressing HO-1, the activity of this enzyme was increased, and conversely, the level of tau protein was significantly decreased when compared with antisense HO-1 or vector transfected cells [63]. The suppression of tau protein expression was almost completely counteracted by zinc-deuteroporphyrin, a specific inhibitor of HO activity [63]. Thus HO-1 is thought to down regulate the production of tau and recently HO-1 polymorphisms have been considered as a possible responsible for susceptibility to AD [69]. Thus, modulation of HO-1 should represent a potential pharmaceutical strategy for the treatment of neurodegenerative disorders [70].

The role of smoking in AD has been debated [71]. In the last years, several case-control studies suggested that smoking was associated with decreased risk of dementia [72,73]. On the other hand, it has been hypothesized that findings obtained in case-control studies were a consequence of survival bias rather than a true protective effect of smoking [74]. However, the mechanisms by which smoking would prevent the risk of AD should be related to the positive nicotinic effects of smoking on cognitive functioning

[72,75]. On the other hand, the increased frequency of cardio-vascular and cerebrovascular illnesses among smokers [71] is likely to increase the risk of AD in later life [76,77]. However, the results of a recent meta-analysis of prospective studies clearly showed that, when compared with people who have never smoked, current smokers have an increased risk of dementia and cognitive decline ranging from 40 percent to 80 percent, depending on the outcome examined [73]. Oxidative stress is a possible mechanism consistent with dangerous effects of smoking since cigarette smoke contains free radicals that activate inflammatory cells with inflammatory mediator production and further oxidative damage by triggering a vicious cycle [2,71].

CONCLUSIONS

A major goal of ongoing research in AD is to improve early detection by developing tools to move diagnosis backward in disease temporal course, i.e. before the clinical manifestation of the disease, where a treatment might play a decisive role in preventing or significantly retarding the manifestation of the disease [1,2]. So, the knowledge of the pathophysiology of the diseases is crucial for its therapeutic prevention. Accordingly to the data discussed in the present review, two lines of preventive therapeutics can be discussed, the first based on anti-inflammatory drugs, the second one on anti-oxidative properties.

Concerning anti-inflammatory drugs, patients who received non-steroidal anti-inflammatory drugs (NSAIDs) for a period of 2 years had less AD incidence with relative risk of 0.2 [78]. The incidence of AD appears to be reduced in some post hoc studies, by about 13%for aspirin and 28% for other NSAIDs [79-81]. However, a recent report based on a longitudinal study of men and women aged 70 years and older with a family history of AD (ADAPT study) showed that use of naproxen or celecoxib did not improve cognitive function [82]. However, several critical issues have been raised concerning the study results, i.e. it has been claimed that the ADAPT study does not indicate that NSAIDs, if taken during adulthood and for an extended period, cannot prevent or delay the onset of dementia [83]. In addition, examining the effects on AD risk of NSAID use for >5 years in a large health care database including 49,349 cases and 196,850 controls, Vlad *et al.* clearly demonstrated that long-term NSAID use was protective against AD [84].

Regarding molecules with anti-oxidant properties, a number of experimental and epidemiological studies have recently supported the beneficial effects of some commonly used natural products in preventing various pathologic conditions ranging from cardio-vascular diseases to cancer. Spices and herbs often contain phenolic substances with potent antioxidative and chemopreventive properties [85]. However, since curcumin studies are a growing area in AD research as well as in other pathological conditions [86,87], we will insight into its possible effect on AD.

Curcumin (1,7-bis[4-Hydroxy-3-methoxyphenyl]-1,6-hepta-diene-3,5-dione), a colouring agent and food additive commonly used in Indian culinary preparations extracted from the rhizome of *Curcuma Longa*, polyphenolic substance has the potential to inhibit lipid peroxidation and to effectively intercept and neutralize ROS [70]. Curcumin has been shown to significantly increase HO-1 expression and activity in vascular endothelial cells [88], in rat astrocytes [89] and in cultured hippocampal neurons [90]. This latter effect on HO-1 can explain, at least in part, the strong anti-oxidant and anti-inflammatory properties of curcumin, that depends more by its action as cellular signals than by its radical scavenger effect [70]. Curcumin has been demonstrated to stimulate the mitogen-activated protein kinase pathway and to activate *heterodimers of NF-E2-related factors 2,* leading to induction of the antioxidant responsive element activated reporter genes [91]. By this pathways curcumin strongly induce the expression of phase II detoxification enzymes and of HO-1, resulting in cell protection and enhancing cell survival [90]. The involvement of curcumin in restoring cellular homeostasis and rebalancing redox equilibrium, suggests that it might be a useful adjunct also in AD treatment. Epidemiological studies suggested that curcumin, as one of the most prevalent nutritional and medicinal compounds used by the Indian population, is responsible for the significantly reduced (4.4-fold) prevalence of AD in India compared to United States [92]. Furthermore, Elderly Singaporeans who ate curry with turmeric had higher MMSE scores than those who did not [93]. However, the relatively short duration of follow-up, cultural factors and other potential confounders suggest caution in interpreting these findings. Consistent with these data, Lim and colleagues have provided convincing evidence that dietary curcumin, given to an Alzheimer transgenic mouse model (Tg2576) for 6 months, resulted in a suppression of indices of inflammation and oxidative damage in the brain of these mice and to reverse Aβ-induced cognitive deficits [94,95]. The same group demonstrated in a successive research that curcumin was a better Aβ40 aggregation inhibitor than ibuprofen and naproxen, and prevented Aβ42 oligomer formation and toxicity at very low concentration (between 0.1 and 1.0 microM) [96]. They also shown that curcumin readily entered the brain to label plaques *in vivo* inhibiting the formation of Aβ oligomers and their toxicity [96]. Among the several mechanisms by which curcumin is able to clear amyloid is the induction of HSPs, that function as molecular chaperones to block protein aggregate formation [97]. Recently curcumin has been evaluate in a pilot clinical trial in AD patients, with preliminary encouraging results [98]. Curcumin is highly lipophilic and might cross the BBB and reach the brain, and although its bioavailability is very low, since the drug is rapidly metabolized by conjugation, curcumin may reach brain concen-trations sufficient to activate signal transduction events and to decrease Aβ aggregation [99]. Other plant-derived phenolic agents with analogous chemical structures to curcumin have been demonstrated to strongly activate HO-1 expression and to defend cells against oxidative stress. In particular, ethyl ferulate, resveratrol (a phitoalexin derived from grape) and caffeic acid phenethyl ester, are able to protect neurons via HO-1 induction [100]. These and other studies identify a novel class of natural substances that could be used for therapeutic purposes as potent inducers of HO-1 in the protection of tissues against inflammatory and neurodegenerative conditions. It needs to be emphasized that curcumin, and other plant constituents eventually become part of the human diet and can be consumed daily as herbal supplements. Further *in vitro* and *in vivo* studies using curcumin-like molecules will give important information on the feasibility of developing new pharmacological strategies for maximizing heme oxygenase activity in targeted tissues as an alternative to or in combination with HO-1 gene therapy.

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ABBREVIATIONS

Aβ	=	beta-amyloid
AD	=	Alzheimer's Disease
APP	=	Aβ Precursor Protein
HNE	=	4-Hydroxynonenal
HO-1	=	Heme Oxygenase-1
Hsps	=	Heat Shock Proteins
IFN	=	Interferon
IL	=	Interleukin
MMSE	=	Mini-Mental State Examination

NSAIDs = Non-Steroidal Anti-Inflammatory Drugs
PS1 = Presenilin-1 gene
PS2 = Presenilin-2 gene
ROS = Reactive Oxygen Species
TNF = Tumor Necrosis Factor

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Chapter 5

Association between the Polymorphisms of TLR4 and CD14 Genes and Alzheimer's Disease

Association between the Polymorphisms of TLR4 and CD14 Genes and Alzheimer's Disease

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Abstract: Alzheimer's disease (AD) is a heterogeneous and progressive neurodegenerative disease which in Western society mainly accounts for clinical dementia. Inflammation plays a key role in AD and dissecting the genetics of inflammation may provide an answer to the possible treatment. Hence, the better understanding of different molecular and cellular inflammatory mechanisms is crucial for complete knowledge of AD pathophysiology, and for its prevention and drug therapy. Accordingly, in the present study we evaluated whether the pro-inflammatory polymorphisms of lipopolysaccaride-receptors, +896A/G Toll-Like Receptor (TLR4) and -260C/T CD14, are risk factors for AD. The study included both 626 AD patients (427 women and 199 men; age range: 53-98 years; mean age: 74.88 ± 8.44) from Northern Italy and age and gender matched controls. Our results demonstrate that the +896A/G TLR4 single nucleotide polymorphism (SNP) is associated with AD, whereas no association has been observed with -260C/T CD14 SNP. Furthermore, no differences have been observed evaluating the combined presence of +896A+TLR4/-260T+CD14 "high responder"(pro-inflammatory-profile). However, our results showing the involvement of TLR4 in AD pathophysiology, strengthen the suggestion that systemic inflammation plays a key role in AD. Carriers of high responder SNP, affected by mild cognitive impairment might, be the ideal target for a preventive treatment with biologics as monoclonal antibodies directed against the pro-inflammatory cytokines to decrease the level of systemic inflammation involved in AD pathophysiology.

Key Words: Alzheimer's disease, inflammation, innate immunity, TLR4, CD14.

INTRODUCTION

Alzheimer's disease (AD) is a heterogeneous and progressive neurodegenerative disease which in Western societies mainly accounts for clinical dementia [1]. The AD prevalence is below 1% in individuals aged 60 years, but shows an almost exponential increase with age, so that, in the Western world, in people aged 85 years or older the prevalence is between 24% and 33% [1]. Neuropathological hallmarks of AD are neuronal and synapses loss, extracellular amyloid deposits (neuritic plaques) and intracellular deposition of neurofibrillary tangles, whereas major clinical manifestations of the disease are memory loss and cognitive impairment [2]. There is currently no cure for AD and its pathogenesis remains the subject of many theories involving genetic as well as environmental factors. To date, it has been demonstrated that inflammation seems to occur in pathologically vulnerable regions of the AD brain, where damaged neurons and neurites, highly insoluble β-amyloid(Aβ)42 peptide deposits and neurofibrillary tangles provide obvious stimuli for inflammatory responses, likely induced by reactive astrocytes and activated microglial cells [2-7]. Cytokines and acute phase proteins are also overexpressed in microglia and astrocytes surround neuropathological lesions [2-7]. These observations emphasise the amyloid cascade/neuroinflammation hypothesis [3-8] which considers the microglial cell activation and the release of inflammatory mediators as key elements of the neurodegeneration. Besides, increasing evidences suggest the involvement of innate immunity receptors in the activation of microglial cells. The CD14 antigen/ Toll like receptor-4 (CD14/TLR4) receptor complex seems to be over-expressed on microglial cells inducing activation through the binding of Aβ peptides [9-11]. This receptor complex is known to be involved in cellular activation by micro-organisms components, as lipopolysaccharide (LPS) or other highly hydrophobic and aggregate structures, or endogenous molecules produced by cell and DNA damage [9-15]. So, in the AD brain all these molecules may contribute to development and progression of neurodegeneration through the TLR4/NF-kB pathway [9-15]. The key role of this receptor complex leads to biologically plausible hypothesis that functional variations in the TLR4 (accession number of GenBank: NM-138554.1) and CD14 (accession number of GenBank: NM-000591) genes might influence the susceptibility to sporadic AD. On the other hand, genetic factors seem to be involved in the complex AD pathophysiology, as demonstrated by molecular genetic and epidemiological studies identifying numerous gene polymorphisms as susceptibility AD modifiers. In particular, several reports have indicated that the risk of AD is substantially influenced by several polymorphisms in the promoter region, and other untranslated regions, of genes encoding inflammatory mediators, since alleles favouring increased expression of some inflammatory mediators or decreased expression of anti-

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inflammatory mediators are more frequent in patients with AD than in controls [16-25]. The polymorphisms are fairly common in the general population, so there is a possibility that any given individual will inherit one or more high-risk alleles and that interactions among them and with environmental stimuli will be responsible for the genetic susceptibility to disease [4,17,25]. This might be the case for the allelic variants of CD14 and TLR4 genes. A common adenine to guanine substitution in TLR4 gene, 896 nucleotides downstream of the transcription start site (+896), causes the replacement of an aspartic acid residue by a glycine at amino acid 299 (Asp299Gly or D299G rs number: 4986790 - http://www.ncbi.nlm.nih.gov/entrez/query.snp or Innate Immunity Programs Genomic Applications Web site: http://innateimmunity.net/.//PGAs/InnateImmunity/TLR4). This missense polymorphism leads to an attenuated efficacy of LPS signalling and a reduced capacity to elicit inflammation [26]. The carriage of at least one minor 299Gly allele has been associated with a diminished risk of vascular events, as well as successful ageing in humans [27]. As regards the CD14 gene, the single nucleotide polymorphism (SNP) (C→T), in position −260 (rs259190) of the promoter has been shown to increase transcriptional activity by lowering the affinity of GC box of Sp3, a factor known to inhibit the activity of a number of promoters [28-30]. This enhanced transcriptional activity has been associated with enhanced expression of CD14 on monocytes [28-30]. Several recent reports suggest that CD14 T/T genotype may be a risk factor for some clinical complications of atherosclerosis [29,30]. However, in spite of the potential importance of these common genetic variants in neurodegeneration, to better of our knowledge only three investigations of their role in relation to AD have been undertaken [31-33]. Besides, no study has been performed to assess the role of the combined pro-inflammatory genotype. Given these considerations, in the present report we evaluated whether the TLR4 and CD14 pro-inflammatory alleles are risk factors for AD and we assessed the combined role of the two pro-inflammatory SNPs.

SUBJECTS AND METHODS

Patients and Controls

The study included 626 AD patients (427 women and 199 men; age range: 53-98 years; mean age: 74.88 ± 8.44) from Northern Italy. All AD subjects were diagnosed as probable AD according to NINCDS–ADRDA [34] and DSM-III-R criteria [35]. Cognitive performances and alterations were measured according to the mini mental state evaluation and the global deterioration scale. All AD cases were defined as sporadic because their family history did not mention any first-degree relative with dementia. Besides, 80% of AD patients showed clinical onset of the disease after 65 years of age (late-onset AD) and 20% before this age (early-onset AD). Controls were 190 unrelated individuals (100 women and 90 men; age range: 65-93; mean age 73.21 ± 8.24) randomly selected from a nursing home. These subjects had complete neurological, laboratory and medical examinations shown that they were free of age-related diseases. The controls were collected from the same population like the patients cohort. Patients and controls belonged to same ethnic group because parents and grandparents were born in Northern Italy. Informed consent was obtained from all carers of patients and controls according to Italian laws.

Genotyping

Blood specimens were collected in tripotassium EDTA sterile tubes. DNA extracted and genotyped for +896 A/G TLR4 and -260C/T CD14 SNPs and ApoE4. In particular, all DNA samples were genotyped for +896 A/G TLR4 SNP and ApoE4 allele, while the -260C/T CD14 SNP was analysed only in 229 patients and 119 controls, due to the reduced amount of available DNA. The procedure for detecting the +896A/G TLR4 SNP was based on Restriction Fragment Length Polymorphism-PCR (RFLP-PCR), restriction cleavage with NcoI (New England Biolabs, USA), and separation of the DNA fragments by electrophoresis, as previously described [36]. The genotyping of -260C/T CD14 SNP was performed using a RFLP-PCR with Hae III (New England Biolabs, USA) restriction enzyme. The PCR mixture contained 50-100 ng of DNA template, 0.5pmol/L of each primer, 0.2 mM of each deoxynucleotide triphosphate, 2.5µL of 10x buffer (10 mM Tri-HCl ph 8.3, 50 mM/L KCl), 1.5 mM/L of $MgCl_2$ and 0.3 U Taq polymerase; water was added to the reaction to achieve a total volume of 25 µL. The amplification protocol consisted of 1 cycle of 10 min at 95° C, 15 min at 85 °C and 4.5 min at 94°C; 42 cycles of 30s at 94°C, 1 min at 55°C and 1 min at 72°C, and 7 min at 72°C. The PCR product (10 µL) was cleaved in an appropriate buffer with 10 U of Hae III restriction enzyme. The DNA fragments were separated by electrophoresis thorough a 2% agarose gel containing 0.5µg/mL of ethidium bromide and visualised under UV light. Digestion of PCR products yielded bands of 418 bp (TT homozygote), 263 and 155 bp (CC homozygote), and all 3 bands in the heterozygote subjects. The ApoeE4 allele was assessed by a PCR-based method [20].

Statistics

Allelic and genotypic frequencies were evaluated by gene count. The data were tested for the goodness of fit between the observed and expected genotype frequencies according to Hardy-Weinberg equilibrium, by $\chi 2$ test. Significant differences in frequency, among the groups, were calculated by χ^2 test (3x2, 2x2 tables, where appropriate). Furthermore, ODD Ratio (OR) with Confidence Interval (CI) and its significance were calculated. In patients and controls a logistic regression analysis was used to investigate the associations of genotypes with AD, after adjustment for ApoE4 allele. We also analyzed the allelic frequencies of the TLR4 and CD14 SNPs according to gender in AD patients and controls, by $\chi 2$ test.

RESULTS

Table **1** shows the genotype distributions and allelic frequencies of +896A/G SNP TLR4. The genotypes were significantly differently distributed between the 2 cohorts (p=0.04). Accordingly, +896A TLR4 pro-inflammatory allele was overrepresented in AD patients and underrepresented in controls (p=0.02). Comparing the genotypes and the alleles of +896A/G TLR4 SNP between the two groups, the ORs for the pro-inflammatory genotype and allele were

Table 1. Genotype Distributions and Allelic Frequencies of +896A/G(Asp299Gly) TLR4 Gene Polymorphism in 626 AD Patients and 190 Controls from Northern Italy

Genotypes	AD Patients (N=626)	Controls (N=190)
TLR4 A/A	569	161
A/G	54	28
G/G	3	1
Alleles	AD Patients (N=626)	Controls (N=190)
TLR4 +896A	1192 (95.2%)	350 (92.1%)
+896G	60 (4.8%)	30 (7.9%)

All the genotypes were in Hardy-Weinberg equilibrium. Significant differences by χ^2 (3x2 table) in the frequency of +896A/G TLR4 SNP genotypes between the patients and controls were found (p=0.04). Significance was obtained by χ^2 (2x2 table) in allele frequency of this SNP; in particular, +896G TLR4 low responder allele was underrepresented in AD patients and overrepresented in controls, while +896A proinflammatory allele was overexpressed in AD patients (p= 0.02).

Table 2. Genotype Distributions and Allelic Frequencies of -260 C/T CD14 Gene Polymorphism in 229 AD Patients and 119 Controls from Northern Italy

Genotypes	AD Patients (N=229)	Controls (N=119)
CD14 C/C	50	25
C/T	91	49
T/T	88	45
Alleles	AD Patients (N=229)	Controls (N=119)
CD14 -260C	191 (41.7%)	99(41.6%)
-260T	267 (58.3%)	139 (58.4%)

All the genotypes were in HWE. The genotypes and the alleles were not significantly differently distributed between the 2 cohorts.

statistically significant (OR=1.4, 95% CI=1.01-3.06, p=0.03; OR=1.9, 95% CI=1.03-3.52, p=0.006, respectively).

Table **2** reports the genotype distributions and allelic frequencies of -260C/T CD14 SNP. The genotypes of -260C/T CD14 SNP were not significantly differently distributed between the 2 cohorts. Accordingly, the allele frequencies of this SNP were not significantly differently distributed in controls and patients.

Searching for a pro-inflammatory genetic risk profile, we assessed the frequency of +896A+TLR4/-260T+CD14 "high responder" genotype in patients and controls. By comparing this frequency with that of the other combinations, the frequency of +896A+TLR4/-260T+CD14 "high responder" (pro-inflammatory profile) genotype was not significantly overrepresented in AD patients (data not shown).

Besides, we performed a logistic regression analysis to test the association of genotypes of +896A/G TLR4 and -260C/T CD14 SPNs with AD after adjustment for the presence of ApoE4 allele. This analysis demonstrated that a significant difference in genotype frequency of +896A/G TLR4 persisted between AD patients and controls [p=0.002, OR 6.7 (5.01-11.9)], whereas no significant difference was obtained for CD14 SNP. These results strengthen previous results suggesting that pro-inflammatory SNP of TLR4 gene under study is an independent risk factor for developing AD in the population of Northern Italy [33].

It is known that women are more susceptible to AD than men [37]. So, we analyzed the allelic frequencies of +896A/G TLR4 and SNP -260C/T CD14 according to gender in AD patients and controls. Concerning CD14, no statistically significant difference was observed in SNP distribution analysed frequencies according to gender (data not shown), whereas the frequency of +896A/G TLR4 SNP was significantly differently distributed only between the AD female patients and female controls and not in males (Table **3**, p=0.025). However, due to smaller number of affected and control men, these results need to be confirmed.

DISCUSSION

Microglial cell activation has a fundamental role in the inflammatory pathogenesis of AD, as stated by the amyloid cascade/neuroinflammation hypothesis [3-8, 25, 38]. The former is responsible for the production of the neurotoxic substances, such as reactive oxygen and nitrogen species, pro-inflammatory cytokines, complement proteins, and other inflammatory mediators that bring important neurodegenerative changes [3-8, 25,38]. Some studies have suggested that activation of microglial cells is induced throughout the binding of Aβ peptides [25,38,39]. Several membrane proteins

Table 3. Genotype Distribution and Allele Frequency of +896A/G TLR4 Gene Polymorphism, Analysed According to Gender, in AD (427 Females and 199 Males) Patients and Controls (100 Females and 90 Males) from Northern Italy

Alleles	Women*		Men	
	AD Patients (N=427)	Controls (N= 100)	AD Patients (N=199)	Controls (N=90)
TLR4 +896A	814 (95.3%)	182 (91%)	378 (95 %)	168 (93.3%)
+896G	40 (4.7%)	18 (9%)	20 (5%)	12 (6.7%)

*p=0.025 by $\chi 2$ test.

expressed on microglial cells seem to be implicated in Aβ peptides binding, in particular the CD14/TLR4 receptor complex binds highly hydrophobic Aβ peptides aggregates suggesting that in the central nervous system the TLR4/CD14 signalling pathway might be necessary for the production of neurotoxic substances [9-15, 25, 27, 38, 39].

More interestingly, a further, not mutually, alternative explanation on the key role of microglial activation may be related to the role of CD14/TLR4 as LPS receptor. In fact, some studies have linked infections to another relevant age-related inflammatory disease, i.e. atherosclerosis, since the total burden of infections, at various sites, may affect the progression of atherosclerosis and elicit clinical symptoms. This can be due to remote signalling by inflammatory mediators that activate immune cells in the atherosclerotic plaques through CD14, TLR4 activation [27,40,41]. A similar mechanism may be envisaged in AD, as demonstrated by an increased 3-fold risk of AD in patients whose serum levels of C reactive protein, was in the upper 3 quartiles 25 years before of the diagnosis since this increase is caused by the individual chronic antigen burden [42]. Hence, as in atherosclerosis, total burden of infections may affect the progression of AD, interacting through LPS receptor(s) [43].

It has been observed, in a vivo model of neurodegeneration, that mice bearing a loss-of function mutation in the TLR4 gene are resistant to LPS-induced damage in different neuronal populations [15]. Besides, the CD14 expression increased in microglia of a transgenic murine model of AD, and microglia derived from CD14-deficient mice demonstrated reduction in activation by Aβ peptide, suggesting that CD14 is necessary for Aβ-induced microglia activation [11]. Accordingly to these data, TLR4 and CD14 might represent candidate genes for susceptibility to or exacerbation of this neurodegenerative disease.

The aim of our study was to evaluate whether the TLR4 and CD14 pro-inflammatory alleles can be considered risk factors for AD and to assess the combined role of the two pro-inflammatory alleles. We found that +896A TLR4 pro-inflammatory allele was overrepresented in AD patients. Furthermore, the stratification for ApoE4 allele clearly demonstrated that +896A/G TLR4 SNP is a risk factor for AD independently on ApoE4 status in Northern Italy population. Our findings confirm and extend the data of a recent study which demonstrated that +896A pro-inflammatory TLR4 allele increases the risk for AD independently on ApoE4 [33]. As regards -260 C/T CD14 polymorphism, we were unable to demonstrate an association between this polymorphism and AD. Other two recent studies have demonstrated no association between-260 C/T CD14 polymorphism and AD [31, 32]. So, this larger sample of patients allows us to conclude that this CD14 SNP is not a susceptibility allele for AD at least in the Italian population, whereas this TLR4 SNP is a genetic marker of susceptibility.

The search for a pro-inflammatory profile is of some importance for a pharmacogenomic approach, i.e. to detect and utilize a risk profile which allows early identification of individuals at risk dose for desired effects. In fact, a major goal of clinical research is to improve early detection by developing tools to promote early diagnosis of disease before the clinical manifestation of the malady, where anti inflammatory treatment might play a decisive role in preventing or significantly retarding the clinical expression of the disease. These tools should enable identification of risk individuals during the preclinical period, accelerate and enhance the accuracy of diagnosis in the early clinical phase to ensure appropriate treatment, and help in the development of drugs that could prevent or at least slow the onset of clinical manifestations of disease [44-46]. Accordingly, carriers of high responder polymorphisms, affected by mild cognitive impairment might be the ideal target for a preventive treatment with biologics as monoclonal antibodies directed against the pro-inflammatory cytokines [47]. Decreasing the level of systemic inflammation might be a suitable chemopreventive treatment for AD, since microglia may be activated by systemic stimuli [25,42].

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ABBREVIATIONS

AD = Alzheimer's disease

Aβ = β-amyloid

CI = Confidence Interval

LPS = Lipopolysaccharide

OR = ODD Ratio

RFLP-PCR = Restriction Fragment Length Polymorphism-PCR

SNP = Single nucleotide polymorphism

TLR4 = Toll-Like Receptor

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Chapter 6

Pathophysiology of vascular dementia

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Review

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Pathophysiology of vascular dementia

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Abstract

The concept of Vascular Dementia (VaD) has been recognized for over a century, but its definition and diagnostic criteria remain unclear.

Conventional definitions identify the patients too late, miss subjects with cognitive impairment short of dementia, and emphasize consequences rather than causes, the true bases for treatment and prevention. We should throw out current diagnostic categories and describe cognitive impairment clinically and according to commonly agreed instruments that document the demographic data in a standardized manner and undertake a systematic effort to identify the underlying aetiology in each case.

Increased effort should be targeted towards the concept of and criteria for Vascular Cognitive Impairment and Post-Stroke Dementia as well as for genetic factors involved, especially as these categories hold promise for early prevention and treatment.

Background

The concept of Vascular Dementia (VaD), has been recognized for over a century, but its definition and diagnostic criteria remain unclear and generate, much confusion and debate althoung several clinical criteria have been used for defining the VaD

The term of VaD substantially means "disease with a cognitive impairment resulting from cerebrovascular disease and ischemic or hemorrhagic brain injury".

Dementia represents only a portion of the burden of cognitive dysfunction associated with cerebrovascular disease. In addition to patients who develop dementia, there are those who develop cognitive impairment that does not fulfill traditional criteria for dementia but that nonetheless has a significant impact on quality of life and ability to carry out activities of daily living. As a result, the older term "vascular dementia" is being replaced with a new one: "vascular cognitive impairment" (VCI). In addition, postmortem pathological studies indicate that 15%

to 34% of dementia cases show significant vascular pathology, either alone or in combination with Alzheimer disease (AD) pathology [1].

In fact, dementia criteria are typically modelled on Alzheimer Disease (AD), wherein involvement of the mesial temporal lobe results in dense episodic memory impairment. [2,3]. Current research has led to the identification of a cognitive syndrome signalling a high risk of further cognitive decline, a group of patients similar to that of mild cognitive impairment as a risk state for AD [4]. The cognitive impairment attributable to cerebrovascular disease is a rapidly escalating public health problem. The heterogeneity of the population of patients with VaD diagnosed using current criteria has raised the need for updating the classification of subtypes

History

The concept of "dementia" originated early in the 16th century., when for the first time Pratensis mentioned the "Dementia stroke correlate". in the "De cerebri morbis - 1549" Subsequently Willis described the most important causes of dementia including ageing and vascular disease and the first accurate clinical observations of patients with vascular dementia In the early 19th century Cooke described "intellectual deficits among the sequelae of apoplexy" The history of vascular dementia during 19th and 20th centuries has been recently reviewed [5].

Binswanger and Alzheimer, began a series of clinico-pathological correlation studies attempting to isolate additional forms of dementia, describing four different form of vascular dementia (arteriosclerotic brain degeneration, perivascular gliosis of the brain cortex, dementia post-apoplexian and Binswanger's chronic progressive subcortical encephalitis) [6-16]. Pierre Marie described in 1901 the "***état lacunaire***" associated with a constant intellectual deficits. Dementia was separated from delirium and other psychiatric diseases at the end of the 19th century, but its etiology remained un cleared [17].

Alzheimer and Binswanger had correctly concluded that "arteriosclerotic dementia" represented a large clinico-pathological spectrum. However, "arteriosclerotic dementia" incorrectly became synonymous with senile dementia, and it was widely held that cortical atrophy in the elderly resulted from progressive decrease in cerebral perfusion leading to hypoxic neuronal death.

Little further progress was made for the next 70 years because of a combination of factors (Freud's work, Kraepelin's classification, syphilis as a common and obscuring cause of dementia). New suggestions and observations were made, but they did not thrive against this background. In 1946 a clear description of a multi-infarct dementia was given, recognizing the possible role of silent infarcts and explicating that the chronic ischemia wasn't he cause of vascular dementia, but the true mechanism was the infarction. This process later termed "multi-infarct dementia" [18]

Today, the importance of vascular lesions in Alzheimer's disease is being increasingly recognized: various data shows that more than 30% of AD exhibit cerebrovascular pathology [5].

A recent review define the AD as a vascular disorder: "...since the value of scientific evidence generally revolves around probability and chance, it is concluded that the data presented here pose a powerful argument in support of the proposal that AD should be classified as a vascular disorder. According to elementary statistics, the probability or chance that all these findings are due to an indirect pathological effect or to coincidental circumstances related to the disease process of AD seems highly unlikely. The collective data presented in this review strongly support the concept that sporadic AD is a vascular disorder." [19].

Discussion

Vascular dementia (VAD) and vascular cognitive impairment (VCI): clinical criteria

Cardinal elements implemented in the clinical criteria for VaD are the definition of the cognitive syndrome of dementia and the objective documentation of vascular lesions capable of causing dementia [20-24]. All the currently clinical criteria, are derived from expert opinion based on prevailing knowledge and pathogenetic hypothesis of dementia's causes [25].

The current clinical criteria for VaD recognize the multiplicity of lesions, but none of these clinical criteria provide guidelines for subtype of VaD [26]. Only the NINDS-AIREN criteria mention the following subtype: cortical vascular dementia, Binswanger's disease and thalamic dementia [27]. Also the brain imaging requirements are not included, or are not complete, in all current clinical criteria.

The systematic heterogeneity of the population of patients with VaD diagnosed by using current criteria has raised the need for updating the classification of subtypes. The main subtype of VaD include: multi-infarct dementia or predominantly cortical VaD, strategic infarct dementia and small vessel dementia or subcortical VaD even if only subcortical VaD is probably a more homogenous group [28-31]

The new criteria are based on *homogeneity* (in aetiologies, brain imaging and clinical syndrome), on *predictability*

(phenomenology and clinical picture, clinical course and natural history, outcomes and treatment responses), on *reproducibility* (intra- and inter-rater reliability). Furthermore, they must be validated by prospective clinical pathological correlation [25,32].

The cornerstone of all criteria of dementia is memory impairment; this criterion works very well for AD, in which the mesial temporal lobes are affected early and prominently with consequent early memory loss, but strokes affecting cognition occur most commonly in the frontobasal systems that subserves judgement, planning and emotion, features seldom tested in cognitive screens.

At present, there is no generally accepted test battery for identifying or classifying patients with VCI. However, there are some basic principles that can be followed in developing such a battery. One of these is that large vessel cortical strokes and subcortical small vessel disease, tend to produce different kinds of deficits. The former typically present with region-specific syndromes such as aphasia, apraxia, and amnesia. The latter present with more subtle and temporally progressive deficits, often described as "executive" in nature. [4,33-37]. These include deficits in speed and so-called "strategic" processing (ie, attention, planning, and monitoring) in tasks such as memory tasks. Patients may perform normally on simple tasks but reveal deficits as tasks increase in complexity. It appears that the majority of VCI patients fall into the class with subcortical small vessel disease. [21,38]. Thus, it seems reasonable that neuropsychological testing for VCI would include tasks testing executive function. In addition, such tests may help to differentiate patients in which either vascular or AD pathologies predominate [1]

The extent of ischaemic disease on neuroimaging, that is both sufficient and necessary to cause cognitive impairment, Leukoaraiosis and atrophy as well as the issue of location [39]. To summarize the literature data, it's clear that there are insufficient data to propose firm cut-offs for the extent of Leukoaraiosis or for extent of infarction.

Anyway, enough scientific rationale already exists for undertaking systematic clinical trials in the prevention of cognitive impairment through the control of vascular risk factors and the use of statins, anti-inflammatory agents, ACE inhibitors, vitamins E and B12 [40].

Epidemiology

Epidemiologic studies of VaD have been hampered by the "lack of clear and universal diagnostic criteria, by the use of different strategies in detecting dementia cases of vascular origin, by the difficulties of developing an effective case-finding strategy, and by the complexity of using imaging or laboratory tests in large scale epidemiologic surveys" [41]. Despite these difficulties, the broad contours of VaD epidemiology are emerging slowly [42-44].

The data from current studies cannot be compared and reconciled easily. Diagnostic criteria for VaD have been a long standing source of disagreement and are perhaps the greatest barrier to reaching consensus on the epidemiology of this disease [42,43,45]. A first problem is the classification of patients with the so-called "mixed" dementia, in whom the aetiology appears to include both cerebrovascular and primary degenerative features. A second problem is the disagreement about criteria and evaluation of tools. A number of new sets of criteria have been introduced in the 1990s, including the ICD-10, the Chui et al., the Roman et al., and the DSM-IV criteria [46,47]. An additional problem with epidemiologic studies is the need to transfer published criteria into clinical instruments or procedures that can be implemented on a large scale. Brief scales to separate VaD from AD have been developed beginning with the Hachinski Ischemic Score (HIS) introduced in 1975. A number of modifications or transformations of this original scale have been proposed in the past 20 years [48]. Some of these scales require imaging tests, whereas others are purely clinical. A third problem is the use of imaging findings in the definition of VaD: in the incidence study of dementia in Rochester, the age-specific incidence rate of VaD by using more restrictive DSM-IV criteria (clinical stroke required) and less restrictive criteria (certain vascular imaging lesion sufficient) diverge beyond 70 years of age, and are more than doubled with the restrictive criteria beyond 85 years of age [49]. A fourth problem is to define the range of severity of VaD to be detected in the study: severity may vary from a mild cognitive impairment to terminal stages of deterioration.

If the prevalence of VaD is greater among women than men of a certain age, it remains uncertain whether women have a higher incidence of VaD or whether the incidence is equal to men, but survival after onset of VaD is better in women [41].

Today new prevalence studies are available) and the breadth of variation for VaD prevalence is now even wider. It remains unclear whether the differences between different countries reflect disagreement over diagnostic criteria for VaD or other methodological differences. For studies investigating the incidence of VaD in different countries the data are similarly disparate [50,34].

The data available to answer the question if the prevalence or the incidence of VaD is increasing or decreasing over time are very few. Because the data from current studies cannot be easy reconciled, it remains very difficult to draw conclusions about the epidemiology of VaD. Our progress

would benefit greatly from the development of a new set of diagnostic criteria sensitive and specific, easy to apply in the field setting and internationally accepted. It appears, however, that the prevalence of VaD tends to be higher in men than in women, and that it increases with age; moreover there seems to have been a decline in both prevalence and incidence of VaD between the 1950s and 1970s [41]. The prevalence of VaD ranged typically between 3% and 6%; but the variation described ranged between 0% to 20%. There are relatively few data regarding incidence in the general population. The incidence of cognitive impairment sufficient to adversely affect outcome but not meeting current criteria for vascular dementia is as high as 35.2% compared to 3.8% with a similar degree of impairment in stroke-free controls [18,35,42].

Pathophysiology

Epidemiological studies have explored widely the potential risk factors or protective factors for different dementing disorders [36,51]. Many studies have focused on AD and more recently, on all types of dementia as a syndrome. Aetiological studies about VaD have been hampered by methodological issues and - the search for risk factors of VaD may potentially be more amenable to prevention than AD [52].

Some common determinants between AD and VaD are:

- risk factors involved in cerebrovascular disease (age, sex, some atherogenic disorders or vascular risk factors, genetic factors and inflammation). Other potential risk factors like occupational exposure to pesticide, psychological stress or life events, dietary fat intake, family history of stroke, etc.);

- potential protective factors (high educational attainment, eating fish or shellfish, physical exercise, use of supplementary antioxidants like Vitamins E and C, use of Vitamin B12, Mediterranean diet, etc.) [36,50,53].

Vascular cognitive impairment is not a regular pathogenetic entity. Multiple small thromboembolic strokes or strokes in strategic locations such as the thalamus, frontal lobe or temporal lobes may cause cognitive impairment and frequently occur without classical stroke-like symptoms. Nonetheless, in VaD, the majority of patients instead present widespread microangiopathy-related cerebral damage, which is often clinically silent or is accompanied by unspecific neurological signs.

Several mechanisms may explain why patients affected by stroke are prone to develop dementia [20,37,54,55]:

- post-stroke dementia may be the direct consequence of vascular lesions in the brain

- post-stroke dementia could be the result of pre-existing neuropathological effects AD's related

- recurrent stroke that is cause by vessel damages and by white matter lesions that may lead to cognitive decline and contribute to post-stroke dementia;

One of the mechanism involved in ischemic VaD is under the control of large vessels disease (atherosclerosis, and other arteriopathies), however, impaired cerebral flow in the absence of infarct as consequence of arterial stenosis has been documented, although its clinical consequences remain to be fully investigated. It is also unclear whether and to what degree large vessel disease contributes to the white matter pathology and lacunes associated with the subcortical type of VaD. Statistical association suggests it may have additive effects to small vessel pathology. [56,57]

Moreover, the alterations of small vessels play a role in causing damage to the cerebral tissue and are potentially responsible for the subsequent development of cognitive alterations. Small vessel lesions are considered related to the deep lacunar infarcts and white matter changes typically observed in subcortical forms of vascular cognitive impairment.

The most common types of diseases affecting cerebral microvessels are: arteriosclerosis, lipohyalinosis, cerebral amyloid angiopathy, basal ganglia calcification, CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leucoencephalopathy), other uncommon intracerebral vasculopathies.

The small vessel alterations could also lead to damage affecting the blood-brain barrier and chronic leakage of fluid and macromolecules in the white matter, even if the neuroimaging methods do not detect diffuse alterations of the blood-brain barrier in vivo [58].

Another mechanism influencing small vessel alterations could be incomplete ischemia and selective tissue necrosis (i.e. incomplete infarction) causing a selective neuronal necrosis with sparing of glial cells and microvessels [59-62].

Recently, it has been suggested a new variety of lacunar infarction, (type Ib), characterized by small areas of perivascular rarefaction and selective neuronal loss, followed in the more advanced stages by a varying amounts of depleted neurons and oligodendrocytes, astroglial response and minor central cavitation. Furthermore, embolic occlusion of small penetrating artery followed by spontaneous lyses of the thrombus could be responsible for these lacunes [63-65].

The concept of Leukoaraiosis, suggested by Hachinski, was introduced to describe morphological abnormalities of the white matter on imaging [66].

The pathogenesis of white matter changes (WMC) is not well established and a number of possible mechanisms have been hypothesized, all mechanisms are reconducible to a form of cerebrovascular disease. It has been proposed that the diffuse changes of the white matter should be considered a form of incomplete infarction. [67-76]

The understanding of the pathobiology of AD and VaD recived an impulse by the discovery of genes that produce monogenic forms of the illness or contribute to polygenic forms; in particular, the identification of genes contributing to VCI would no doubt provide insight into the cellular and molecular basis of VCI.

Genetic factors play an important role in the aetiology of VaD, in particular, it's seems to be more important in large-vessel stroke and small vessel stroke than in cryptogenic stroke, and there is no epidemiological evidence for a genetic component in cardioembolic stroke.

The genes underlying VaD must be of 2 nonmutually exclusive classes: *(1)* genes that predispose individuals to cerebrovascular disease, and *(2)* genes that determine tissue responses to cerebrovascular disease (eg, genes conveying ischemic tolerance or susceptibility, or the ability to recover from ischemic insult). With regard to the first class of genes, some progress has been made in the past few years in identifying genes that confer susceptibility to hypertension and stroke [77]. In addition, several monogenic forms of cerebrovascular disease have been identified. The two best studied of these are cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy (CADASIL: a subcortical small vessel disease accompanied by lacunar strokes, migraine, and dementia) and hereditary cerebral hemorrhage with amyloidosis-Dutch type (HCHWA-D) [1].

The CADASIL condition is a heritable small-vessel disease caused by mutations in NOTCH3 gene which is normally expressed in vascular smooth muscle cells and pericytes (including those of the cerebral vasculature) and that encodes a cell-surface receptor, which has a role in arterial development and is expressed on vascular smooth-muscle cells. appears to be involved in directing smooth muscle cell proliferation and differentiation. The NOTCH3 receptor is a heterodimer composed of a large extracellular fragment and a smaller transmembrane intracellular fragment. About 95% of patients have missense mutations that cluster in exons 3- and consist in change of cysteine residues amount, but the pathogenic meccanism is still unknown [78,79].

Whit regard to HCHWA-D (a syndrome of primarily hemorrhagic strokes and dementia), it is caused by a mutation in the gene for amyloid precursor protein (APP) that causes abnormal deposition of amyloid in the walls of leptomeningeal arteries and cortical arterioles (a mpathological condition known as cerebral amyloid angiopathy [CAA]). Mouse models have been developed for CADASIL and HCHWA-D and have contributed critical insights into the cell biology of the pathogenic processes underlying them. [80]

In contrast, little attention has been paid to the second class of genes: those that render the brain more or less susceptible to injury in response to cerebrovascular disease. Evidence for the existence of such response genes is that patients with apparently similar loads of vascular pathology (with regard to lesion type, number, and location) may range from no cognitive impairment to severely cognitively impaired. The association studies using the candidate gene approach, has identified a number of genetic variants possibly involved in risk factor development and, on the other hand, suggests that the risk of stroke has a substantial genetic component. Nonetheless, the genes underlying this risk in the general population remain undetermined. Genetic factors can act at several levels. They can contribute to conventional risk factors such as hypertension, diabetes, or homocysteine concentrations, which have a known genetic components. They might further interact with environmental factors or contribute directly to an intermediate phenotype [78,81]

It is also possible that some genetic factors contribute, by interaction with conventional risk factors, to the development of subcortical injury of vascular origin in non familiar cases.

Most single-gene disorders are associated with specific stroke subtypes, especially in young stroke patients without known risk factors. Table 1; ref. [79,82-87].

In this group of diseases we take in consideration only Fabry's disease for its special association with stroke. The Fabry's disease is an X-linked systemic disorder caused by deficiency of the lysosomal enzyme α-galactosidase A, that results in progressive accumulation of glycosphingolipids in the myocardium, renal epithelium, skin, eye, and vasculature. The symptoms are acroparesthesia, angiokeratoma, hypoidrosis, which start during the childhood or adolescence and are present in the majority of the affected individuals and many young patients are affect by cryptogenetic stroke. The disease occurs either in large-artery either in small-vessel disease, with a preference for posterior circulation. Small-vessel disease in Fabry's disease is associated with white-matter changes and evidence suggests that the extent of such lesions is influenced by gene

Table 1: Single-gene disorders associated with ischaemic stroke (for reference see the text)

Gene	Disease	Mode of inheritance	Stroke mechanism
GAL	*Fabry's disease*	*X-linked*	*Large-artery disease and small-vessel disease*
NOTCH3	*CADASIL*	*AD*	*Small-vassel disease*
HBB	*Sickle-cell disease*	*AR*	*Large-artery disease, small-vessel disease, haemodynamic insufficiency*
CBS and others	*Homocystinuria*	*AR*	*Large-artery disease, cardioembolism, small-vessel disease, arterial dissection*
mtDNA	*MELAS*	*Maternal*	*Complex (microvascular and neuronal factors)*
FBN1	*Marfan syndrome*	*AD*	*Cardioembolism and arterial dissection*
COL3A1	*Ehlers-Danlos syndrome type IV*	*AD*	*Arterial dissection*
ABCC6	*Pseudoxanthoma elasticum*	*AR*	*Large-artery disease and small-vessel disease*

AD = autosomal dominant. AR = autosomal recessive. HBB = haemoglobin beta. CADASIL = cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy. MELAS = mitochondrial myopathy, encephalopathy, lactacidosis, and stroke. mtDNA = mitochondrial DNA.
For references, see text

polymorphisms as interleukin 6, endothelial nitric oxide synthase, factor V, and protein Z [78,82]. These findings are suggestive but need further confirmation (Duro's data not published).

The genetic contribution to common multifactorial stroke seems to be polygenic: there are many alleles with small effect, due to their wide distribution, however, on a population basis the impact on stroke is large. Several genes and polymorphisms were selected for a significant association with ischaemic stroke: in particular polymorphisms in the genes encoding MTHFR (enzyme in homocysteine metabolism), ACE (enzyme in renin-angiotensin-aldosterone system), Factor V Leiden, prothrombin and PAI 1 (Haemostatic system) [78]

Recently, studies for the analyses of ischemic stroke assessed two unsuspected common SNPs on chromosome 12p13 (rs11833579 and rs12425791), consistently associated with total ischemic, and atherothrombotic stroke in caucasic population although there is association with ischemic and atherothrombotic strokes (as compared with total stroke), but no association with non ischemic stroke. The SNPs are on the gene NINJ2 that encodes an adhesion molecule expressed in glia cells and shows increased expression after nerve injury Some mutations are associated with a slightly more aggressive phenotype. [81].

One class of genes that must influence tissue responses to cerebrovascular disease are the AD genes. There is an additive or synergistic interaction between AD and cerebrovascular pathologies, such that individuals with both of these pathologies show greater cognitive impairment than those exhibiting either pathology alone. In addition, at least three sets of genes in the AD pathway, the presenilins, APP, and apolipoprotein E (apoE), are known to interact with the VCI disease pathway.

The presenilins, mutations of which cause AD, have been shown to interact directly with Notch proteins, including Notch 3 (mutations of which cause CADASIL).

Mutations in the APP gene can lead either to AD or to hemorrhagic stroke and dementia (as in HCHWA-D) depending on the site of the mutation and the subsequent cellular site of amyloid accumulation.

Variants of the apoE gene appear to affect not only susceptibility to cerebrovascular disease but also recuperative responses to it (see below). Thus, there appear to be links in the biochemical pathways underlying VCI and AD pathologies, which could be responsible for the observed interactive effects of these pathologies on cognitive function.

Genes that influence brain responses to cerebrovascular disease do not appear to be limited to those within AD pathway. First, it has been shown that VCI can occur in the complete absence of AD pathology in sporadic VCI and in hereditary forms. In addition, the cognitive sequelae of pathogenic processes associated with VCI are different from those seen in "pure" AD, in that executive function appears more strongly affected in VCI than is memory. Consistent with these observations, different brain regions seem differentially affected in VCI and AD, with prefrontal circuits being more affected in VCI and the hippocampus in AD.

An important aspect of VaD's (AD's particular) pathophysiology, is the role of inflammation: the incidence is influenced by the gene polymorphisms of the inflammatory mediators. Among the most widely investigated genes are those involved in inflammation (interleukin 1, interleukin 6, TNFα, toll-like receptor 4, P-selectin and E-selectin, C-reactive protein), lipid metabolism (apolipoprotein E, paraoxonase, epoxide hydrolase), nitric oxide

release, and extracellular matrix (matrix metalloproteinases) [88,89].

Infact, in recent years, an increasing set of evidence has stress the role of inflammation in the brain, particularly in the microglia-rich amyloid deposits, where the microglias tend to release a wide variety of proinflammatory mediators including cytokines (IL 1β, IL 6, TNF α, acute phase proteins) complement components, various free radicals and nitric oxide (NO), all of which potentially contribute to further neuronal dysfunction and eventually result in cellular death. In addition, apolipoprotein E (ApoE) is strongly associated with AD in terms of cognitive decline and disease onset: ApoE, especially the ε4 allele, has been observed promoting an inflammatory reaction[14]. The AD's pathomechanism is related to the accumulation of toxic amyloid-β (Aβ), which precipitate along the vessel walls and in brain parenchymal plaques, as well as the formation of neurofibrillary tangles (NFTs); the development of cognitive impairment in VaD may be related to a number of different pathological processes including multiple infarctions resulting from occlusion of major brain vessels or their branches and lypohyalinosis. The latter is a degeneration of small arterial vessel walls supplying the subcortical white matter, thalamus, and basal ganglia, or cerebral amyloid angiopathy (CAA is the accumulation of amyloid protein in the walls of cerebral blood vessels): less commonly, vascular dementia is related to deposition of Aβ in brain vessels. CAA can be either sporadic or familial. The progressive cognitive impairment in CAA results from multiple brain hemorrhages and/or ischemia related to narrowing of vessel lumen [88,90]

Conclusion

We should throw out current diagnostic categories and describe cognitive impairment clinically and according to commonly agreed instruments that document the demographic data in a standardized manner and undertake a systematic effort to identify the underlying aetiology in each case (imaging and DNA should be obtained whenever possible.

However, further empirical research and international debate is needed to define the syndrome and stages of vascular subcortical cognitive impairment, validate the brain imaging criteria for subcortical Vascular Dementia by clinical-pathological correlation, as well as the natural history and outcomes of the syndrome.

Increased effort should be targeted towards the concept of and criteria for Vascular Cognitive Impairment and Post-Stroke Dementia as well as for genetic factors involved, especially as these categories hold promise for early prevention and treatment.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors drafted, read and approved the final manuscript.

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Chapter 7

GENERAL DISCUSSION

Biomarkers of Aging

Whereas the average life span of a population is relatively stable, on the level of the individuals, it has a big variation. On the other hand, the rate of aging in humans is not uniform, due to genetic heterogeneity and the influence of environmental factors. Thus, the aging rate, measured as the decline of functional capacity and stress resistance, seems to be different in each individual (A.L. Goldberger, et al 2002). Therefore, attempts have been made to analyse this individual age, the so-called biological age, in comparison to chronological age.

Age-related changes in body function or composition that could serve as a measure of biological age and predict the onset of age-related diseases and/or residual lifetime are termed biomarkers of aging. Many candidate biomarkers have been suggested but in all cases their variability in cross-sectional studies is considerable, and therefore no single measurement has been proven so far to yield a useful biomarker of aging on its own, probably due to the multi-causal and multi-system nature of aging.

Such biomarkers of aging should help on the one hand to characterise this biological age and, as age is a major risk factor in many degenerative diseases, could be subsequently used on the other hand to identify individuals at high risk of developing age-associated diseases or disabilities. However, these biomarkers might be more than disease risk factors, and represent individual indicators of functional status.

We discuss functional parameters, including anthropometric data, functional challenge tests, physiological tests, oxidative stress assessment, genomic and proteomic tests. Such a set of functional biomarkers of aging would help to identify tests that had the greatest predictive value when matched against functional outcome and morbidity patterns. Those with the highest predictive value would be defined as functional biomarkers of aging.

These biomarkers could be used to develop personalized medicine or other interventions which effectively reduce morbidity and improve organ-specific function by delaying the necessity for costly hospitalization or social support of the aging populations (D.F. Terry, et al 2006; T.E. Johnson 2006; E. Jirillo, *et al* 2008).

Inflammation, Cytokines, Immune Response, Apolipoprotein E, Cholesterol, and Oxidative Stress in Alzheimer's Disease: Therapeutic Implications

A better understanding of the interplay among inflammatory mediators, oxidative stress, and acquired immunity is necessary to understand AD development.

AD prevention is an important goal of ongoing research. Two objectives must be accomplished to make prevention feasible:

i) individuals at high risk of AD need to be identified before the earliest symptoms become evident, by which time extensive neurodegeneration has already occurred and intervention to prevent the disease is likely to be less successful and ii) safe and effective interventions need to be developed that either reduce or slow the accumulation of AD neuropathology or lead to a decrease in clinical expression of this pathology (Vasto S, et al 2007).

APOE, cholesterol, and AD has been the subject of intense scrutiny by numerous groups. Since cholesterol levels at midlife are a considerable risk factor for dementia/AD in most of long term follow-up studies.

The best studied role of cholesterol in AD is in amyloid precursor protein (APP) processing and Aβ generation. Increased cholesterol

leads to increased cleavage of APP and increased Aβ production, whereas reduction of cellular cholesterol decreases the γ-secretase activity, which is responsible for Aβ generation (Vasto S, et al 2008; Kivipelto M, et al 2006; de Chaves EP, et al 2008).

Epidemiological studies have suggested a possible protective effect for the cholesterol-lowering statin drugs that inhibit 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase the enzyme that catalyzes the rate-limiting step in cholesterol, in AD patients numerous studies have examined the role of statins in the prevention of dementia and treatment of established AD.

Besides cognitive impairment can be influenced by a number of other factors. The potential effect of nutrition has become a topic of increasing scientific and public interest.

In particular, there are arguments that nutrients (food and/or supplements), such as vitamins, trace minerals, and lipids, can affect the risk of cognitive decline and dementia, especially in frail elderly people at risk of deficiencies.

Aging is associated with cognitive dementia and AD; concomitantly, aging is also associated with malnutrition and reduced intake of micro- and macronutrients. The role of diet in cognitive decline has not been looked into broadly, but because several dietary factors affect the risk

of cardiovascular disease and some studies have suggested a link between cognitive decline and cardiovascular disease, diabetes mellitus, and hypertension, it might be assumed that dietary intake might also influence the risk of dementia (Breteler MM et al 1994; Naor M et al 1997; Guo Z et al 1997).

Diets enriched with anti-oxidant and anti-inflammatory agents (curcumin, green tea, and ferulic acid) may lower the risk of developing age-related neurodegenerative diseases such as AD and PD. Many studies indicate that dietary supplementation with fruit or colored vegetable extracts can decrease the age-enhanced vulnerability to oxidative stress and inflammation. Additional studies indicate that polyphenolic compounds found in red wine and fruits such as blueberries may exert their beneficial effects through signal transduction and neuronal communication, delaying dementia (Ruitenberg et al., 2002; Lau et al., 2007; Joseph et al., 2007).

Other food-based antioxidants (such as vitamins C and E, b carotene, curcumin, and green tea) may modulate primary as well as secondary processes of aging by neutralizing free radicals. An imbalance between free radical production and anti-oxidant defense leads to an oxidative stress state, which may be involved in aging processes and even in some pathologies. Therefore, diet enrichment

with antioxidants may protect brain tissue and result in successful aging (Cabrera et al., 2006).

Immune-Inflammatory Responses and Oxidative Stress in Alzheimer's Disease: Therapeutic Implications

Increasing evidence has supported the possibility that neuroinflammation significantly contributes to the pathogenesis of AD. Knowledge about the roles of both microglia and astrocytes, and the action of proinflammatory mediators in neurodegenerative diseases, supports the hypothesis that neuroinflammation causes progression of AD pathology.

In particular, the production and secretion of proinflammatory mediators may interact with various mechanisms, with the initiation and progression of neurodegeneration.

Variations in immune-inflammatory genes such as cytokine, cyclo-oxygenase, lipo oxygenease, toll-like receptors have also been considered to be important in the risk for AD (Vasto S et al 2008).

In particular, cytokine gene polymorphisms have been claimed to play a key role in pathophysiology of AD, as demonstrated by two

recent meta-analysis performed by our group on IL-1β and TNF-α (Di Bona DI et al 2009; Di Bona DI et al 2008).

Therefore, we present data on a meta-analysis focused on IL-6. IL-6 is a pleiotropic cytokine involved in the regulation of the acute inflammatory response. Microglia, astrocytes, neurons, and endothelial cells seem capable of synthesizing IL-6. Although IL-6 overexpression is generally detrimental, there are evidences that it may have also trophic properties under restricted conditions and levels of exposure. For example, it has been suggested that IL-6 plays a key role in regulating neuronal survival and function and it may be essential for neuron and astrocyte differentiation and increased IL-6 levels in the brain have been implicated in early stages of plaque formation. Polymorphisms in the genes regulating the expression of IL-6 have been hypothesized to enhance the risk of developing AD. In particular, the most studied polymorphism of IL-6, the G/C polymorphism at the position -174 in the 5' flanking region of IL-6 gene, was shown to be associated with a decreased expression and also with a reduced plasma level of IL-6 (Di Bona DI et al 2008; Bradt BM et al 1998; Di Bona DI et al 2009).

The studies were selected and analysed according to criteria and methods discussed and in particular the putative risk genotype in

homozygosity (GG) was compared to the other two aggregated genotypes data (CG+CC), condensing the results into one statistic. The current meta-analysis summarizes the evidence regarding the association between IL-6 -174 SNP and AD, representing a pooled total of 3811 cases and 10716 controls. The analysis of pooled data ruled out the role of IL-6 -174 SNP in modifying AD risk that was hypothesized by some investigators. However, a remarkable heterogeneity was observed between the results of individual studies, suggesting differences among the enrolled populations, and a possible population specific genetic effect of the -174 IL-6 SNP. Also genetic or environmental factors may play a contributing role and may explain differences between the results of individual studies.

So, these data do not apparently support a role for this IL-6 SNP in pathophysiology of AD. However, it is to take into account that inflammatory mediators do not act alone but they act in a complex network reciprocally interacting (Vasto S et al 2007).

Association between the Polymorphisms of TLR4 and CD14 Genes and Alzheimer's Disease

Several membrane proteins expressed on microglial cells seem to be implicated in Aβ peptides binding. It has been demonstrated that CD14/TLR4 receptor complex binds highly hydrophobic Ab peptides aggregates suggesting that production of neurotoxic substances. This receptor complex is known to be involved in cellular activation by micro-organisms components, as lipopolysaccharide (LPS) or other highly hydrophobic and aggregate structures, or endogenous molecules produced by cell and DNA damage.

The key role of this receptor complex leads to biologically plausible hypothesis that functional variations in the TLR4 and CD14 genes might influence the susceptibility to sporadic AD.

Evidence discussed here indicates the involvement of genetic TLR4 variations in the attainment of longevity and in the susceptibility to these age-related diseases, since TLR4- mediated inflammation from bacterial and viral infection or other endogenous molecules can influence the development of these diseases. Given the complex inheritance patterns of these polygenetic traits, the impact of any single allele on the trait under study is obviously modest.

The Asp299Gly SNP might result in an increased chance of longevity in a modern environment with reduced pathogen load and improved control of severe infections by antibiotics. Finally, our data prompt consideration of the role that antagonistic pleiotropy plays in diseases and longevity (Capri M et al 2008; Licastro F et al 2005).

The search for a pro-inflammatory profile is of some importance for a pharmacogenomic approach, i.e. to detect and utilize a risk profile which allows early identification of individuals at risk dose for desired effects. Accordingly, carriers of high responder polymorphisms, affected by mild cognitive impairment might be selected for a clinical trial on antibiotic prophylaxis for the prevention of AD (EL,Gross H. 2008).

Pathophysiology of Vascular Dementia

Alzheimer's disease (AD) and vascular dementia are the most common forms of cognitive impairment in the elderly. The pathogenic mechanisms underlying these two conditions have traditionally been considered separate, even mutually exclusive (Erkinjuntti T, Gauthier S 2009).

Diagnostic criteria were drafted and widely applied, establishing AD as the predominant cause of senile cognitive impairment, a course of action aptly referred to as "alzheimerization" of dementia (Libon DJ et al 2006).

On the other hand, vascular dementia evolved from the concept of ''arteriosclerotic dementia'', in which hardening of cerebral arteries leads to diffuse ischemia and neuronal loss, to "multiinfarct dementia", caused by multiple infarcts resulting in cognitive impairment due to progressive brain loss (Hachinski VC et al 1974). In the early 1990s, the broader term "vascular cognitive impairment" (VCI) was introduced to encompass the wide spectrum of cognitive alterations associated with cerebrovascular pathologies, including more subtle deficits that would not fulfill AD criteria (Hachinski VC, Bowler JV 1993).

These observations, together with epidemiological studies indicating that AD and cerebrovascular diseases share the same risk factors, have revived the interest in the idea that vascular factors may play a role in the pathogenesis of AD (Petersen RC: 2000; Leblanc GG, et al 2006; Rockwood K, et al 2000).

Chapter 8

CONCLUSIONS

Alzheimer's disease (AD) is a progressive neurodegenerative disorder affecting aged people; AD prevalence is approximately 1% between 65 and 69 years and it is higher than 50% in individuals above 95 years. It is characterized by irreversible cognitive and physical deterioration.

With increasing life expectancy across the world, dementia is a rapidly growing socioeconomic and medical problem. The confirmatory diagnosis of AD is based on the recognition and quantification of senile plaques and neurofibrillary tangles, which are the hallmarks of the disease (Vasto S, et al 2008).

AD prevention is an important goal of ongoing research. Two objectives must be accomplished to make prevention feasible: i) individuals at high risk of AD need to be identified before the earliest symptoms become evident, by which time extensive neurodegeneration has already occurred and intervention to prevent the disease is likely to be less successful and ii) safe and effective interventions need to be developed that either reduce or slow the accumulation of AD

neuropathology or lead to a decrease in clinical expression of this pathology (Vasto S, et al 2007).

The aim of this thesis was to illustrate the role of immune-inflammatory response in AD and that the treatment might play a decisive role in preventing or significantly delaying the onset of the disease. So, the knowledge of the pathophysiology of the diseases is crucial for its therapeutic prevention. Accordingly to the data discussed in the present thesis, two lines of preventive therapeutics can be discussed, the first based on anti-inflammatory drugs, the second one on anti-oxidative properties.

Concerning anti-inflammatory drugs, patients who received non-steroidal anti-inflammatory drugs (NSAIDs) for a period of 2 years had lower AD incidence with relative risk of 0.2. The incidence of AD appears to be reduced in some post hoc studies, by about 13% for aspirin and 28% for other NSAIDs (in t' Veld BA, et al 2001; McGeer PL, et al 1996; Breitner JC. 2003; Etminan M, et al 2003).

On the other hand, recent clinical trials showed that use of naproxen or celecoxib did not improve cognitive function (Martin BK, Szekely C, Brandt J, Piantadosi S, Breitner JC, et al 2009).

This failure could be due to a number of factors, including the pharmacological characteristics of the drugs, their brain penetration

properties, and the dosing schedule. It has been claimed that the ADAPT study does not indicate that NSAIDs, if taken during adulthood and for an extended period, cannot prevent or delay the onset of dementia (Bregman N et al 2009).

Regarding molecules with anti-oxidant properties, a number of experimental and epidemiological studies have recently supported the beneficial effects of some commonly used natural products in preventing various pathologic conditions ranging from cardiovascular diseases to cancer.

According to free radical theory of aging, an elevation in reactive oxygen species (ROS) and reactive nitrogen species (RNS) damages neural membranes and induces oxidative and nitrosative stress. The increase in oxidative and nitrosative stress is accompanied by the concomitant decline in cognitive and motor performance in the elderly population, even in the absence of neurodegenerative diseases. Markedly increased rates of oxidative and nitrosative stress are the major factors associated with the pathogenesis of neurodegenerative diseases. Diet is a key environmental factor that affects the incidence of chronic neurodegenerative diseases.

Dietary supplementation with polyphenols, resveratrol, ginkgo biloba, curcumin, ferulic acid, carotenoids, flavonoids, and n-3 fatty

acids exerts beneficial effects not only through the scavenging of free radicals, but also by modulating signal transduction, gene expression, and restoring optimal neuronal communication. Collective evidence from studies suggests that these compounds have great potential to combat against normal human brain aging and age-related neurodegenerative diseases.

Spices and herbs often contain active phenolic substances endowed with potent antioxidative and chemopreventive properties and recently a series of papers focused on specific neuroprotective effects of some of those polyphenols derived from nutritional sources (Nakatani, N. 2000; Go´ mez-Pinilla. 2008).

Curcumin (1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione), a coloring agent and food additive commonly used in Indian culinary and traditional medical preparations from time immemorial, is extracted from the rhizome of Curcuma longa (Ammon HPT, Wahl MA 1991).

It is a polyphenolic substance that has the potential to inhibit lipid peroxidation and to effectively intercept and neutralize ROS (superoxide, peroxyl, hydroxyl radicals) and nitric oxide (NO)-based free radicals (nitric oxide and peroxynitrite) (Priyadarsini KI et al 1998; Martin-Aragon S et al 1997; Sreejayan A, Rao MN. 1997).

In this regard, curcumin has been demonstrated to be several times more potent than vitamin E. It is generally assumed that the phenol moiety is responsible for the antioxidant properties of any plant phenolic compound. Consequently, the free radical chemistry of curcumin has focused on its phenol ring. The possible involvement of the β-diketone moiety in the antioxidant action of curcumin has been considered and H-atom donation from the β-diketone moiety to a lipid alkyl or a lipid peroxyl radical has been reported as the potentially more important mechanism underlying its antioxidant action.

Of particular interest is the ability of curcumin to inhibit COX-1 and COX-2 enzymes and to reduce the activation of nuclear transcription factor NF-kB (Masuda T et al 1999; Jovanovic SV et al 2001; Ramos-Gomez M 2001; Singh S, Aggarwal BB.1995; Huang MT et al 1997).

Its anti-inflammatory properties and cancer-preventive activities have been consistently reported using in vitro and in vivo models of tumor initiation and promotion. In addition to its ability to scavenge carcinogenic free radicals, curcumin also interferes with cell growth through inhibition of protein kinases. Although the exact mechanisms by which curcumin promotes these effects remains to be elucidated, the electrophilic properties of this yellow pigment appear to be an essential

component underlying its pleiotropic biological activities (Abe Y et al 1999; Awasthi S et al 2000).

It has been then demonstrated the ability of curcumin to induce HO-1 in cultured hippocampal neurons. The results indicate that curcumin activates HO-1 and phase II enzymes expression in astrocytes and neurons, probably by activation of transcription factor Nrf2, and this activation is able to effort a significant cytoprotection in cultured neurons exposed to oxidative stress (Rajakrishnan V et al 1999).

The involvement of curcumin in restoring cellular homeostasis and rebalancing redox equilibrium suggests that it might be a useful adjunct also in the treatment of neurodegenerative illnesses characterized by inflammation, such as AD. This idea has been reinforced by epidemiological studies showing that, in India where this spice is widely used in daily diet, there is a reduced age-adjusted prevalence of AD (in patients between 70 and 79 years of age is 4.4-fold less than that of the United States) (Chandra V et al 2001).

Consistent with its possible use in neurodegenerative diseases, curcumin has been reported to decrease oxidative damage and amyloid deposition in a transgenic mouse model of Alzheimer's disease, and to reverse Aβ-induced cognitive deficits and neuropathology in rats (Lim GP et al 2001; Frautschy SA et al 2001).

Although clinical studies on cognitive impairment and specific berry supplementation have yet to be completed, a number of studies have indicated that inclusion of antioxidant-rich foods in the diet can improve cognitive functioning in humans. In elderly non-demented people, elevated dietary intake of flavonoid-rich foods was associated with better cognitive function (Shukitt-Hale B, et al 2009). Additionally, high flavonoid consumption was associated with attenuation of cognitive decline over a period of 10 years. Other studies have revealed that general dietary patterns, such as daily fruit and vegetable consumption and adherence to a 'Mediterranean diet' emphasizing vegetables, fruits, beans and nuts, can decrease the risk of developing dementia in aging humans (Scarmeas N, et al 2009).

Dietary supplementation with fruit or vegetable extracts decreases the age-enhanced vulnerability to oxidative stress and inflammation. Several studies have indicated that polyphenolic compounds found in fruits such as blueberries may exert their beneficial effects through signal transduction and neuronal communication. Collective evidence suggests that nutritional supplementation with polyphenolic compounds may exert therapeutic protection against age-related deficits and neurodegenerative diseases (Lau et al., 2007).

The use of anti-aging remedies along with physical activity stimulate the regeneration of neurons in the old brain, and boost the performance of mental and physical tasks. Collective evidence suggests that physicians already have ‘‘anti-aging’’ treatments at their disposal. However, the influence of such treatments on life span of humans has not been studied.

The increase in human life expectancy at birth in the second half of the last century is mostly caused by enhanced survival at old age. The use of neuroprotective and regenerative drugs is increasing in the elderly population of the Western world, and it is suggested that the use of medicines exerting anti-aging properties may contribute to an increase in human longevity.

Chapter 9

SOMMARIO E DISCUSSIONE

L'invecchiamento è un processo multifattoriale prodotto dell'interazione tra fattori genetici, ambientali e stile di vita. Nell'uomo, l'età avanzata è caratterizzata dall'insorgenza di alcune patologie che, benché non siano di per sé espressione dell'invecchiamento, sono però strettamente associate alla vecchiaia, in quanto il declino fisiologico dovuto alla progressione dell'età conduce inesorabilmente l'organismo ad una condizione di maggiore suscettibilità e vulnerabilità nei confronti delle malattie, con conseguente aumento della mortalità in maniera età-dipendente.

Nel corso del tempo molti studi sono stati indirizzati all'identificazione degli eventi biologici a cui potrebbe essere imputabile il deterioramento progressivo che si verifica in concomitanza con l'invecchiamento.

Gran parte delle caratteristiche fenotipiche osservate nel processo di invecchiamento sono frutto dell'insorgenza, con l'età, di uno stato pro-infiammatorio cronico di basso grado detto *"Inflamm-aging"*, in parte sotto controllo genetico: tale stato sembra essere la conseguenza della

continua stimolazione antigenica che continua oltre l'età riproduttiva e quindi largamente non prevista dall'evoluzione .

L'aumentato carico antigenico, oltre a determinare gli effetti sulla risposta immune specifica finisce per instaurare un'infiammazione cronica che contribuisce al deterioramento dei vari organi, diventando un grande fattore di rischio per tutte le malattie croniche tipiche dell'età avanzata, dal diabete alle malattie cardiovascolari o alle demenze.

Infatti, gli anziani che presentano livelli ematici più alti di una proteina di fase acuta, la PCR, sono più soggetti ad ammalarsi delle patologie infiammatorie croniche e hanno un maggior rischio di disabilità e morte.

Le conseguenze sistemiche determinano un pattern di modificazioni che va sotto il nome di "fragilità" (*chronic inflammatory disease or frailty*) e gli studi epidemiologici suggeriscono che l'infiammazione di modico grado osservabile nei processi d'invecchiamento promuova un profilo aterogeno e sia correlata alle malattie infiammatorie croniche tipiche dell'invecchiamento (Alzheimer, Diabete di tipo 2) e in ultima analisi a un aumentato rischio di morte.

All'interno di questo contesto, interazioni neuroendocrine, immunologiche e infiammatorie sono state chiamate in causa nella patogenesi della malattia di Alzheimer. L'invecchiamento cerebrale è

normalmente associato ad una infiammazione cronica di basso grado, quindi l'infiammazione ha un ruolo rilevante specialmente nei confronti dell'AD, che rappresenta una malattia progressiva e neurodegenerativa che nel mondo occidentale è la causa più comune di demenza senile.

Nell'AD, i mediatori dell'infiammazione sono prodotti dagli astrociti e dalle cellule microgliali e concorrono a sostenere l'ipotesi neuro-infiammatoria come causa della AD. La proteina beta amiloide, elemento patognomico della malattia, sembra essere indotta e incentivata dall'ambiente infiammatorio incrementando, a sua volta, i livelli della stessa infiammazione attraverso la generazione di specie reattive dell'ossigeno e dell'azoto, citochine pro-infiammatorie, proteine del complemento ed altri composti.

Gli studi riportati nella presente tesi sui polimorfismi implicati nella regolazione della produzione delle citochine hanno dimostrato come possedere un genotipo caratterizzato da un'altra produzione di citochine pro-infiammatorie (IL-1, IL-6, TNF-α) o da una bassa produzione di citochine anti-infiammatorie (IL-10) aumenti significativamente il rischio di ammalarsi di AD.

Inoltre, diverse prove sperimentali ipotizzano il coinvolgimento dei recettori dell'immunità innata nell'attivazione delle cellule microgliali.

Il complesso antigene CD14/TRL4 sembra essere maggiormente espresso nelle cellule microgliali causando così l'attivazione attraverso il legame del peptide Aβ. Il ruolo chiave di questo complesso di recettori porta all'ipotesi, plausibile dal punto di vista biologico, che le variazioni funzionali nei geni di TLR4 e CD14 possano influire sulla suscettibilità all'AD sporadico.

Poiché la variabilità genetica esercita una grande influenza nello sviluppo dei processi infiammatori, l'analisi di tali fattori nell'ambito della complessa fisiopatologia dell'Alzheimer, permette l'identificazione di un profilo di rischio utile nell'individuare i soggetti suscettibili alla malattia.

Questo ha indirizzato il nostro interesse verso lo studio dei geni immuno-infiammatori, in modo tale da poter effettuare un adeguato trattamento terapeutico in quei soggetti con un profilo genetico a rischio, permettendo una diagnosi precoce della malattia; il trattamento antinfiammatorio potrebbe avere un ruolo decisivo nel prevenire o nel ritardare notevolmente la manifestazione clinica della malattia.

Inoltre, le specie reattive dell'ossigeno (ROS) sono in grado di indurre profondi effetti sull'espressione genica e sono implicate nella patogenesi dell'AD e il danno ossidativo probabilmente è uno dei primi segni di disfunzione neuronale nella malattia. Lo stress ossidativo può

essere definito come uno squilibrio tra l'aumentata produzione di ROS e le capacità di riparazione delle cellule.

La produzione di ROS è un fenomeno che avviene normalmente nei tessuti come ad esempio durante la produzione di energia dai mitocondri, ma vi è un'aumentata produzione a causa di fenomeni eccitatori, ischemici oltre che ovviamente infiammatori. D'altronde, l'invecchiamento si accompagna ad un declino dei meccanismi di difesa cellulare verso gli effetti dei ROS. L'equilibrio finale può essere, inoltre, modificato dallo stile di vita, quale il consumo di vitamine, fumo di sigarette e l'esercizio fisico.

È noto che un regime alimentare ipocalorico, adeguatamente integrato con elementi nutritivi essenziali, prolunga la durata media della vita negli animali da laboratorio. Considerando lo stress ossidativo come una delle principali cause dell'invecchiamento e delle patologie neurodegenerative ad esso connesse, il beneficio apportato dalla restrizione calorica andrebbe collegato a un abbassamento dei livelli di stress ossidativo e a un aumento del potenziale metabolico. I risultati sperimentali confermano tale ipotesi.

Allo stesso modo anche sostanze dotate di proprietà antiossidanti o in grado di potenziare i sistemi endogeni di difesa dai radicali liberi

dovrebbero prevenire l'insorgenza di stati di demenza, inclusa la malattia di Alzheimer.

Anche nell'uomo è stata evidenziata una possibile correlazione tra fattori dietetici, invecchiamento cerebrale, disfunzioni cognitive e rischio di demenze.

In particolare una dieta ricca di acidi grassi e una contemporanea carenza di sostanze antiossidanti potrebbero aggravare e potenziare il danno ossidativo.

Al contrario, una corretta integrazione alimentare con vitamina C, vitamina E, beta-carotene e polifenoli (tutte sostanze in grado di neutralizzare l'azione dei radicali liberi) porterebbe a un miglioramento delle capacità cognitive.

Queste osservazioni portano a pensare che qualsiasi trattamento capace di ridurre o di rallentare il processo ossidativo si inserisce, in questo contesto, come un possibile approccio preventivo, più che terapeutico, al trattamento delle patologie neurodegenerative. Se si pensa che una dieta ricca in antiossidanti è probabilmente una dieta bilanciata, ricca in vegetali e fibre, potenzialmente utile anche nella prevenzione di malattie cardiovascolari, al di là degli specifici meccanismi e delle prove scientifiche di efficacia, costituisce un suggerimento di stile di vita sicuramente proponibile.

Si potrà così impostare una terapia ed una dieta appropriata, mirate alla prevenzione della malattia o a ritardarne anche di molti anni la manifestazione e di poter quindi godere di un maggior numero di anni di vita sana e indipendente.

Ancora molti studi devono essere effettuati in questo settore, non solo allo scopo di approfondire la comprensione dei meccanismi fini che regolano i processi biologici legati all'infiammazione nell'invecchiamento, ma anche e soprattutto per rendere disponibili all'uomo degli strumenti terapeutici e preventivi attendibili in un ambito di straordinaria importanza come quello della longevità umana.

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