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Inflammation and oxidative damage in Alzheimer's disease: friend or foe?

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1. ABSTRACT

The two major neuropathologic hallmarks of AD are extracellular Amyloid beta plaques and intracellular neurofibrillary tangles. A number of additional pathogenic mechanisms have been described, including inflammation and oxidative damage. Regarding inflammation, several cytokines and chemokines have been detected both immunohistochemically and in Cerebrospinal Fluid from patients. Some of them, including Tumor Necrosis Factor-alpha, Interferon-gamma-inducible Protein-10, Monocyte Chemotactic Protein-1 and Interleukin-8, are increased in AD and in Mild Cognitive Impairment, considered the prodromal stage of AD, suggesting that these modifications occur very early during the development of the disease, possibly explaining the failure of trials with anti-inflammatory agents in patients with severe AD. Further evidence suggests that cytokines and chemokines could play a role in other neurodegenerative disorders. These disorders are considered multifactorial diseases, and genetic factors influence pathological events and contribute to change the disease phenotype from patient to patient. Gene polymorphisms in crucial molecules, including cytokines, chemokines and molecules related to oxidative stress, may act as susceptibility factors, or may operate as regulatory factors, modulating the severity of pathogenic processes.

2. INTRODUCTION

Alzheimer's disease is the most common cause of dementia in the elderly, with a prevalence of 5% after 65 years of age, increasing to about 30% in people aged 85 years or older. It is characterized clinically by progressive cognitive impairment, including impaired judgment, decision-making and orientation, often accompanied, in later stages, by psychobehavioural disturbances as well as language impairment. Mutations in genes encoding for *APP*, *PSEN1* and *PSEN2* account for about 5% of cases, characterized by an early onset (before 65 years of age). So far, 32 different mutations, causing amino acid changes in putative sites for the cleavage of the protein, have been described in the *APP* gene in 89 families, together with 179 mutations in *PSEN1* and 14 in *PSEN2* (http://www.molgen.ua.ac.be/).

The two major neuropathologic hallmarks of AD are extracellular Abeta plaques and intracellular NFTs. The production of Abeta, which represents a crucial step in AD pathogenesis, is the result of cleavage of APP, that is overexpressed in AD (1). Abeta forms highly insoluble and proteolysis resistant fibrils known as SP. In contrast to the low-fibrillar Abeta plaques (diffuse plaques), highly fibrillar forms of Abeta plaques (dense-core plaques, showing all the classical properties of amyloid including

beta-sheet secondary structure) (2) are associated with glial and neuritic changes of the surrounding tissue (neuritic-plaques) (3). NFTs are composed of the tau protein. In healthy subjects, tau is a component of microtubules, which represent the internal support structures for the transport of nutrients, vesicles, mitochondria and chromosomes within the cell. Microtubules also stabilize growing axons, which are necessary for the development and growth of neurites (1). In AD, tau protein is abnormally hyperphosphorylated and forms insoluble fibrils, originating deposits within the cell.

A number of additional pathogenic mechanisms, possibly overlapping with Abeta plaques and NFTs formation, have been described, including inflammation, oxidative damage, iron dysregulation, cholesterol metabolism. In this review, mechanisms related to inflammation and oxidative stress will be discussed and treatments under development to interfere with these pathogenic steps presented.

3. PATHOGENESIS OF ALZHEIMER'S DISEASE: THE AMYLOID HYPOTHESIS

The APP plays a central role in AD pathogenesis and in AD research, as it is the precursor of Abeta, which is the heart of the amyloid cascade hypothesis of AD.

3.1. APP gene family

The human *APP* gene was first identified in 1987 by several laboratories independently. The two *APP* homologous, *APLP1* and *APLP2,* were discovered several years later. APP is a type I membrane protein. Two predicted cleavages, one in the extracellular domain (beta-secretase cleavage) and another in the transmembrane region (gamma-secretase cleavage) are necessary to release Abeta from the precursor protein. Notably, *APP* is located on chromosome 21, and this provided an immediate connection to the invariant development of AD pathology in trisomy 21 (Down's syndrome) individuals. The first mutations demonstrated to be causative of inherited forms of familial AD were identified in the *APP* gene (4), providing an evidence that APP plays a central role in AD pathogenesis. Importantly, only *APP* but not its homologous *APLP1* and *APLP2* contain sequences encoding the Abeta domain.

3.2. APP processing

Full-length APP undergoes sequential proteolytic processing. It is first cleaved by alpha-secretase (non-amyloidogenic pathway) or beta-secretase (amyloidogenic pathway) within the luminal domain, resulting in the shedding of nearly the entire ectodomain and generation of alpha- or beta-CTFs. The major neuronal beta-secretase, named BACE1 is a transmembrane aspartyl protease which cleaves APP within the ectodomain, generating the N-terminus of Abeta (5). Nevertheless, several zinc metalloproteinases such as TACE/ADAM17, ADAM9, ADAM10 and MDC-9, and the aspartyl protease BACE2, can cleave APP at the alpha-secretase site (6) located within the Abeta domain, thus precluding the generation of intact Abeta.

The second proteolytic event in APP processing involves intramembranous cleavage of alpha- and beta-CTFs by gamma-secretase, which liberates a 3kDa protein (p3) and Abeta peptide into the extracellular milieu. The minimal components of gamma-secretase include presenilin (PS)1 or PS2, nicastrin, APH-1 and PEN-2 (7). Protein subunits of the gamma-secretase assemble early during biogenesis and cooperatively mature as they leave the endoplasmic reticulum. Biochemical evidence is consistent with PSEN1 (or PSEN2) as the catalytic subunit of the gamma-secretase. APH-1 and PEN-2 are thought to stabilize the gamma-secretase complex, and nicastrin to mediate the recruitment of APP CTFs to the catalytic site of the gamma-secretase. Major sites of gamma-secretase cleavage correspond to positions 40 and 42 of Abeta.

Amyloidogenic processing is the favored pathway of APP metabolism in neurons, due to the greater abundance of BACE1, whereas non-amyloidogenic pathway predominates in other cell types.

It seems that none of the above mentioned secretases have unique substrate specificity towards APP. Besides APP, a number of other transmembrane proteins undergo ectodomain shedding by enzymes with alpha-secretase activity. Regarding BACE1, its low affinity for APP leads to the hypothesis that APP is not its sole physiological substrate. Similarly, PS1 and PS2 play a crucial role in intramembranous gamma-secretase cleavage of several type I membrane proteins other than APP, including Notch1 receptors and its ligands (8).

3.3. APP role

A number of functional domains have been mapped to the extra- and intracellular region of APP, including metal (copper and zinc) binding, extracellular matrix components (heparin, collagen and laminin), neurotrophic and adhesion domains. Thus far, a trophic role for APP has been suggested, as it stimulate neurite outgrowth in a variety of experimental settings. The N-terminal heparin-binding domain of APP also stimulates neurite outgrowth and promotes synaptogenesis. In addition, an "RHDS" motif near the extraluminal portion of APP likely promotes cell adhesion, possibly acting in an integrin-like manner. Similarly, APP colocalizes with integrins on the surface of axons at sites of adhesion (9,10).

Despite APP was initially proposed to act as a cell surface receptor, the evidence supporting this hypothesis has been unconvincing. Only recently, aside of from interactions with extracellular matrix proteins, a candidate ligand has been proposed. In was in fact reported that F-spondin, a neuronal secreted signaling glycoprotein that may function in neuronal development and repair, binds to the extracellular domain of APP as well as of APLP1 and APLP2 (11). This binding reduces beta-secretase cleavage of APP, suggesting therefore that F-spondin binding may regulate APP processing.

APP-deficient animals are a useful model to better understand the role of APP. Deficient APP mice did not show major phenotypic abnormalities (12). However,

APLP2$^{-/-}$/*APLP1*$^{-/-}$ and *APP*$^{-/-}$/*APLP2*$^{-/-}$ mutants, but not *APP*$^{-/-}$/*APLP1*$^{-/-}$ animals, showed early postnatal lethality, indicating that members of the APP gene family are essential genes, which exhibit partial overlapping functions. Deficiency of all the *APP* genes lead to death shortly after birth. The majority of animals studied showed cortical dysplasia suggestive of migrational abnormalities of the neuroblasts and partial loss of cortical Cajal Retzius cells (13). Taken together, these findings presented a convincing picture that members of the *APP* family play essential roles in the development of the nervous system related to synapse structure and function as well as in neuronal migration.

Given the trophic properties of APP, it would be natural to predict that overexpression of APP would lead to phenotypes related to the enhanced neurite outgrowth and cell growth, which in fact was demonstrated (14). However, convincing negative phenotypes, in which APP does not act as trophic factor, has been reported as well. For example, over-expression of APP in cells induced to differentiate into neurons lead to cell death (15). In transgenic mice, the overexpression of familial AD-associated APP mutations resulted in the development of Abeta deposition and Abeta associated changes in the brain, including loss of synaptic markers, thus confirming the pathogenic nature of these mutations. A detailed examination also showed axonal swellings and varicosities, which were observed months before any evidence of Abeta deposition (16). In this model, tau deposition occurs as a consequence of a deregulation of its phosphorylation induced by Abeta deposition (17).

4. TAU AND ALZHEIMER'S DISEASE

Tau is relatively abundant in neurons but is present in all nucleated cells and functions physiologically to bind microtubules and stabilize microtubule assembly for polymerization. Tau encoding gene (*MAPT*) consists of 16 exons. In the adult brain, alternative splicing of tau nuclear RNA transcribed on exons 2, 3, and 10, results in six tau isoforms, having either three or four peptide repeats of 31 or 32 residues in the C terminal region encoded on exon 10, comprising the microtubule binding domain or differing in the expression of zero, one or two inserts encoded on exon two and three. During neurodegeneration, tau is abnormally phosphorylated . The profile of alternative splicing differs among pathological phenotypes, such that tau accumulation in AD is a mixture of 3R and 4R tau, Pick disease tends to be 3R tau, corticobasal degeneration and progressive supranuclear palsy tends to be 4R tau, and so-called argyrophilic grain disease accumulates small inclusions comprised of 3R tau (18).

5. ROLE OF INFLAMMATION IN ALZHEIMER'S DISEASE

The identification of pathogenic molecules as well as potential biomarkers in AD can be theoretically achieved via two different strategies: the first strategy can be defined as "knowledge-based" (deductive method based on the current knowledge of AD pathophysiology), while the second one is more "unbiased" (inductive strategy). The "knowledge-based" approach relies on a direct understanding of the pathophysiological processes that underlie the development of AD. It consists of biochemical assays (ELISA) aiming to assess attractive novel candidate markers informed by the biology of the disease process. On the other hand, the "unbiased" approach involves the use of modern techniques including proteomics, peptidomics, metabolomics, and bioinformatics that have allowed unbiased investigations of putative markers that may be informative with regard to AD. Notably, the majority of inflammation and/or oxidative stress biomarkers in AD described in detail in the following sections have been thus far identified using a "knowledge-based" approach".

5.1. Cytokines

The fibrillar deposition of extracellular Abeta is closely associated with a neuroinflammatory response, which includes a local up-regulation of acute-phase proteins, complement fragments, cytokines and other inflammatory mediators (19). So far, epidemiological studies suggested that inflammatory processes play a role in the pathogenesis of AD. Prospective case-cohort studies showed that higher serum levels of certain acute-phase proteins are a risk factor for the development of AD (20-22). Moreover, epidemiological studies indicate that longstanding use of non-steroidal anti-inflammatory drugs can prevent or delay the development of AD (23).

Microglial cells are the major producers of inflammatory factors. During the early stages of AD pathogenesis, activated microglia were clustered within classic (dense-cored) plaques in the AD neocortex (24). These plaques showed strong immunostaining for complement factor C1q and serum amyloid P component (SAP). Plaque-associated factors C1q and SAP may trigger microglia to secrete high levels of proinflammatory cytokines (3).

Activated microglial cells colocalize with Abeta, and *in vitro* studies demonstrated that Abeta induces the production of TNFa in such cells (25). This cytokine is a pleiotropic factor acting as an important mediator of inflammatory responses in a variety of tissues. Levels of TNFa in CSF from AD patients are 25-fold higher than in CSF from age-matched controls (26), suggesting a role for inflammation in neurodegeneration. Nevertheless, other findings demonstrated a protective effect of TNFa, as it likely protects neurons against Abeta triggered citotoxicity (27). In APP23 transgenic mice overexpressing beta APP, deletion of the TNFR1, which contributes to apoptosis, leads to Abeta generation and plaque formation inhibition in the brain. Moreover, deletion of TNFR1 resulted in reduced beta-secretase levels and activity (28).

IL-1 exerts pleiotropic actions by binding to its receptor IL-1R and has been identified as a mediator in several forms of neurodegeneration. In AD, an increased production of IL-1 has been demonstrated by immunohistochemistry. In particular, it is expressed by microglia localized around amyloid deposits, possibly participating to plaque formation (29).

Conflicting results have been reported with regard to IL-6 levels in serum and CSF of AD patients. However, it has been shown that its mRNA levels are increased in the entorhinal cortex and the superior temporal gyrus of AD patients (29). Interestingly, previous studies demonstrated a large increase in endogenous IL-6 bioactivity in response to ischemia, as well as a marked neuroprotection produced by exogenous IL-6, thus suggesting that this cytokine is an important inhibitor of neuronal death during cerebral ischemia (30).

Additional cytokines of the IL-6 family are IL-11 and LIF (31). Interleukin-11 mean CSF levels were significantly increased in AD and FTLD (a neurodegenerative dementia causing behavioural and language dysfunctions) as compared with controls, whereas CSF LIF levels were not detectable either in patients or controls (32). In accordance with previous results (33), in AD patients, a significantly positive correlation between MMSE scores and IL-11 CSF concentration was observed (32).

In contrast with the previously described cytokines, TGFb has mainly an anti-inflammatory action. Several data show that its levels are increased in the brain of AD patients, as well as in plasma and CSF. Moreover, TGFb was also found both in amyloid plaques and tangles (21). TGFb1 drives the production of Abeta40/42 by astrocytes, leading to Abeta production in TGFb1 transgenic mice. Notably, TGFbeta1 induces the overexpression of the APP in astrocytes but not in neurons, involving a highly conserved TGFb1 responsive element in the 5'-UTR. In addition, TGFb1 potentiates Abeta production also in human astrocytes, possibly enhancing the formation of Abeta plaques in the brain of patients with AD (34).

As a general comment, microglial-produced "inflammatory" cytokines have neuropathic as well as neuroprotective actions. For instance, whereas excess levels of TNFa might cause neurotoxicity, low-dose TNFa could, alternatively, trigger the neuroprotective and/or anti-apoptotic genes (35). The role of glial cells is to support and sustain proper neuronal function and microglia are no exception to this general principle. In acutely injured CNS, microglia have a neuroprotective and pro-regenerative role (36). Therefore, the primary mode of action of microglia seems to be the protection of the central nervous systems. Nevertheless, upon excessive or sustained activation, microglia could significantly contribute to chronic neuropathologies, leading to neurotoxicity (3).

5.2. Chemokines

Chemokines are low molecular weight chemotactic cytokines that have been shown to play a crucial role in early inflammatory events. Based on the arrangement of cysteine residues, chemokines are divided into two main families: CXC or alpha-chemokines, i.e IP-10 and IL-8, responsible for attracting neutrophils, and CC or beta-chemokines, i.e MCP-1 and MIP-1a and b, which act basically on monocytes (37).

Upregulation of a number of chemokines has been associated with AD pathological changes (38). IP-10 immunoreactivity was markedly increased in reactive astrocytes in AD brains, as well as the level of its expression. Astrocytes positive for IP-10 were found to be associated with senile plaques and showed an apparently coordinated up-regulation of MIP-1b (39,40). Significant increased IP-10 levels were observed in CSF from patients with mild AD as compared with severe AD. Similarly to mildly impaired AD, IP-10 increased levels were also found in subjects with amnestic MCI (33). The deposition of Abetąknown to activate microglia to produce proinflammatory cytokines, may be responsible for the accumulation of IP-10 found in AD patients. In fact, the genomic organization of IP-10 gene shows the presence, in the promoter region, of critical regulatory sequences responding to IFNg and TNFa independently activated factors, which lead to the transcriptional activation of the gene. Therefore, it is conceivable that the increased IP-10 levels observed in mild AD patients could be linked to amyloid deposition (33). IP-10 increase is likely restricted to an early stage of AD, when proinflammatory events are thought to play a more important role in disease development. The same trend has been observed also considering MCP-1 and IL-8 levels, strengthening the hypothesis that intrathecal inflammation precedes the development of AD, and represents an initiating factor of the disease rather than a late consequence (33).

With regard to a possible use of chemokine to easily predict evolution from MCI to AD, few investigation in serum have been so far carried out, despite a growing body of evidence supporting the hypothesis that some peripheral biochemical modifications also occur very early during AD pathogenesis. For instance, serum MCP-1 levels have been demonstrated to be increased in MCI subjects (41). Conversely, IP-10 serum levels were not increased in AD patients, but were found to correlate with aging (42).

5.3. Oxidative damage

Oxidative stress is supposed to play a relevant role in the pathogenesis of several neurodegenerative diseases, including AD. Abeta and other lesion-associated proteins are a major source of ROS and other toxic radicals (43). Increasing evidence supports a role of oxidative stress and impaired energy metabolism in the pathogenesis of the disease: an increase in DNA, lipid and protein oxidation metabolites has been observed in blood as well as post-mortem brain samples from AD patients compared with healthy subjects (44). Free radicals are produced by mitochondria, as a side product, during the reduction of molecular oxygen. The production of radicals is thought to be higher in cerebral tissue, particularly vulnerable to free radical damage because of its low content of antioxidants, high content of polyunsaturated fatty acids in neuronal membranes and high oxygen requirements for its metabolic process (45). Further observations indicate reduced cerebral metabolism in AD (45) as well as reduced activities of specific mitochondrial enzyme complexes, such as cytochrome oxidase (46-48). Alterations in these key enzymes can favor the aberrant production of ROS. Intracellular oxidative balance is tightly regulated and, therefore, an upregulation of antioxidant compensatory mechanisms would be expected in AD. The induction of

Cu/Zn superoxide dismutase, catalase, GSHPx, GSSG-R, peroxiredoxins and a number of heat shock proteins (49) suggests that vulnerable neuronal cells mobilize antioxidant defense in the face of increased oxidative stress (43). On the other hand, the TAC (including glutathione, ascorbic acid, uric acid and bilirubin) was shown to be reduced by 24% in plasma samples from AD patients (50). A link between oxidative stress and hyperhomocysteinemia, which is a known risk factor for the development of AD (51), has been hypothesized, as Hcy influences DNA repair, promoting the accumulation of DNA damage caused by oxidative stress (52). Recent *in vitro* studies demonstrate that Hcy increases levels of thiobarbituric acid reactive substances, which represent an index of peroxidation, and decreases levels of total-trapping antioxidant potential in a model of rat hippocampus (53). High tHcy levels are at present considered one of the major risk factors for the development of AD as a strong, graded association between tHcy levels and the risk of dementia and AD has been demonstrated (51). In this regard, there are evidences that tHcy levels are increased in late onset AD (LOAD; disease onset >65 years), but not in early onset AD (EOAD; disease onset <65 years), suggesting an influence on this parameter of other pathological conditions, mainly vascular diseases, which often co-occur with LOAD (54).

Similarly to inflammation, emerging evidence indicates that oxidative damage to neuronal RNA and protein is an early event in AD pathogenesis (55). Oxidative imbalance is likely to be present in subjects with MCI. Both in MCI and in AD patients, plasma mean levels of non-enzymatic antioxidants and lower activity of antioxidant enzymes appeared to be lower than in controls, with no parallel induction of antioxidant enzymes (56). In this regard, it has been recently shown that subjects with MCI have plasma, urine and CSF levels of the isoprostane 8,12-iso-iPF2a -VI, which is a marker of *in vivo* lipid peroxidation, higher than healthy subjects (57). This evidence clearly indicates that oxidative imbalance and subsequent oxidative stress are early events in AD evolution, and are probably secondary to other mechanisms specific to AD but not present in other neurodegenerative diseases (58).

On the basis of these studies, suggesting that oxidative imbalance may help to understand whether MCI is a prodromal stage of AD and whether a common pathogenesis between AD and MCI occurs, ROS, tHcy, and TAC were evaluated in samples from patients with AD, MCI and VaD, compared with age-matched healthy subjects. Total Hcy levels were significantly increased in AD as well as in VaD patients compared with controls. Notably, tHcy levels slightly increased were found in MCI patients compared with controls. As regards ROS levels, no significant differences were shown between patients and controls. TAC was significantly lower in AD patients than in either healthy subjects or VaD patients. No correlation between ROS and TAC levels in each subject was observed (59). In conclusion, an alteration of some biochemical factors involved in oxidative stress occurs in AD patients. Both tHcy and TAC modifications seem to be early events in the pathogenesis of AD, whereas ROS levels appear to be correlated with age rather than with a specific dementing disorder. This consideration leads to the hypothesis that oxidative imbalance observed in AD is mainly due to a decreased TAC rather than to an increased production of ROS (59).

5.4. Role of innate immunity

As previously mentioned, activated microglia, which represents innate immune cells in the CNS, play a pivotal role in the development of AD, either clearing Abeta deposits by phagocytic activity or releasing cytotoxic substances and proinflammatory cytokines. TLRs are a family of pattern-recognition receptors in the innate immune system. Exogenous and endogenous TLR ligands activate microglia. In mouse models homozygous for a destructive mutation of TLR4, an increase in diffuse and fibrillar Abeta deposits in the brain was observed as compared with wild type mice (60). The Asp299Gly polymorphism of TLR4 gene has been associated with an attenuated receptor signaling and a blunted inflammatory response. The frequency of the minor 299Gly allele was significantly higher in healthy elderly people than in patients with AD (61), suggesting a protective role of the variant towards the development of AD. A subsequent study demonstrated that the +896A/G SNP is associated with AD, strengthening the hypothesis that systemic inflammation plays a role in AD (62).

Another factor likely implicated in AD is the RAGE, Administration of soluble (s)RAGE in mouse models of AD reduced the development of cerebral beta-amyloidosis. Given this evidence, plasma levels of sRAGE were studied in patients with AD and VaD compared with controls, demonstrating a specific decrease of sRAGE plasma levels in AD (63). Moreover, sRAGE levels were reduced in MCI, suggesting a potential use of sRAGE levels as early biomarker (64). sRAGE plasma levels are likely influenced by a polymorphism in PPAR-g gene. It has in fact been demonstrated that AD patients carrying the Pro/Ala genotype of the PPAR-g Pro12Ala polymorphism had significantly lower plasma sRAGE levels than patients with Pro/Pro genotype, despite this polymorphism was nor associated with the risk to develop AD (65).

6. GENETIC INFLAMMATORY RISK FACTORS FOR ALZHEIMER'S DISEASE

Genetic variability is a prominent characteristic of many neurodegenerative disorders. Risk genes are likely to be numerous, displaying intricate patterns of interaction with each other as well as with non-genetic variables, and - unlike classical Mendelian ("simplex") disorders - exhibit no simple mode of inheritance. Mainly due to this reason, the genetics of these diseases has been labeled "complex" (66). The ability to genetically map complex disorders has been facilitated by technological improvement in identifying and genotyping polymorphic DNA markers. The current trend is to use SNPs, the most frequent genetic variants. The search for susceptibility genes in complex diseases centers on two major techniques: linkage mapping and candidate gene approach. Linkage analysis attempts to identify a region (locus) of the chromosome or regions

(loci) in the genome associated with the disease or trait by identifying which alleles in the loci are segregating with the disease in families, whereas genetic association analysis examines whether affected individuals share the variant allele more often than control subjects do (67).

6.1. Apolipoprotein E

The gene mainly related to sporadic AD is the *APOE* (68) which is located in chromosome 19q13.32 and was initially identified by linkage analysis (69). The relationship between *APOE* and AD has been confirmed in more than 100 studies conducted in different populations. The gene has three different alleles: *APOE*2, APO*3* and *APOE*4*. The *APOE*4* allele is the variant associated with AD. Longitudinal studies in Caucasian populations have shown that carriers of one *APOE*4* allele have at least a two-fold increased risk for AD (70). The risk increases in homozygote for the *APOE*4* allele, and this allelic variant is also associated with an earlier onset of the disease. Interestingly, *APOE*4* likely interacts with other genes to increase the susceptibility to AD. In particular, an interaction with two polymorphisms in APH-1a and APH-1b, both subunits of the gamma-secretase complex, have been demonstrated (71). *APOE*4*, has also been analyzed to test whether it acts as a risk factor for sporadic FTLD. A number of studies suggested an association between FTLD and *APOE*4* allele (72-77). Other Authors however, did not replicate these data (78-80). Recent findings demonstrated an association between the *APOE*4* allele and FTLD in males, but not females (81), possibly explaining the discrepancies previously reported. An increased frequency of the *APOE*4* allele was described in patients with SD compared to those with FTD and PPA (79).

6.2. Cytokines

A large number of candidate genes studies have been performed in order to search a robust risk factor for the sporadic form of the disease. These studies were mainly focused on genes clearly involved in the pathogenesis of AD, such as genes encoding for inflammatory molecules or genes involved in the oxidative stress cascade, both considered major factors in AD pathology. One of the strongest evidence of the role played by genetic variants in inflammatory agents to increase the risk of AD involves the IL1 complex which is located on chromosome 2q14-21 and includes IL1a, IL1b, and Il-1Ra, all of which have significant polymorphisms found to be associated with AD in several case- controls studies carried out in different populations (82-84). Several polymorphisms in *IL-6* which is a potent inflammatory cytokine have also been investigated. The *IL6* gene is located on chromosome 7p21 and polymorphisms exist in the -174 promoter region and in the region of a VNTR which is located in the 3'untraslated region. Both of them have been found associated with AD in case-controls studies (85,86). Investigation of *TNFa* polymorphisms was initiated because genome screening had suggested a putative association of AD with a region on chromosome 6p21.3, which lies within 20 centimorgans of the *TNFα* gene. Furthermore, other polymorphisms located in the promoter region of *TNFα* have been associated with autoimmune and inflammatory diseases (87). α1-Antichymotrypsin (*ACT*) is an acute-phase reactant produced by activated astrocytes and is elevated in brains from patients affected with AD. The combined effect of *ACT* and *IL1beta* polymorphisms have been demonstrated by Licastro *et al.* (86).

As with TNFα, investigation, of the role of alpha-2macroglobulin (*A2M*) was initiated as a result of screening studies of the genome. In this case, linkage was found in the region of chromosome 1p, where *A2M* and its low-density lipoprotein receptor are located. Blacker *et al.* (88) tested for association of polymorphisms with AD showing a strong involvement of this gene in AD.

6.3. Chemokines

In addition, polymorphisms in chemokines have been investigated with regard to susceptibility of AD. In particular, MCP-1 and RANTES have been widely screened in different neurodegenerative diseases (89). The distribution of the *A-2518G* variant was determined in different AD populations with concordant results (90,91) showing no evidence for association of this variant in AD compared with controls. Moreover, a significant increase of MCP-1 serum levels in AD carrying at least one *G* polymorphic allele was found (91). Therefore, the *A-2518G* polymorphism does not seem to be a risk factor for the development of AD, but its presence correlates with higher levels of serum MCP-1.

RANTES promoter polymorphism *-403 A/G*, found to be associated with several autoimmune diseases, was examined in AD population, failing to find significant differences between patients and controls (89).

The genes *CCR2* and *CCR5*, encoding for the receptors of MCP-1 and RANTES, respectively, have been also screened for association with AD. The most promising variants involve a conservative change of a valine with an isoleucine at codon 64 of *CCR2* (*CCR2-64I*) and a 32-bp deletion in the coding region of *CCR5* (*CCR5Δ32*), which leads to the expression of a non functional receptor. A decreased frequency and an absence of homozygous for the polymorphism *CCR2-64I* were found in AD, thus suggesting a protective effect of the mutated allele on the occurrence of the disease (86); conversely, no different distribution of the *CCR5Δ32* deletion in patients compared with controls were shown (92,93).

CCL8/MCP-2 rs1133763 SNP was studied in both patients with AD and FTLD, but no differences with controls were found (94). A further chemokine recently tested for susceptibility with AD is IP-10. A mutation scanning of the gene coding region has been performed in a restricted number of AD patients searching for new variants. The analysis demonstrated the presence of two previously reported polymorphisms in exon 4 (*G/C* and *T/C*), which are in complete linkage disequilibrium, as well as a novel rare one in exon 2 (*C/T*). Subsequently, these SNPs have been tested in a wide case-control study, but no differences in haplotype frequencies were found (95).

6.4. Oxidative damage

Other genes under investigation are related to oxidative stress. In this regard, genes coding for the NOS complex have been screened. The common polymorphism consisting in a *T/C* transition (*T-786C*) in *NOS3,* previously reported to be associated with vascular pathologies, has been tested in AD, but no significant differences with controls were found (96). Nevertheless, the expression of *NOS3* in PBMC either from patients or controls seems to be influenced by the presence of the *C* allele, and is likely to be dose dependent, being mostly evident in homozygous for the polymorphic variant. The influence of the polymorphism on *NOS3* expression rate supports the hypothesis of a beneficial effect exerted in AD by contributing to lower oxidative damage (96).

An additional variant in *NOS3* gene has been extensively investigated in AD patients, although results are still controversial. It is a common polymorphism consisting in a single base change (*G894T*), which results in an aminoacidic substitution at position 298 of *NOS3* (Glu298Asp). Dahiyat *et al.* (97) determined the frequency of the Glu298Asp variant in a two-stage case-control study, showing that homozygous for the wild-type allele were more frequent in late onset AD. However, studies in other populations failed to replicate these results (98-101).

Recently Guidi *et al* (102) correlated this variant with tHcy levels in 97 patients and 23 controls, on the basis of a previous study from Brown *et al.* (103) who demonstrated, in two independent healthy populations, that subjects homozygous for the mutation tend to have higher tHcy concentrations compared with Glu/Asp and Glu/Glu subjects. The Glu/Glu genotype was correlated with higher levels of tHcy and its frequency was increased in AD patients (102). Thus, the mechanism by which this genotype contributes to increase the risk in developing AD could be mediated by an increase of tHcy levels.

However, *NOS-1* is the isoform most abundantly expressed in the brain. Genetic analyses demonstrated that the double mutant genotype of the synonymous *C276T* polymorphism in exon 29 of the *NOS1* gene represents a risk factor for the development of early onset AD (104), whereas the dinucleotide polymorphism in the 3'UTR of *NOS1* is not associated with AD (105). To date, the promoter region of *NOS1*, located approximately 200 kb upstream of this polymorphism, has not been investigated for susceptibility to AD. Due to this reason and to further explore a possible association of *NOS1* polymorphisms with AD, the distribution of a functional polymorphisms and a variable number of tandem repeats (VNTR) was analyzed in a case-control study, which tested 184 AD patients as well as 144 healthy subjects (106). The functional variant considered is located in exon 1c, which is one of the nine alternative first exons (named 1a-1i), resulting in *NOS1* transcripts with different 5'-untraslated regions (107). Three SNPs have been identified in exon 1c, but only the *G-84A* variant displays a functional effect, as the *A* allele decreases transcription levels by 30% in in-vitro models (108). Regarding exon 1f, a VNTR) polymorphism has been recently reported in its putative promoter region, termed *NOS1* Ex1f-VNTR. This VNTR is highly polymorphic and consists of different numbers of dinucleotides (B-Q), which, according to their bimodal distribution, have been dichotomized in short (B-J) and long (K-Q) alleles for association studies (109). Both Ex1c *G-84A* and Ex1f-VNTR are associated with psychosis and prefrontal functioning in patients with schizophrenia (108). Notably, both Ex1c and Ex1f transcripts are found in the hippocampus and the frontal cerebral cortex (109), i.e. brain regions implicated in the pathogenesis of schizophrenia as well as AD. The presence of the short (*S)* allele of *NOS1* Ex1f-VNTR resulted to be a risk factor for the development of AD (106). The effect is cumulative, as in *S/S* carriers the risk is doubled. Most interestingly, the effect of this allele is likely to be gender specific, as it was found in females only. In addition, the *S* allele was shown to interact with the APOE*4 allele both in males and females, increasing the risk to develop AD by more than 10 fold (106). Thus, *NOS1* seems to be a risk factor for AD, but only in female population. This could be explained by a possible interaction with other genes or with additional environmental factors present in females but not males.

6.5. Progranulin

An intriguing factor related with inflammatory processes is named *GRN*. It has attracted significant attention in the scientific community following the recent discoveries of mutations causative for FTLD with ubiquitin-immunoreactive neuronal inclusions (FTLD-U) (110,111). A number of pathogenic *GRN* variants are predicted to result in a premature termination codon, leading to the degradation of the mutant mRNA through the process of nonsense mediated decay, resulting in a null allele (110).

GRN is a multifunctional secreted growth factor encoded by *GRN* gene on chromosome 17q21. *GRN* encodes for a 593 amino acid glycoprotein containing 7.5 tandem repeats of 12 cysteinyl granulin motifs. Progranulin and the various granulin peptides derived by elastase cleavage are implicated in a range of biological functions (112). Progranulin is widely expressed in several tissues and has been implicated in development, wound repair, inflammation and tumorigenesis (113). It is highly expressed in neurons of the cerebral cortex, the hippocampus and the cerebellum but its role in the CNS has not been investigated extensively (114). Intriguingly, *GRN* expression has been found increased in activated microglia as well as in peripheral blood in AD suggesting a potential role in this pathology (110,115). Furthermore, several patients carrying mutations in *GRN* exhibited a clinical presentation indistinguishable from AD (116-118). Recently the same Authors investigated the genetic variability within the *GRN* locus in a Belgian population of AD patients and found *GRN* haplotypes associated with increased risk for AD (119). Progranulin was tested for association with AD in an Italian population but this study did not confirm the role of this gene as susceptibility factor for the disease (120). Nevertheless, rs5848 polymorphism was consistently associated with decreased *GRN* mRNA levels in the parietal lobe of patients with AD. This effect was observed also in PBMC from patients (120). *GRN*

variability was shown to contribute instead to sporadic FTLD in an Italian population (121).

6.6. Wide Genome Analyses

Several linkage studies have been performed, giving rise to additional candidate susceptibility loci at chromosomes 1, 4, 6, 9, 10, 12 and 19. In particular, promising loci have been found at chromosome 9 and 10 (122,123). Very recently, a wide genome analysis identified variants at CLU (which encodes clusterin or ApoJ) on chromosome 8 and PICALM in chromosome 11 associated with AD (124).Data on CLU were replicated in an independent study, which, in addition, demonstrated that CR1, encoding the complement component (3b/4b) receptor 1 and located on chromosome 1, is associated with AD (125).

7. ANTI-INFALMMATORY DRUGS IN ALZHEIMER'S DISEASE

A large body of epidemiologic evidence suggested that long-term use of NSAIDs protects against the development of AD (23,126). Nevertheless, prospective studies of Rofecoxib (127), Naproxen (128), diclofenac (129), celecoxib (130), dapsone (131), hydroxychloroquine (132), nimesulide (133) failed to slow progression of cognitive decline in patients with moderate AD. In contrast, Indomethacin may delay cognitive decline in this subset of patients, but gastrointestinal toxicity is treatment-limiting (134,135). Because of general concerns about lack of efficacy, gastrointestinal toxicity, myocardial infarction and stroke, NSAIDs are not considered to be viable treatment options for patients with AD.

A promising compound is Rosiglitazone (AVANDIA®), an anti-diabetic agent with anti-inflammatory properties, which was tested in two small clinical trials. Rosiglitazone treatment for 24 weeks resulted in a modest but significant improvement in cognition in non-ApoE*4 carriers, but no improvement and rather a decline in cognition in *e4* carriers was demonstrated (136). A phase III trial has recently been completed (http://clinicaltrials.gov), although results are not available yet.

8. CONCLUDING REMARKS

As demonstrated by data previously discussed, a number of factors implicated in inflammation and oxidative damage play a role in the pathogenesis of AD. The ongoing research on biomarkers of AD is becoming extremely complex, thus a systematic organization of data that may facilitate the online sharing of biomarker metadata among researchers is mandatory.

Importantly, inflammation is an early event in the pathogenesis of AD, thus explaining the failure of all drugs so far tested in mild to severe AD. Future therapeutic trials should be carried out as early as possible during the course of the disease, implying the need to identify more accurate tools for early diagnosis. In this regard, new research diagnostic criteria have been proposed in 2007 (137), introducing the use of CSF analysis, structural (CT scan, MR) and functional (PET, SPECT) imaging and genetics, together with classical neuropsychological testing, for early and specific diagnosis. Large-scale international controlled multicenter trials are engaged in phase III development of the core feasible imaging and CSF biomarkers candidates in AD (US, European, Australian, and Japanese AD Neuroimaging Initiative, and the German Dementia Network). If the validation of these new criteria will be achieved, they should be considered in the setting of future clinical trials to identify more homogeneous study groups.

Lastly, indicators useful as surrogate outcome measures (surrogate biomarkers) should be identified in order to: 1) have substitutes for clinical endpoints (i.e. neuropsychological testing) 2) have tools able to predict clinical benefit, or the opposite 3) demonstrate whether there are disease-modifying properties. So far, none among biomarkers proposed for early diagnosis has been validated as a surrogate marker for monitoring treatments.

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Abbreviations: AD**:** Alzheimer's disease; APP: Amyloid Precursors Protein; PSEN: presenilin; Abeta: Amyloid beta; NFTs: neurofibrillary tangles; SP: senile plaques; CTFs: C-terminal fragments; BACE1: beta site APP cleaving enzyme; MAPT: Microtubule Associating Protein Tau; TNFa: Tumor Necrosis Factor alpha; CSF: Cerebrospinal Fluid; TNFR1: TNF type 1 death receptor; IL: Interleuchin; LIF: Leukaemia Inhibitory Factor; MMSE: Mini Mental State Examination (MMSE); TGFb: Tumor Growth Factor; UTR: untranslated region; CNS: central nervous system; FTLD: Frontotemporal Lobar Degeneration; IP-10: Interferon-gamma-inducible Protein-10; MCP-1: Monocyte Chemoattractant Protein-1; MIP:1a and 1b: Macrophage Inflammatory Protein-1alpha and 1beta; MCI: Mild Cognitive Impairment; tHcy: total plasma homocysteine; ROS: Reactive Oxygen Species; GSHPx: glutathione peroxidase; GSSG-R: glutathione reductase; TAC: Total Antioxidant Capacity; VaD: Vascular Dementia; TLRs: Toll-like receptors; RAGE: receptor for advanced glycation end products; PPARg: peroxisome proliferator-activated receptor gamma; SNPs: Single Nucleotide Polymorphisms; APOE: Apolipoprotein E; VNTR: variable number of tandem repeats; IL1R: IL1 receptor antagonist; NOS: Nitric Oxide Synthase; PBMC: Peripheral Blood Mononuclear Cells; GRN: progranulin.

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