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REVIEW ARTICLE

The Search for Biomarkers in Alzheimer's Disease

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Abstract

BACKGROUND: As population demographic shift and the number of individuals with Alzheimer Disease (AD) continue to increase, the challenge is to develop targeted, effective treatments and our ability to recognize early symptoms. In view of this, the need for specific AD biomarker is crucial.

CONTENT: In recent years it has become evident that CSF concentrations of some brain – specific proteins are related to underlying disease pathogenesis and may therefore aid clinical investigation. Among several, we have focused on three candidates that have been suggested to fulfil the requirements for biomarkers of AD: β - amyloid 42 (Aβ42), total Tau (T-tau) and tau phosphorylated at various epitopes (P-tau). An increasing number of studies suggest that supplementary use of these CSF markers, preferably in combination, adds to the accuracy of an AD diagnosis.
More recently visinin – like protein (VLP-1), a marker for neuronal cell injury has been studied. CSF VLP-1 concentrations were 50% higher in AD patients than in the control population.

SUMMARY: The number of studies aimed at the identification of new biomarkers for AD is expected to increase rapidly, not only because of the increasing insights into the pathological mechanisms underlying this disease, but also because new therapies have been developed or are under consideration now, which warrant an early and specific diagnosis for effective treatment of the patients.

KEYWORDS: Dementia, Amyloid Plaque, Neurofibrillary Tangels, Amyloid β-peptide 42 (Aβ42), Total Tau (T-tau), Phosphorylated Tau (P-tau), visinin–like protein 1 (VLP-1).

Introduction

Alzheimer's disease is a progressive and fatal neurodegenerative disorder manifested by cognitive and memory deterioration, progressive impairment of activities of daily living, and a variety of neuropsychiatric symptoms and behavioral disturbances (1).

According to the World Health Organization, an estimated 37 million people worldwide currently have dementia; Alzheimer disease affects about 18 million of them (2). Increasing age is the greatest risk factor for Alzheimer disease. Its prevalence approximately doubles every five years after the age of 60—one in 10 individuals over 65 years and nearly half of those over 85 are affected by the disease. So, although the incidence rate of Alzheimer disease is not thought to be changing, Alzheimer disease poses one of the greatest threats to the future of healthcare systems, owing to the anticipated demographic shift to an aging population—the number of people worldwide above the age of 60 years is expected to double over the next 25 years (3).

Alois Alzheimer first described plaques and tangles that characterize the diseased brain nearly 100 years ago. The dense tangles are a feature in many different dementias, but amyloid plaques in the brain are unique to Alzheimer

disease (4). Thus the major hallmarks of Alzheimer disease are amyloid-β (Aβ)-containing plaques, tau containing neurofibrillary tangles (NFTs) and progressive neuronal loss accompanied by cognitive decline. Although plaques and NFTs are pathognomic, it would be misleading to create the impression that these are the only significant pathological changes occurring in the AD brain. In fact, numerous other structural and functional alterations ensue, including inflammatory responses and oxidative stress (6-8). The combined consequences of all the pathological changes, including the effects of the Aβ and tau pathologies, is severe neuronal and synaptic dysfunction and loss; at the time of death, the brain of a patient with AD may weigh one-third less than the brain of an age-matched, non-demented individual (9).

These figures underscore the urgency of seeking more effective therapeutic interventions for patients with Alzheimer's disease (1). Treatment requires accurate diagnosis and increasingly is based on an understanding of the pathophysiology of the disease (1).

The diagnosis of Alzheimer's disease is most often based on the criteria developed by the National Institute of Neurologic and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association (NINCDS–ADRDA) (10), according to which the diagnosis is classified as definite (clinical diagnosis with histologic confirmation), probable (typical clinical syndrome without histologic confirmation), or possible (atypical clinical features but no alternative diagnosis apparent; no histologic confirmation). Typical sensitivity and specificity values for the diagnosis of probable Alzheimer's disease with the use of these criteria are 65% and 75%, respectively (1,11).

Definitive diagnosis of Alzheimer disease can only be performed by examining the neuropathological features of the disease—amyloid plaques and neurofibrillary tangles—at autopsy. Nevertheless, in the day-to-day clinical setting, a variety of methods are used, and research has suggested that this can be considered 87% effective compared with autopsy. Early diagnosis is beneficial for the patients, as they can be treated early and any comorbidities can be monitored, as well as for their families, who can receive additional support (3,12).

Recent research on CSF biomarkers has focused on early diagnosis, and several studies have shown a high predictive value for identification of prodromal Alzheimer's disease in mild cognitive impairment (MCI) (13). A large study with extensive clinical follow-up that assessed the ability of

CSF biomarkers to predict incipient Alzheimer's disease in MCI cases reported a sensitivity of 95% at a specificity of 83–87% for different combinations of biomarkers (14).

Epidemiology and Risk Factors

Alzheimer's disease is the most common form of dementia, accounting for 50–60% of all cases. The prevalence of dementia is below 1% in individuals aged 60–64 years, but shows an almost exponential increase with age, so that in people aged 85 years or older the prevalence is between 24% and 33% in the Western world. Representative data from developing countries are sparse, but about 60% of patients with dementia are estimated to live in this part of the world. Alzheimer's disease is very common and thus is a major public health problem. In 2001, more than 24 million people had dementia, a number that is expected to double every 20 years up to 81 million in 2040 because of the anticipated increase in life expectancy. (15).

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 (16,17). 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 (16). Moreover, several epidemiological studies have shown that head injury could be a risk factor (18). Whether brain trauma initiates the pathogenic cascade leading to plaque and tangle formation or whether it simply reduces the brain reserve capacity is unclear (33).

Other risk factors are associated with vascular disease, including hypercholesterolaemia, hypertension, atherosclerosis, coronary heart disease, smoking, obesity, and diabetes (16). 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. Some evidence suggests that dietary intake of homocysteine-related vitamins (vitamin B12 and folate); antioxidants, such as vitamin C and E; unsaturated fatty acids; and also moderate alcohol intake, especially wine, could reduce the risk of Alzheimer's disease (19), but data so far are not conclusive to enable any general dietary recommendations to be made. 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% (20).

Familial Alzheimer's disease is an autosomal dominant disorder with onset before age 65 years. The first mutation causing the familial form of the disease was identified in the amyloid precursor protein (*APP*) gene on chromosome 21 (21). When investigating other families with the familial disease, several additional *APP* mutations were found. However, these mutations explain only a few familial cases. Instead, mutations in the highly homologous presenilin 1 (*PSEN1*) and presenilin 2 (*PSEN2*) genes account for most cases of familial disease (22,23). However, the familial form of the disease is rare, with a prevalence below 0.1% (24).

In 1993, two groups independently reported an association between the apolipoprotein E (*APOE*) ε4 allele and Alzheimer's disease (25,26). Meta-analysis shows that the *APOE* ε4 allele increases the risk of the disease by three times in heterozygotes and by 15 times in homozygotes (27). The *APOE* ε4 allele operates mainly by modifying age of onset (28), with each allele copy lowering the age at onset by almost 10 years (25). The molecular mechanism for the disease-promoting effect has been difficult to pinpoint. ApoE acts as a cholesterol transporter in the brain with ApoE4 being less efficient than the other variants in reuse of membrane lipids and neuronal repair (29). On the other hand, ApoE is essential for amyloid β (Aβ) deposition, promoting Aβ fibrillisation and plaque formation (30) possibly by acting as a pathological chaperone. The gene-dose dependent reduction in CSF Aβ42 could be associated with this process (31). The *APOE* ε4 allele has been calculated to account for most of the genetic risk in sporadic Alzheimer's disease (32).

Molecular Pathogenesis

Slowly but surely, Alzheimer's disease (AD) patients lose their memory and their cognitive abilities, and even their personalities may change dramatically. These changes are due to the progressive dysfunction and death of nerve cells that are responsible for the storage and processing of information. Although drugs can temporarily improve memory, at present there are no treatments that can stop or reverse the inexorable neurodegenerative process. But rapid progress towards understanding the cellular and molecular alterations that are responsible for the neuron's demise may soon help in developing effective preventative and therapeutic strategies (34).

Alzheimer's disease is the most common cause of dementia in the elderly. Extracellular amyloid plaques and intracellular neurofibrillary tangles are defining lesions in AD (33,35). Mounting genetic and biochemical data support the hypothesis that amyloid-β (Aβ) accumulation and aggregation in the brain are early and central events in the pathogenesis of AD (33,36). Aβ is derived from sequential proteolytic processing of amyloid precursor protein (*APP*) by β- and γ-secretases. Mutations associated with early-onset familial AD (FAD) are dominantly inherited and are found in the APP gene itself or in the presenilin 1 (*PSEN1*) and *PSEN2* genes, the products of which, together with nicastrin, APH1 and PSENEN2, are essential components of a protein complex that is responsible for γ-secretase activity (37). A common feature of most FAD mutations is that they increase the generation of Aβ peptides or increase the proportion of the longer Aβ42 form, which has a higher tendency to aggregate and is more toxic than the shorter Aβ40 form (36). Because γ-secretase cleavage of a number of substrates is important for synaptic function and neuronal survival, a loss-of-function hypothesis for PSEN mutations in AD pathogenesis has also been proposed (38).

One hundred years after Alois Alzheimer's description of the plaques and tangles in the first reported case of Alzheimer disease, we have looked at the proteins that make up these deposits as pathologies and have not extensively investigated their physiologic roles. Perhaps we should consider the possibility that Aβ has a function that relates directly to its involvement in vascular pathology (39). We know, for example, that APP is involved in blood clotting (40) and that Aβ drains from the brain along the walls of the microvasculature (41). Perhaps we should consider the possibility that Aβ has complementary damage-response roles: (i) as an emergency sealant of the vasculature during hemorrhage and (ii) as a neuronal depressant (42).

The aggregates of amyloid β-peptide (Aβ) in the brain parenchyma (amyloid plaques) also in the walls of small brain arteries, leading to cerebral amyloid angiopathy (CAA). The degree of amyloid deposition ranges from a thin ring of amyloid in the vessel wall to large plaque-like extrusions into the brain parenchyma. CAA is also associated with local loss of neurons, synaptic abnormalities, microglial activation and microhaemorrhage. Clearly, such defects will alter neuronal and synaptic function and even at its earliest stage, amyloid deposits around brain vessels could certainly interfere with the dynamic adaptation of cerebral blood flow (CBF) to changing brain function (23).

Bell *et al*. provide a molecular mechanism that could explain how vascular defects may lead to reduced amyloid

clearance, and thus Alzheimer's pathology, by showing that hypoxia in vascular smooth muscle cells (VSMCs) of meningeal arterioles induces transcription factors that regulate the expression of the low-density lipoprotein receptor related protein 1 (44), a major efflux transporter for Aβ across the blood brain barrier (45).

Alterations in the microcirculation precede the appearance of amyloid plaque deposits and is followed by cognitive deficits (46), indicating that an excess of Aβ could lead to CAA through direct perturbation of amyloid clearance by VSMCs. The study by Bell *et al*.(44) provides a molecular model that explains how a general and quite common circulatory problem may lead to failure of an essential brain detoxification process (that is, the removal of Aβ from the brain. Their findings also strengthen the vascular hypothesis of AD, showing how vascular defects may underlie the occurrence of sporadic AD (47-49).

Cerebrovascular disease and Alzheimer disease are common diseases of aging and frequently coexist in the same brain. Accumulating evidence suggests that the presence of brain infarction, including silent infarction, influences the course of Alzheimer disease. Conversely, there is evidence that β-amyloid can impair blood vessel function. Vascular β-amyloid deposition, also known as CAA, is associated with vascular dysfunction in animal and human studies. Alzheimer disease is associated with morphological changes in capillary networks, and soluble β-amyloid produces abnormal vascular responses to physiological and pharmacological stimuli (50).

APP Processing and Aβ Generation

Brain regions involved in learning and memory processes, including the temporal and frontal lobes, are reduced in size in AD patients as the result of degeneration of synapses and death of neurons. Central to the disease is altered proteolytic processing of the amyloid precursor protein (APP) resulting in the production and aggregation of neurotoxic forms of Aβ. Neurons that degenerate in AD exhibit increased oxidative damage, impaired energy metabolism and perturbed cellular calcium homeostasis; Aβ appears to be an important instigator of these abnormalities (34).

APP is an integral membrane protein with a single membrane-spanning domain, a large extracellular glycosylated N terminus and a shorter cytoplasmic C terminus—Aβ is located at the cell surface (or on the lumenal side of ER and Golgi membranes), with part of the peptide embedded in the membrane.

The normal functions of APP are not fully understood, but increasing evidence suggests it has important roles in regulating neuronal survival, neurite outgrowth, synaptic plasticity and cell adhesion (51). APP is transported along axons to presynaptic terminals where it accumulates at relatively high levels, which can result in Aβ deposition at synapses. One possible function of full-length APP is as a cell surface receptor that transduces signals within the cell in response to an extracellular ligand (52). Physiological roles for sAPPα are supported by data showing that sAPPα is released from presynaptic terminals in response to electrical activity, and that sAPPα regulates neuronal excitability and enhances synaptic plasticity and learning and memory, possibly by activating a cell surface receptor that modulates the activity of potassium channels and also activates the transcription factor NF-kβ (53).

Synapses may be particularly susceptible to the adverse effects of aggregating forms of Aβ, as is suggested by the ability of Aβ to impair synaptic ion and glucose transporters and by electrophysiological studies showing that Aβ impairs synaptic plasticity (51,54). Aβ may damage neurons by inducing oxidative stress and disrupting cellular calcium homeostasis (51). Coincident with the increased production of Aβ in AD is a decrease in the amount of sAPPα produced, which may contribute to the demise of neurons because sAPPα is known to increase the resistance of neurons to oxidative and metabolic insults (51)

Synapses are likely to be the sites at which neuronal death is initiated in AD because they contain most of the biochemical machinery for the initiation and execution of apoptosis, and Aβ can induce apoptotic cascades in synapses (55).

The amyloid-β (Aβ) peptide is derived via proteolysis from a larger precursor molecule called the amyloid precursor protein (APP), a type 1 transmembrane protein consisting of 695–770 amino acids. APP can undergo proteolytic processing by one of two pathways. Most is processed through the nonamyloidogenic pathway, which precludes Aβ formation. The first enzymatic cleavage is mediated by α-secretase, of which three putative candidates belonging to the family of a disintegrin and metalloprotease (ADAM) have been identified: ADAM9, ADAM10 and ADAM17. Cleavage by α-secretase occurs within the Aβ domain, thereby preventing the generation and release of the Aβ peptide. Two fragments are released, the larger ectodomain (sAPPα) and the smaller carboxy-terminal fragment (C83). Furthermore, C83 can also undergo an additional cleavage mediated by γ-secretase to generate P3. APP molecules that are not cleaved by the non-amyloidogenic pathway become a substrate for β-secretase (β-site APP-cleaving

enzyme 1; BACE1), releasing an ectodomain (sAPPβ), and retaining the last 99 amino acids of APP (known as C99) within the membrane. The first amino acid of C99 is the first amino acid of Aβ. C99 is subsequently cleaved 38–43 amino acids from the amino terminus to release Aβ, by the γ-secretase complex, which is made up of presenilin 1 or 2, nicastrin, anterior pharynx defective and presenilin enhancer 2. This cleavage predominantly produces Aβ1–40, and the more amyloidogenic Aβ1–42 at a ratio of 10:1 (9,56,57).

The toxicity associated with accumulation of Aβ suggests that activation of endoproteolytic enzymes capable of preventing generation of Aβ might provide a realistic target for pharmacotherapy of Alzheimer disease. On the other hand, cleavage of APP by β- and γ-secretase generates Aβ peptides. β-secretase, which initiates cleavage of APP, cuts the protein at the N terminus and has been successfully cloned. γ-secretase is the second enzyme that cleaves APP, and full understanding of its mechanism of action has long been lacking. Reconstitution of γ-secretase activity illuminates the interaction between the various protein components of the γ-secretase complex that leads to formation of Aβ (58).

Either presenilin-1 (PS1) or presenilin-2 (PS2) makes up the first component of the γ-secretase complex. Mutations in the genes that encode PS1 and PS2 cause a subset of early-onset, familial Alzheimer disease. Presenilin mutations probably act upstream of APP or tau to cause Alzheimer disease pathology, including deposition of Aβ and accumulation of hyperphosphorylated tau. For example, mutant presenilins have been shown to increase formation of the longer Aβ species, Aβ42. This species is important for Alzheimer disease pathology because it accelerates deposition of Aβ, which presumably precipitates early-onset Alzheimer disease. Thus, disease-related mutations in presenilin are considered to shift cleavage of APP by γ-secretase toward increased Aβ42 production (53).

In fact, presenilin is a part of a large, high-molecular-weight complex with γ-secretase activity. Nicastrin, the product of a recently cloned gene, is a component of this γ-secretase complex (59). Overexpressing the two genes together, however, does not ramp up γ-secretase activity (60). Instead, other proteins are required. Studies of Notch signaling in C. elegans, which depends on γ-secretase activity (61), provide additional information about γ-secretase activity. Aph-1 and PEN-2 are two

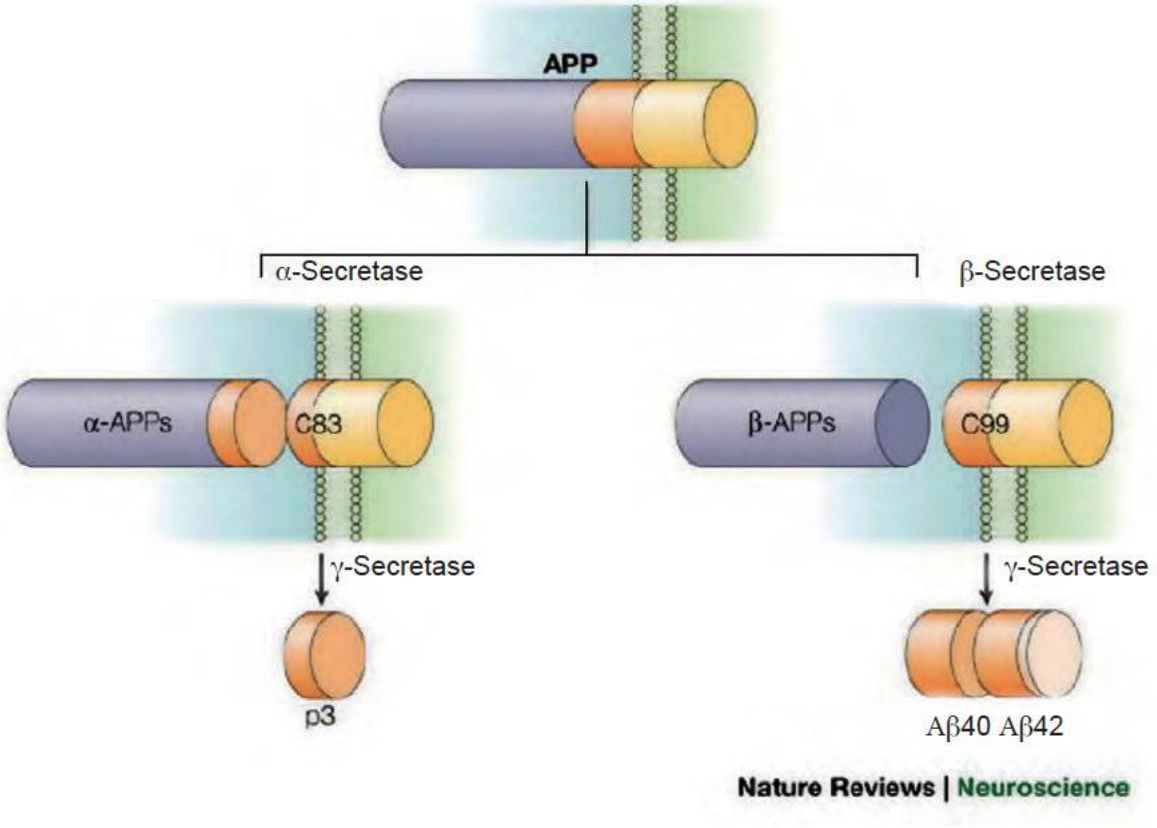

Figure 1. Amyloid – precursor protein (APP) and its metabolites (Adapted with permission from Nature Publishing Groups).

transmembrane proteins that are expressed upstream of release of the Notch intracellular domain (62,63). When these proteins are knocked down, γ-secretase activity decreases, which suggests that these two proteins may also be involved in γ-secretase activity (62,64).

The results described above indicate that four proteins (presenilin, nicastrin, Aph-1 and PEN-2) are required for γ-secretase activity, but none of them generate γ-secretase activity on their own; an increase in activity is only possible when the four proteins are overexpressed together (58,64). Thus, it is clear that these four proteins are essential components needed for γ-secretase activity (65).

Fahihi *et al*. (66) report that expression of the noncoding antisense RNA for BACE1—which is the rate limiting enzyme in Aβ synthesis—is elevated in the brains of individuals with Alzheimer's disease. *BACE1* antisense RNA increases Aβ production by stabilizing the *BACE1* mRNA and results in increased BACE1 protein expression and activity. Aβ in turn subtly induces the expression of this antisense RNA. *In vitro*, at least, this induction sets up a feed forward mechanism, which reiteratively accelerates Aβ production and then BACE1 expression. If the same holds true in vivo, this feed-forward could theoretically cause an ever accelerating tempo of disease (67).

Tau Phosphorylation and NFT

The accumulation of proteinacious fibrillary substances (such as senile plaques (SPs) made of β-amyloid (Aβ), or neurofibrillary tangles (NFTs) made of tau), but significant circumstantial evidence also clearly implicates these aggregates in the onset and progression of most aging-related neurodegenerative disorders that manifest clinically with progressive cognitive and/or motor impairments. In the case of neurodegenerative tauopathies — a group of disorders that includes Alzheimer's disease (AD) and the frontotemporal dementias (FTDs) — NFTs consisting of aggregated straight or paired helical filaments (SFs and PHFs, respectively), twisted ribbons or other conformations (68) of aberrantly phosphorylated forms of the microtubule-associated protein (MAP) tau are the diagnostic hallmark lesions in the CNS (69). It is increasingly evident that tau-mediated neurodegeneration may result from the combination of toxic gains-of-function acquired by the aggregates or their precursors and the detrimental effects that arise from the loss of the normal function(s) of tau in the disease state.

The primary function of the MAP tau, which is particularly abundant in the axons of neurons, is to stabilize MTs. There are six major isoforms of tau expressed in the adult human brain, all of which are derived from a single gene by alternative splicing. From a structural stand-point, tau is characterized by the presence of a MT-binding domain, which is composed of repeats of a highly conserved tubulin binding motif (70) and which comprises the carboxyterminal (C-terminal) half of the protein, followed by a basic proline-rich region and an acidic amino-terminal (N-terminal) region, which is normally referred to as the 'projection domain'.

Interestingly, although the primary function of the MT-binding domain of tau is the stabilization of MTs, various lines of investigation have indicated that it may also engage with other structures and enzymes, including RNA (71) and presenilin 1 (PS1) (72). Similarly, numerous possible binding partners have been proposed for both the proline-rich and the projection domains (the SH3 domains of src-family tyrosine kinases such as FYN, and the plasma membrane (74,75), respectively). Collectively these findings support the notion that tau might be a rather promiscuous binder that is prone to heterogeneous interactions — particularly when disengaged from the MT — which may lead to protein misfolding and aggregation (76).

Under pathological conditions, the equilibrium of tau binding to the MTs is perturbed, resulting in an abnormal increase in the levels of the free (unbound) tau fraction. It is likely that the resultant higher cytosolic concentrations of tau increase the chances of pathogenic conformational changes that in turn lead to the aggregation and fibrillization of tau (76).

Under physiological conditions, single tau molecules are typically phosphorylatedat a subset of potential phosphate-acceptor amino-acid residues. During late stage neurodegeneration, the phosphorylation state of a single tau molecule can reach such high levels that many or most of these residues are phosphorylated and, at the same time, a higher proportion of tau molecules are in this hyperphosphorylated state. Although several kinases have been found to be capable of phosphorylating tau *in vitro*, it is not yet clear whether all of them participate in tau phosphorylation under physiological or pathological conditions *in vivo1*. Nonetheless, glycogen synthase kinase 3 (GSK3), cyclin-dependent kinase 5 (CDK5) and the microtubule-affinity-regulating kinase (MARK) have received particular attention as potential targets for disease-modifying therapies using inhibitory compounds (77).

The overall effect of the increased rate and/or state of phosphorylation appears to be the abnormal disengagement of tau from the MTs. Furthermore, it is likely that various other pathological events, including Aβ-mediated toxicity, as well as oxidative stress and inflammation, may be able to trigger or contribute (independently or in combination) to an abnormal detachment of tau from the MTs (78-81). As described above, in AD and related neurodegenerative disorders that are collectively referred to as tauopathies (82,83), tau no longer binds to the MTs; instead it becomes sequestered into NFTs in neurons, and into glial tangles in astrocytes or oligodendroglia (69).

The discovery that the total level of NFTs correlates with the degree of cognitive impairment (85,86) provided the initial circumstantial evidence to suggest that toxic gains-of-function by NFTs might play an important part in then progression of the disease.

Mitochondrial Dysfunction and Oxidative Stress

Many lines of evidence suggest that mitochondria have a central role in aging-related neurodegenerative diseases. Mitochondria are critical regulators of cell death, a key feature of neurodegeneration. Mutations in mitochondrial DNA and oxidative stress both contribute to aging, which is the greatest risk factor for neurodegenerative diseases. In all major examples of these diseases there is strong evidence that mitochondrial dysfunction occurs early and acts causally in disease pathogenesis (87).

There is extensive literature supporting a role for mitochondrial dysfunction and oxidative damage in the pathogenesis of AD. Oxidative damage occurs early in the AD brain, before the onset of significant plaque pathology (88). Oxidative damage also precedes Aβ deposition in transgenic APP mice (89), with upregulation of genes relating to mitochondrial metabolism and apoptosis occurring even earlier and co-localizing the neurons undergoing oxidative damage (90). Moreover, such oxidative damage and mitochondrial dysfunction probably contribute causally to AD-related pathology.

Several pathways connecting oxidative stress and AD pathology have recently been uncovered. Oxidative stress may activate signaling pathways that alter APP or tau processing. For example, oxidative stress increases the expression of β-secretase through activation of c-Jun amino-terminal kinase and p38 mitogen-activated protein kinase (MAPK) (91), and increases aberrant tau phosphorylation by activation of glycogen synthase kinase 3 (92). Oxidant-induced inactivation of critical molecules may also be important. In a proteomic study, the prolyl isomerase PIN1 was found to be particularly sensitive to oxidative damage (93). PIN1 catalyses protein conformational changes that affect both APP and tau processing.

Functional complexes with γ-secretase activity, which is essential to cleave APP and create amyloid-β, have been found in mitochondria (94). Insulin degrading enzyme (IDE), which is important for amyloid-β removal, can be targeted to mitochondria by alternative translation initiation (95). The presequence peptidase PreP, which is localized to the mitochondrial matrix and is responsible for degrading presequences and other short peptides, can also degrade amyloid-β (96).

The calcium overload of mitochondria results in the opening of the mitochondrial permeability transition pore (mPTP) (97), a large channel in the inner mitochondrial membrane. Its opening allows uncontrolled bidirectional passage of large molecules, which results in the functional and structural disintegration of mitochondria – akin to an activation of a natural self destruction facility built into the complicated mitochondrial fabric (98).

Park *et al* report that NADPH oxidase, the major source of free radicals in blood vessels, is responsible for the cerebrovascular dysregulation induced by Aβ. Thus, the free-radical production and the associated alterations in vasoregulation induced by Aβ are abrogated by the NADPH oxidase peptide inhibitor gp91ds-tat and are not observed in mice lacking the catalytic subunit of NADPHoxidase (gp91phox). Furthermore, oxidative stress and cerebrovascular dysfunction do not occur in transgenic mice overexpressing the amyloid precursor protein but lacking gp91phox. The mechanisms by which NADPH oxidase-derived radicals mediate the cerebrovascular dysfunction involve reduced bioavailability of nitric oxide. Thus, a gp91phox-containing NADPH oxidase is the critical link between Aβ and cerebrovascular dysfunction, which may underlie the alteration in cerebral blood flow regulation observed in AD patients (99).

Finally, several recent reports suggest that many of the proteins implicated in AD pathogenesis have direct physical involvement with mitochondria or mitochondrial proteins (87).

Inflammation

In addition to Aβ deposition, neurofibrillary tangle

accumulation, and neuronal loss, the end-stage pathology of AD is also notable for the presence of numerous cellular and molecular markers of an inflammatory response that are often associated with the Aβ deposits (100). The cellular inflammatory response consists of widespread astrogliosis and microgliosis. A large number of molecular markers of inflammation are also increased, including multiple cytokines, interleukins, other acute-phase proteins, and complement components. Aβ aggregates appear capable of inciting an inflammatory response, and there is evidence that inflammation can promote increased Aβ production and also enhance Aβ deposition (100). Thus, an Aβ-induced inflammatory response could promote further Aβ accumulation and increased inflammation. Alternatively, it is possible that under certain circumstances the inflammatory response is beneficial and may actually promote Aβ clearance (101).

Inflammation clearly occurs in pathologically vulnerable regions of the AD brain, and it does so with the full complexity of local peripheral inflammatory responses. In the periphery, degenerating tissue and the deposition of highly insoluble abnormal materials are classical stimulants of inflammation (100).

Tesseur et al. report that the expression of TGF-β type II receptor (TβRII) by neurons is reduced very early in the course of AD and that reduced TGF-β signaling increased Aβ deposition and neurodegeneration in a mouse model of AD (102). Thus, reduced TβRII levels indicate a likely dysfunction in TGF-β–mediated neuroprotective signaling events in the AD brain. Reduced TGF-β signaling, therefore, may lead to neurotrophic factor deficiencies and thus neuronal dysfunction (103).

It has been hypothesized that neurodegeneration results from a chronic inflammatory response to deposited amyloid (100,104). Alternatively, the various forms of Aβ aggregates may be directly neurotoxic (105,106).

Cholesterol Metabolism in the Brain

Emerging from the established genetic dispositions of AD is an association between plasma cholesterol and AD (107,108). Retrospective analysis of the effect of cholesterol lowering HMG-CoA reductase inhibitors (statins) on plasma cholesterol levels and coronary heart disease suggests that statins significantly reduce AD development. One study of 57,104 patients over 60 years of age who were taking lovastatin or pravastatin showed a 60–73% lower incidence of AD (109). Another study concluded that individuals 50 years and older who were treated with statins had a substantially lower risk of developing dementia, independent of the presence or absence of hyperlipidemia (110). These suggestive clinical observations correlate with in vivo and in vitro evidence, indicating a role for cholesterol in APP processing and Aβ generation (111).

Consistent with the in vivo observations, plasma membrane cholesterol levels modulate APP processing by the α-secretase pathway in vitro (112). Treatment of neuronal and nonneuronal cell lines with either cholesterol-extracting agents or with statins dramatically increased α-secretase activity and the release of the neurotrophic APPsα fragment, and concomitantly decreased β-secretase activity. Moreover, cellular sites with increased APPs α production were membrane regions with low cholesterol concentrations and high fluidity. Statin-induced reduction of cellular cholesterol levels resulted in reduced generation of Aβ-42 and Aβ-40 both in vitro and in vivo (113). Collectively, these studies support a role for cellular cholesterol in modulating Aβ production.

The mechanism by which cholesterol modulates the proteolytic cleavage of APP is unclear. However, the effect of cholesterol on membrane fluidity is potentially important. As first suggested by in vitro studies, increased plasma membrane fluidity may enhance APP/α-secretase interactions and α-secretase enzymatic activity (112). In contrast, rigid cholesterol-enriched membranes may reduce APP/α-secretase interactions and promote β- and γ-secretase processing (113). In support of this suggestion, γ-secretase activity has been identified in cholesterol- and sphingolipidrich membrane microdomains known as lipid rafts (113,114). Lipid rafts appear to promote the accumulation of Aβ and may initiate Aβ aggregation (115).

Intriguingly, apolipoprotein J, which is also secreted by glia and is believed to be a major carrier of amyloid-β peptides in biological fluids (116), was transported efficiently across the BBB in an Loco – density lipoprotein Receptor – Related Protein 2 (LRP2) -dependent manner. Furthermore, complexing amyloid-β42 to apolipoprotein J enhanced amyloid-β42 clearance rates by 83% (117). This important study shows that various transport pathways are required to clear amyloid-β from the brain, and highlights the quantitative and temporal contribution of apolipoprotein E, apolipoprotein J, LRP1 and LRP2 in mediating amyloid-β efflux across the BBB.

Several studies have suggested that high intracellular cholesterol concentrations increase the amyloidogenic processing of amyloid precursor protein (APP), leading

to greater amyloid-β production (114,118-120). On the other hand, low cellular cholesterol levels have been associated with reduced amyloid-β generation (112,121-125). Interestingly, APP and all of the components of secretases (the enzymes that cleave APP), are integral membrane proteins. Furthermore, the proteolytic activity of γ-secretase takes place within the hydrophobic membrane environment (125). These observations suggest that the ABCA and ABCG classes of the ATP-binding cassette transporter superfamily, which modulate intracellular cholesterol trafficking and homeostasis, may play a key role in amyloid-β metabolism (126).

The brain contains about 2% of the total body cholesterol, of which most is unesterified. It is found in the plasma membranes of glial cells, which provide structural and metabolic support to neurons, in neuronal membranes, and in the myelin sheaths that insulate and protect neurons. Under normal conditions, essentially all of the cholesterol in the brain is synthesized locally (127). The blood-brain barrier prevents any real contribution from plasma lipoproteins. Thus, mechanisms that remove cholesterol from the brain are required for cholesterol homeostasis.

To be transported across the blood-brain barrier, most cholesterol is thought to be converted to 24(S)-hydroxycholesterol, a soluble oxysterol that can diffuse across the barrier, enter the blood circulation, and be taken up directly by the liver for excretion (128,129). The enzyme suggested to perform this conversion is cholesterol 24-hydroxylase or Cyp46, a new sub-family member of the cytochrome P450 enzymes. Cyp46 is highly expressed in the brain (130) and is expressed in neurons in the cerebral cortex, hippocampus, and dentate gyrus (131) (the same neurons that are preferentially targeted in AD).

Most of the 24-hydroxycholesterol in circulation originates from the brain (131). Since neurodegeneration involves degradation of neuronal cell membranes and release of cholesterol, the relationship of plasma concentrations of this oxysterol to brain cholesterol metabolism was examined. In a study comparing AD subjects with healthy age-matched controls, depressed subjects, and subjects with vascular dementia not related to AD, the plasma levels of 24-hydroxycholesterol were significantly elevated only in subjects with AD or vascular dementia (132). Another study showed increased 24-hydroxycholesterol levels in the CSF of AD subjects (133). These results suggest that neuronal death is coupled with increased flux of cholesterol from the brain. In addition, 24-hydroxycholesterol is neurotoxic and may directly contribute to neurodegeneration (134). However, 24-hydroxycholesterol concentrations are decreased in cases of advanced AD (135). In a recent study, three statins (lovastatin, simvastatin, and pravastatin) and niacin reduced plasma concentrations of 24-hydroxycholesterol in AD subjects (136).

Adipoprotein E and Its Receptor

The vast majority of AD cases are late-onset (LOAD) and their development is probably influenced by both genetic and environmental risk factors. A strong genetic risk factor for late-onset AD is the presence of the ε4 allele of the apolipoprotein E (*APOE*) gene, which encodes a protein with crucial roles in cholesterol metabolism. There is mounting evidence that APOE4 contributes to AD pathogenesis by modulating the metabolism and aggregation of amyloid-β peptide and by directly regulating brain lipid metabolism and synaptic functions through APOE receptors. Emerging knowledge of the contribution of APOE to the pathophysiology of AD presents new opportunities for AD therapy.

It is widely believed that impaired Aβ clearance is a major pathogenic event for LOAD. Aβ has a relatively short half-life in the brain. Using in vivo microdialysis and a γ-secretase inhibitor, it has been shown that Aβ has a half-life of ~2 h and ~4 h in young and aged mice, respectively (138). In human brains the Aβ clearance rate is 8.3% per hour (139), indicating that Aβ is actively and efficiently cleared from the brain.

There are two major pathways by which Aβ is cleared from the brain: receptor-mediated clearance by cells in the brain parenchyma (microglia, astrocytes and neurons), along the interstitial fluid drainage pathway or through the blood–brain barrier (BBB); and through endopeptidase-mediated proteolytic degradation. Receptor-mediated clearance of Aβ in the brain is likely to be mediated by the APOE receptors LRP1, LDLR and VLDLR, which are widely expressed in neurons, astrocytes and microglia of the brain parenchyma, as well as in endothelial cells, astrocytes and smooth muscle cells at the BBB and cerebral arteries. APOE as well as LRP1 and several other LRP1 ligands (for example, α2-macroglobulin and lactoferrin) are present in amyloid plaques. These receptors can bind Aβ directly (141) or indirectly through Aβ chaperones. APOE is the best characterized Aβ chaperone. APOE immunoreactivity is found in amyloid plaques (140,142), suggesting that APOE interacts with Aβ directly in AD brains.

APOE is a major apolipoprotein and a cholesterol carrier in the brain8. In humans, the APOE gene exists as three different polymorphic alleles (ε2, ε3 and ε4),

which engender six different genotypes (ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4 and ε4/ε4). ε3 is the most (77%) and ε2 the least (8%) common allele8. The ε4 allele frequency is ~15% in general populations but is □40% in patients with AD. Individuals with one ε4 allele are three to four times as likely to develop AD than those without ε4 alleles (25,144). This odds ratio is much greater than those for other AD risk alleles, which are typically <1.5 (144). The effects of the ε4 allele on AD risk are maximal between 60 and 70 years of age, and the prevalence of the ε4 allele in AD patients is >50%. Interestingly, the rare ε2 allele is associated with protection against LOAD compared with the ε3 allele (25).

APOE3–lipoprotein binds to Aβ with higher affinity than APOE4–lipoprotein (146). Accordingly, APOE3 clears Aβ through APOE receptors on the cell surface more efficiently than APOE4. Indeed, several studies using different amyloid mouse models expressing either human APOE3 or human APOE4 demonstrated that APOE3-expressing mice develop fewer amyloid plaques than APOE4-expressing mice (30,147,148). Post-mortem studies have demonstrated increased amyloid plaque load in the brains of carriers of the ε4 allele for both sporadic (149) and genetic AD cases (150), and this notion has been confirmed by positron emission tomography imaging studies from 'cognitively normal' controls (151). An emerging body of data has identified multiple pathways that could explain the pathogenic nature of APOE4. These include Aβ production, Aβ clearance, Aβ fibrillization, tangle formation, cholesterol homeostasis, synaptic plasticity and repair, and neuronal toxicity

Neuronal Cell Death

Neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease trigger neuronal cell death through endogenous suicide pathways. Surprisingly, although the cell death itself may occur relatively late in the course of the degenerative process, the mediators of the underlying cell-death pathways have shown promise as potential therapeutic targets (152).

Neurodegenerative diseases are associated with a number of insults that may trigger PCD: misfolded proteins, reactive oxygen and nitrogen species, mitochondrial-complex inhibition, calcium entry, excitotoxicity, trophic-factor withdrawal, and death-receptor activation to name a few. In some cases, however, deaths occur that do not fit neatly into any of the three classes of PCD, and these more controversial forms of death are also discussed below (152).

The biochemical activation of classical apoptosis occurs through two main pathways. These are the extrinsic pathway, which originates through the activation of cell-surface death receptors such as Fas, and results in the activation of caspase-8 or -10 (153), and the intrinsic pathway, which originates from mitochondrial release of cytochrome c and associated activation of caspase-9. A third, less well-characterized pathway — essentially a second intrinsic pathway — originates from the endoplasmic reticulum (ER) and also results in the activation of caspase-9 (154-156). Other organelles, such as the nucleus and Golgi apparatus, have damage sensors that link to apoptotic pathways (158).

Autophagy (referring to macroautophagy herein) is an intracellular process that allows cells to engulf cytoplasmic contents — both soluble molecules and large organelles — in specialized double membranes and deliver them to lysosomes for degradation (158). This self-eating process is often a nonselective stress response to many extracellular and intracellular stimuli. Autophagy is highly dynamic and involves multiple steps, including the initial formation of double membranes and autophagosomes and their maturation into autolysosomes. Whether autophagosomes are beneficial or detrimental to a cell depends on the context (159).

The amyloid β (Aβ) peptide is thought to be a major culprit in AD, and its production and degradation have been intensely investigated. Nevertheless, it remains largely unknown how Aβ pathology is modulated by the autophagy pathway. The study by Pickford and colleagues shows that beclin 1, a multifunctional protein that also plays an important role in the autophagy pathway, affects some aspects of Aβ pathology in aged but not young transgenic mice expressing amyloid precursor protein (APP). These findings further support the notion that modulation of autophagy, in this case through beclin 1, may represent a novel therapeutic strategy for AD (159).

The novel data of Grimm *et al* (160) suggest that the amyloid and associated neurodegenerative pathologies of AD result from a self-amplifying cascade of membrane-associated events (Fig. 2). Altered γ-secretase activity, resulting from mutations in APP or presenilin and/or oxidative stress, increases the Aβ42/Aβ40 ratio which, in turn, increases SMase and HMG-CoA reductase activities. As a consequence, levels of ceramides and cholesterol are increased and levels of sphingomyelin are decreased in the membranes of neurons. These alterations may then promote further production of Aβ42. Aβ42-induced membrane-associated oxidative stress and changes in lipid

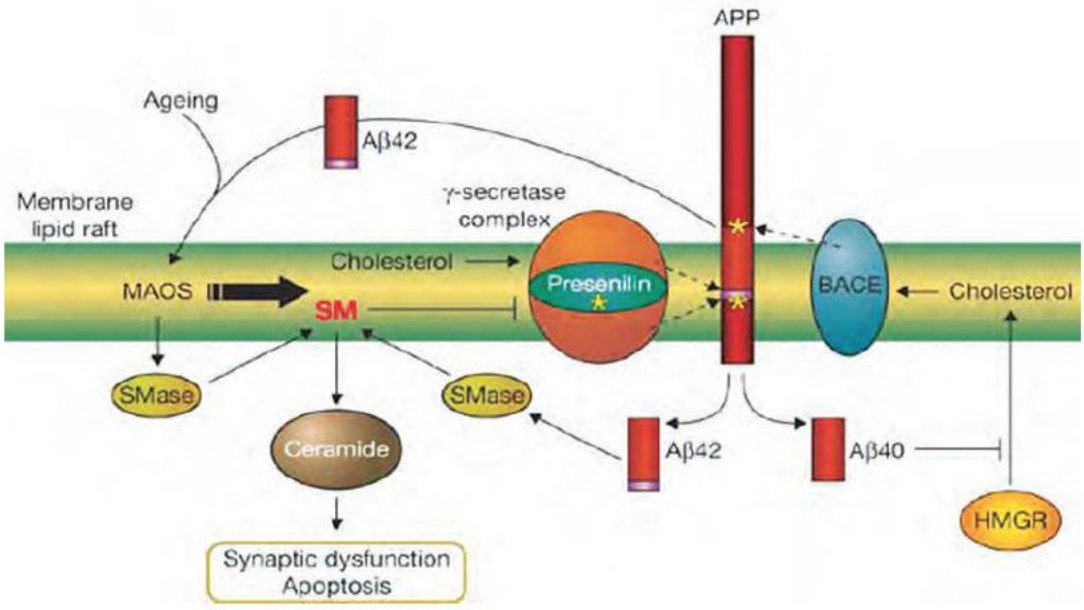

Figure 2. Interactions between membrane – lipid metabolism, APP processing and Aβ neurotoxicity in AD (Adapted with permission from Nature Publishing Groups).

metabolism, together with altered ceramide production, may cause dysfunction of synapses and render neurons vulnerable to apoptosis and excitotoxicity (34,161).

An increase in the size of the amyloid β-peptide (Aβ42 versus Aβ40) may be a key factor in the pathogenesis of Alzheimer's disease. By altering the activities of enzymes involved in the metabolism of cholesterol and sphingomyelin, an increase in the Aβ42:Aβ40 ratio may cause dysfunction and death of neurons (161).

AD Biomarkers

The 'amyloid cascade theory' is the prevailing hypothesis on the cause of Alzheimer disease. It holds that an imbalance between production and clearance of Aβ in the brain is the initiating event in the disease, ultimately leading to neuronal degeneration and dementia (36).

Substantial efforts have been made to translate the understanding of pathogenic mechanisms into therapeutic strategies. A major focus has been to inhibit production and aggregation of Aβ and to increase its clearance from the brain—for example, by inhibiting Aβ-generating enzymes and by using Aβ immunotherapy (33). A number of promising drug candidates are now under development. Such drugs with disease-modifying potential are likely to have the best efficacy in the early phase of the disease, when the neuronal degeneration has not become too widespread. This has initiated an intense search for Alzheimer disease biomarkers.

The first clinical phase in Alzheimer disease, typically characterized by isolated memory disturbances, is called mild cognitive impairment (MCI) (162). Only around 40–60% of individuals with MCI have incipient Alzheimer disease that will progress to Alzheimer disease with full-blown dementia, whereas others will develop different forms of dementia or have a benign form of MCI. As there is no clinical method to determine which MCI cases have incipient Alzheimer disease, there is a great need for biomarkers to identify these cases.

Another potential use for biomarkers is in clinical trials. At present, trials for new Alzheimer disease therapies often involve people with MCI. But these studies are impeded by the insufficiency of current criteria to identify MCI cases with incipient true Alzheimer disease (163).

The accuracy of the clinical diagnosis at the primary care level and in general hospitals is probably even lower, especially in the early stages of the disease when the symptoms are indistinct. In view of this, the need for specific AD markers is great. According to a proposal of a consensus group on molecular and biochemical markers of AD (164), an ideal marker of AD should be able to detect a fundamental feature of neuropathology and should be validated against neuropathologically confirmed cases.

Furthermore, its sensitivity for detection of AD as well as its specificity for discrimination of AD from other dementia disorders should exceed 80%. A marker for AD should also be reliable, reproducible, noninvasive, simple to perform in clinical routine and inexpensive (165).

β-amyloid 42 (Aβ42), Total Tau (T-tau) and Phosphorylated Tau (P-tau)

Underlying neuropathological changes in AD are the accumulation of senile plaques (SPs) and neurofibrillary tangles (NFTs). SPs are made up mainly of β-amyloid, especially the 42-amino-acid isoform, β-amyloid 42 (Aβ42) (166). The major constituent of NFTs is a cytoskeleton-associated protein called tau, which is hyperphosphorylated in NFTs (167). The golden standard of diagnosis is the identification of typical neuropathological changes in the brain of a patient who has suffered from clinical AD.

Among several, we have focused on three candidates that have been suggested to fulfill the requirements for biomarkers of AD: β-amyloid42 (Aβ42) , total tau (T-tau) and tau phosphorylated at various epitopes (P-tau). The cerebrospinal fluid (CSF) levels of these proteins reflect the metabolism of these proteins in the central nervous system (165).

Aβ42

The central protein in SPs is Aβ42. It is produced and secreted from human cells as a result of normal cellular processing of the larger transmembrane protein APP (168). In this processing, APP is cleaved in several steps and Aβ is produced. In, AD, APP is first cleaved by an enzyme called β-secretase, which results in the release of a large N-terminal fragment called β-secretase-cleaved soluble APP. In a second step, APP is cleaved by the γ-secretase complex, which results in the release of free Aβ. In this processing, various isoforms of Aβ, for example, Aβ42, are produced; all of which are secreted into the CSF.

Using four different ELISA methods that are specific to Aβ42 (169-172), more than 30 studies have consistently demonstrated a moderate to marked decrease in CSF Aβ42 in AD. The principle for the ELISA that is most commonly used to measure Aβ42 in CSF, INNOTEST™ β-AMYLOID(1 – 42) (172. There are 13 studies, including a total of about 600 AD cases and 450 controls, in which sensitivity and specificity figures have been given or can be calculated from graphs. These studies show that, for CSF Aβ42, the mean sensitivity for discrimination between AD and normal aging is approximately 86%, while the specificity is approximately 91% and the mean level of decrease in AD patients compared with controls is about 50%

On the other hand, the specificity for discrimination of AD from other disorders is moderate. Low levels of Aβ42 in CSF have, for example, been found in Lewy body dementia (173,174), a disorder also characterized by the presence of SPs. Low levels have also been found in a small percentage of patients with frontotemporal dementia and vascular dementia (175,176) and also in Creutzfeldt–Jakob's disease (177,178) and amyotrophic lateral sclerosis (179). These studies question the putative relation between low CSFAβ42 levels and the accumulation of SPs. There are several possible causes of low CSF-Aβ42 levels, for example, axonal degeneration (179,180) and entrapment in narrow interstitial and subarachnoid drainage pathways (179).

T-tau

Tau is a microtubule-associated protein which is located mainly in neuronal axons. By binding to microtubules, it promotes the stability and function of these. In the normal human brain, six different isoforms of tau are found, all of which have numerous phosphorylation sites (182). As tau is a major constituent of NFTs, CSF T-tau has been suggested as a marker for AD. Using monoclonal antibodies that detect all isoforms of tau independent of degree of phosphorylation, enzyme-linked immunosorbent assays (ELISAs) have been developed that measure the T-tau levels in CSF (183-185). Using these ELISAs, more than 50 studies have consistently demonstrated a moderate to marked increase in CSF T-tau as well as high sensitivity and specificity of CSF-tau in AD patients when compared with controls. So far, CSF from about 2400 AD patients and 1250 controls has been investigated in this way. The mean degree of increase is about 300% in AD compared with controls. The high sensitivity and specificity make CSF T-tau a good candidate for the designation biochemical marker for AD, or AD biomarker. However, high levels of T-tau in the CSF have also been found in a proportion of cases with other dementia disorders, such as frontotemporal dementia (186,187) and Lewy body dementia (173), but in several other disorders, for example, alcohol dementia, Parkinson's disease and depression, the CSF levels of T-tau seem to be normal and only occasionally increased (184,187-189).

It has been suggested that the CSF T-tau levels reflect the degree of neuronal (especially axonal) degeneration

and damage (184). Some evidence for this has been found, for instance, a transient increase in CSF T-tau after acute stroke, with a positive correlation between CSF T-tau and infarct size as measured by computerized tomography [21], a very marked increase in CSF T-tau in Creutzfeldt–Jakob's disease (191), and a correlation between premortem CSF T-tau levels and the postmortem density of neurofibrillary tangles in the brain (192). Indirect evidence is that, in AD and controls, there is a positive correlation between the CSF levels of T-tau, GAP-43 and amyloid precursor protein (APP), all proteins located in the axon of neurons (193).

P-Tau

Tau is normally in a phosphorylated state. Over 70 phosphorylation sites are found on the human tau molecule and, in AD, tau is usually in a hyperphosphorylated state. In AD, this hyperphosphorylation involving certain epitopes on the tau molecule has the consequence that tau loses its ability to promote microtubule assembly and stability, which in turn leads to cytoskeleton instability and diminished transport ability (194,195). A consequence of this is aggregation of tau with subsequent formation of NFTs (182). Several ELISAs have been developed that use monoclonal antibodies directed toward sites that are phosphorylated in AD. The principle for one of these ELISAs, INNOTEST™ PHOSPHO-TAU$_{(181P)}$, which measures tau phosphorylated at threonine 181 (P-Tau$_{181}$) (197). Other ELISAs identify tau phosphorylated at the epitopes threonine 181 and 231 (P-tau$_{181\ +\ 231}$) (184), threonine 231 and serine 235 (P-tau$_{231\ +\ 235}$), serine 199 (P-tau$_{199}$) (184), threonine 231 (P-tau$_{231}$) (199) and serine 396 and 404 (P-tau$_{396\ +\ 404}$) (200). All these assays have shown increased CSF levels of P-tau in AD patients compared with controls. The sensitivity of CSF P-tau for discrimination between AD and normal aging is about the same or slightly lower as that of CSF T-tau, that is, about 75%. Interestingly, the specificity of CSF P-tau for discrimination of AD from other dementias seems to be higher than those of CSF T-tau and CSF Aβ42. Normal CSF levels of P-tau have been found in vascular dementia, frontotemporal dementia (201) and Lewy body dementia (202), which suggests that the above ELISAs may help to discriminate between AD and these dementias. In addition, while there is a marked increase in CSF T-tau after acute stroke, the CSF P-tau does not change (203). This suggests that the origin of increased CSF P-tau levels is more closely related to AD pathology, for instance, the formation of NFTs.

Combination of CSF Markers

The rationale for using the CSF levels of T-tau, Ah42 and P-tau in combination to detect AD is very clear. Because the concentrations of any one of these substances is believed to reflect central pathogenetic processes in the disorder, that is, according to the leading hypothesis on the development of AD, the amyloid cascade hypothesis, the combination might result in increased sensitivity and specificity. In fact, some large studies have shown that both sensitivity and specificity increase when, for instance, CSF T-tau and CSF Aβ42 are used in combination instead of being used alone (173,175,204,205). Moreover, in a community-based setting, the sensitivity for AD was more than 90%, when combinations of the above CSF markers for AD were used in routine clinical chemistry analyses. The sensitivity and specificity figures were based on the values for all consecutive patients admitted for investigation of cognitive disturbances during 1 year (173).

High CSF levels of T-tau and low CSF levels of Ah42 in the early stages of AD have been found in several studies (175,204,206-209). For more severely demented AD cases, the sensitivity figures are 80–90%, suggesting that the two CSF markers are workable in the early stages of the disease process. Several studies have also found high CSF levels of T-tau and low CSF levels of Aβ42 in patients with mild cognitive impairment (MCI) who later developed AD (206,210,211). Increased CSF levels of T-tau were also found to discriminate, with high sensitivity and specificity, MCI patients whose disturbances later progressed to AD from the others (210). Other studies have also found increased CSF levels of P-tau in a high proportion of MCI cases (210,211). These findings suggest that all three CSF markers may be of use in the clinical identification of AD in the very early phases of the disease and thus facilitate early intervention (165).

To simultaneously study several biomarkers for Alzheimer disease (AD), the xMAPTM technology has been develop and evaluate a multiparametric bead-based assay for quantification of β-amyloid$_{(1-\ 42)}$ [Aβ$_{(1-42)}$], total tau (T-TAU), and hyperphosphorylated tau [P-TAU$_{(181P)}$] in cerebrospinal fluid (CSF). The new multiparametric method may be able to replace the corresponding ELISA methods (212).

Visinin-like Protein 1 (VLP-1)

Another class of biomarkers that may have utility in the diagnosis of AD are those that reflect neuronal death rather than specific markers of disease pathogenesis. Such markers may provide information about disease progression related to functional outcome and may have utility in future clinical trials testing therapeutic efficacy. Several reports have demonstrated the lack of correlation between amyloid plaque load and degree of dementia, suggesting that the former may not directly relate to the latter (85,213). Therefore, a neuronal death biomarker might have greater correlation with dementia severity than the well-studied pathological biomarkers (214).

Quantified the levels of a brain injury marker, visinin-like protein 1 (VLP-1, also abbreviated as VILIP-1 or VSNL-1), in CSF of AD patients and age-matched controls. VLP-1 belongs to the family of neuronal calcium sensor proteins involved in calcium-dependent signal transduction mechanisms in neurons. VLP-1 increases neuronal cyclic adenosine monophosphate levels by inducing protein kinase A. VLP-1 is expressed in neurons (215) and its immunoreactivity is decreased in brains of AD patients compared to controls (216). Remarkably, VLP-1 expression is associated with neurofibrillary tangles in AD brains (217). The investigation of the concentration of VLP-1 in CSF reported by Lee et al. was based on findings they reported (218). VLP-1 appeared to be a protein that was relatively brain specific; its concentration was increased in plasma of stroke patients and in CSF in a rat model for stroke, suggesting that VLP-1 is a marker for (rapid) neuronal cell injury. In the present study, CSF VLP-1 concentrations were 50% higher in AD patients than in the control population. An interesting aspect of the studies of Lee and colleagues is that their original approach to find novel markers of brain injury, i.e., mRNA profiling and selection for products that were highly enriched in brain tissue (218), resulted in the identification of VLP-1, which was not picked up by comparable fishing expeditions using a proteomics approach with human CSF from AD patients (219).

Interestingly, VLP-1 concentrations in AD patients with an apolipoprotein E (*APOE*) *ε4/ε4* genotype were approximately double those in ε3/ε3 carriers. Although the current study includes a relatively small patient series and the results await confirmation in larger cohorts and fro m independent studies, this association of VLP-1 with the *APOE* genotype seems to be remarkably different from the association of the *APOE* genotype with T-tau concentrations (214).

Another remarkable finding by Lee *et al*. is the correlation between Mini Mental Status Examination (MMSE) scores as a marker for disease severity and CSF VLP-1 concentrations. Many reported studies have found no correlation of CSF Aβ42 and T-tau with MMSE score [summarized in (220) and confirmed in the small cohort described by Lee et al. (214). VLP-1 is negatively correlated to MMSE scores, suggesting VLP-1 may also have a role as a biomarker of disease severity, and role in monitoring disease activity (loss of neurons and cognition per period of time) can also be envisioned. The findings of MMSE correlation with VLP-1, however, should be confirmed in wider ranges of MMSE values and larger groups.

Finally, as Lee *et al*. point out in their report, the real clinical challenge is not the differentiation of patients with AD from controls but of patients with AD from patients with other types of dementia, including vascular dementia, dementia with Lewy bodies, or frontotemporal lobe degeneration, and also in patients whose dementia is attributable to treatable disorders such as vitamin deficiencies, depression, alcohol abuse, and normal-pressure hydrocephalus (221).

Miscellaneous Brain-Spesific Proteins

A variety of other brain-injury biomarkers have been examined in the CSF of patients with dementia, including neuron-specific enolase (223,224), S100β protein (225), and glial fibrillary acidic protein (GFAP) (226), all with variable diagnostic specificity and sensitivity. More recently, proteomic profiling has resulted in the identification of several candidate biomarkers (227), including heart-fatty acid binding protein (228,229), Park 7, and nucleoside diphosphate kinase A (230). The effectiveness of a fluid biomarker is dependent on a multitude of factors, including organ specificity, accumulation in accessible body fluids, stability, clearance, and detectability. Direct comparisons between biomarker candidates will be important to identify such an ideal biomarker.

APOE genotype is the strongest known genetic risk factor for the development of late-onset AD, with the ε4 allele incurring greatest risk (25,142,231). The molecular mechanism for this risk is not known; however, it is believed that ApoE protein may play a role in Aβ transport/clearance (232), and that the genotype may also impart increased vulnerability to a variety of central nervous system injuries (233).

With the increasing clinician awareness that CSF biomarkers have additional value in the diagnostic work-up of dementia patients and that CSF analysis appears likely to gain a position in the diagnostic (research) criteria for AD, this study will motivate other researchers in their quest to find specific biomarkers for dementia syndromes (221).

Plasma Signaling Proteins

Because the brain controls many body functions via the release of signaling proteins, and because central and peripheral immune and inflammatory mechanisms are increasingly implicated in Alzheimer's (7) and related diseases4, Ray et al hypothesized that the pathological processes leading to Alzheimer's would cause characteristic changes in the concentrations of signaling proteins in the blood, generating a detectable disease-specific molecular phenotype (235).

The computational gene network prediction tool Ingenuity Pathway Analysis (Ingenuity Systems) identified two independent regulatory networks connecting the 18 signaling proteins. One network centered on tumor necrosis factor (TNF)-a and monocyte-colony stimulating factor (M-CSF), whereas the other centered on epidermal growth factor (EGF). Consistent with these findings, gene ontology (Kyoto Encyclopedia of Genes and Genomes; http://www.genome.jp/kegg/) and BioCarta (http://www.biocarta. com/) pathway analyses indicated involvement of the 18 markers in immune response, hematopoiesis and apoptosis (235).

A decrease in the abundance of factors linked to hematopoiesis would be particularly noteworthy in light of recent data suggesting that hematopoietic cells can enter the brain in Alzheimer's disease or in Alzheimer's mouse models at increased frequencies and modulate the disease (7, 236, 237). Dysfunction of apoptotic pathways has also been linked to Alzheimer's disease (238).

The observed dysregulation of the signaling pathways represented by the 18 signaling proteins in blood plasma may point to changes in the periphery, the central nervous system or both that are relatively specific to Alzheimer's disease and occur early in the disease process.

Studied by Ray *et al* found 18 signaling proteins in blood plasma that can be used to classify blinded samples from Alzheimer's and control subjects with close to 90% accuracy and to identify patients who had mild cognitive impairment that progressed to Alzheimer's disease 2–6 years later. Biological analysis of the 18 proteins points to systemic dysregulation of hematopoiesis, immune responses, apoptosis and neuronal support in presymptomatic Alzheimer's disease (235).

Conclusions

For the time being, a presumptive diagnosis of Alzheimer's can be made clinically using various cognition tests, neurological exams, and patient history. A definitive diagnosis is possible only through post – mortem brain analysis. Unfortunately, by the time symptoms appear and a clinical diagnosis is made, the disease has been simmering for decades and intractable neurological damage occurred (239).

Development of disease-specific CSF, serum and urine biomarkers will undoubtedly add to the process of differential diagnosis early in the course of the disease (220).

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