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Candidate Bio-Markers of Alzheimer's Disease

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1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder of the central nervous system characterized by a progressive loss of short-term memory accompanied by a gradual loss of cognitive functions (Ross et al., 2004). AD is among the most frequently encountered diseases in aging societies with an estimated 5million people in the United States and 17 million people worldwide suffering from the disease. It is expected that these numbers will quadruple by the year 2040, by which 1 out of 45 Americans will be affected, leading to a considerable public health burden (Fratiglioni et al., 1999). AD pathogenic mechanisms contributing to neuronal loss and brain dysfunction are still unclear. However, remarkable advances have taken place in understanding of both the genetics and molecular biological aspects of the intracellular processing of amyloid and tau and the changes leading to the pathologic formation of extracellular amyloid plaques and the intraneuronal aggregation of hyperphosphorylated tau into neurofibrillary tangles. This progress in our understanding of the molecular pathology has set the stage for clinically meaningful advances in the development of biomarkers.

Proper diagnosis is essential for instituting appropriate clinical management. While diagnostic accuracy for the disease has improved, the differential diagnosis of the disorder is still problematic. In the very early stages of the disease, frequently classified as mild cognitive impairment (MCI), delineating disease process from "normal ageing" may be difficult; in later stages of the disease, distinguishing AD from a number of neurodegenerative diseases associated with dementia may also be difficult. Furthermore, the disease progression is slow and there is variability of performance on clinical measures, making it difficult to monitor change effectively. Since disease modifying therapy is likely to be most effective early in the course of disease, early diagnosis is highly desirable before neurodegeneration becomes severe and widespread.

In clinical practice, the diagnosis of AD is still largely based on consensuscriteria combined with the exclusion of secondary causes of memory loss (Knopman et al., 2001; McKhann et al., 1984).Thus, there is an urgent and desperate need for a biomarker that can reliably prognose the disease. Biomarkers of AD occupy an essential place in recently formulated diagnostic criteria for AD, in which their role is to identify the pathophysiological processes underlying cognitive impairment or to help predict time to reach up to dementia. Criteria for a useful biomarker have been proposed by an international consensus group on molecular and biochemical markers of AD in 1998 (The Ronald and Nancy Reagan Research Institute of the Alzheimer's Association and the National Institute on Aging Working Group, 1998). According to these guidelines, a biomarker for AD should detect a manifestation of the fundamental neuropathology and be validated in neuropathologically-confirmed cases. Its sensitivity for detecting AD should exceed 80% and its specificity in differentiating between AD and other dementias should be higher than 80%. Ideally, a biomarker should also be reliable, reproducible, non-invasive, simple to perform, and inexpensive. One further role of particular interest to patients and clinicians dealing with AD is its ability to detect the disease at the earliest possible stage.

Based on growing body of evidence concerning the pathophysiology of AD, a number of putative biological markers of disease have been evaluated against clinical and neuropathological standards. Biomarkers are very useful for diagnosing and monitoring disease progression (Ward et al., 2007) and are important for patient selection, monitoring side-effects, aiding selection of appropriate patient treatment, and helping new drug discovery. For the clinical studies of AD therapeutics, there is an increasing need for diagnostic markers to ensure that therapies are targeted at the right patient population, to initiate early treatment when disease-modifying drugs will be available, and to monitor disease progression (Hye et al., 2006).

2. Biomarkers in CSF

One of the most promising sources of biomarkers in AD is the cerebrospinal fluid (CSF).The molecular changes in the brain extracellular and interstitial environments are reflected in CSF. The single-cell layer epithelium separating the two compartments allows a virtually unhindered flow of molecules from the brain towards the CSF. CSF biomarkers for AD should reflect the central pathogenic processes in the brain. Furthermore the CSF is accessible to trained clinicians using a relatively simple lumbar puncture (Fenton et al., 1994). Several studies have investigated CSF inflammatory markers, immunological mediators, neurotrophins, metalloproteinases or isoprostenes. Candidate CSF biomarkers include total tau (T-tau) as a marker for the neuronal degeneration (table 1), phosphorylated tau (P-tau) as a marker for tau hyperphosphorylation (table 2) and formation of tangles $A\beta 42$ as a marker for $A\beta$ metabolism and plaque formation (table 3, Blennow et al.,2003).

Category	Reference	Sensitivity range (100%) for AD versus controls	Methods	Study Title	Study population
Tau	Arai et al., 1995	80-90	ELISA	Tau in cerebrospinal fluid: a potential diagnostic marker in Alzheimer's disease	AD (n=70), non-AD (n=96) control (n=19)
Tau	Riemenschneider et al., 1996	90-100	ELISA	Cerebrospinal protein tau is elevated in early Alzheimer's disease.	AD(n=22), dementia(n=3) Healthy controls(HC)(n=19)
Tau	Shoiji et al., 1998	20-30	ELISA	Combination assay of CSF tau, A beta 1-40 and A beta 1-42(43) as a biochemical marker of Alzheimer's disease	sporadic AD(n=55), controls(n=34), non-AD dementia(n=23), other neurological diseases(n=45)
Tau	Kanai et al., 1998	30-40	ELISA	Longitudinal study of cerebrospinal fluid levels of tau, A beta1-40, and A beta1-42(43) in Alzheimer's disease: a study in Japan	AD(n=93), non-AD dementia(n=33) other neurological diseases (n=56), HC(n=54)
Tau	Tapiola et al., 1998	50-60	ELISA	CSF tau is related to apolipoprotein E genotype in early Alzheimer's disease.	Early AD(n=81), other dementia (n=43), non demented neurologic HC(n=33)
Tau	Kahle et al., 2000	50-60	ELISA	Combined assessment of tau and neuronal thread protein in Alzheimer's disease CSF	Probable AD(n=25), definite AD(n=5), non demented with PD (n=29), HC(n=16).
Tau	Sjögren et al., 2000	60-70	ELISA	Decreased CSF -amyloid42 in Alzheimer's disease and amyotrophic lateral sclerosis may reflect mismetabolism of -amyloid induced by separate mechanisms	AD (n = 19), FTD (n = 14), ALS (n = 11) PD(n = 15) HC(n = 17)
Tau	Shoji et al., 2002	50-60	ELISA	Cerebrospinal fluid tau in dementia disorders:a large scale multicenter study by a Japanese study group	AD(n=366), 168 non-AD dementia(n=168) HC(n=181).
Tau	Buerger et al., 2002	70-80	ELISA	Differential diagnosis of Alzheimer's disease with cerebrospinal fluid levels of tau protein phosphorylated at threonine 231	AD(n=82) FTD(n=26) VD(n=20) HC(n=21)
Tau	Riemenschneider et al., 2002	80-90	ELISA	Tau and Abeta42 protein in CSF of patients with frontotemporal degeneration	FTD(n=34), AD(n=74), HC(n=40).
Tau	Schönknecht et al., 2003	50-60	ELISA	Levels of total tau and tau protein phosphorylated at threonine 181 in patients with incipient and manifest Alzheimer's disease	manifest AD (n=43) Incipient AD(n=8) VD(n=16) HC(n=16)

Data from Blennow K, Hampel H (2003)

Table 1. CSF total tau (T-tau) as a diagnostic marker for AD

Catagory	Reference	Sensitivity (100%) for AD versus controls	Methods	Study Title	Study population
p- tau	Ishiguro etal., 1999	80-90	ELISA	Phosphorylated tau in human cerebrospinal fluid is a diagnostic marker for Alzheimer's disease.	AD (n=36) , Controls (n=30)
p- tau	Kohnken et al., 2000	80-90	ELISA	Detection of tau phosphorylated at threonine 231 in cerebrospinal fluid of Alzheimer's disease patients	AD(n=27), non-AD(n=31)
p- tau	Sjögren et al., 2001	40-50	ELISA	The cerebrospinal fluid levels of tau, growth-associated protein-43 and soluble amyloid precursor protein correlate in Alzheimer's disease, reflecting a common pathophysiological process	FTD(n = 14), AD(n = 47) VAD(n = 16), controls (n = 12)
p- tau	Itoh et al.,2001	90-100	ELISA	Large-scale, multicenter study of cerebrospinal fluid tau protein phosphorylated at serine 199 for the antemortem diagnosis of AD	AD(n = 236), non-AD (n = 239), controls (n = 95)
p- tau	Parnetti et al., 2001	80-90	ELISA	CSF phosphorylated tau is a possible marker for discriminating AD from dementiawith Lewy bodies. Phospho-Tau International Study Group	AD (n=80), DLB (n=43) Controls (n=40)
p-tau	Sjögren et al., 2002	50-60	ELISA	Decreased CSF -amyloid42 in Alzheimer's disease and amyotrophic lateral sclerosis may reflect mismetabolism of -amyloid induced by separate mechanisms.	AD (n = 19), FTD (n = 14), ALS (n = 11) PD(n = 15)
p- tau	Buerger et al., 2002	90-100	ELISA	CSF tau protein phosphorylated at threonine 231 correlates with cognitive decline in MCI subjects	MCI(n=77), probable AD (n=55) Control (n=30)
p-tau	Hu et al., 2002	90-100	ELISA	Levels of nonphosphorylated and phosphorylated tau in cerebrospinal fluid of Alzheimer's disease patients: an ultrasensitive bienzyme-substrate-recycle enzyme-linked immunosorbent assay.	AD (n = 30), VaD, (n = 18) non-AD (n = 13): depression (n = 3), malignant lymphoma (n = 2) control (n = 24)
p-tau	Schönknecht et al.,2003	60-70	ELISA	CSF phosphorylated tau is a possible marker for discriminating Alzheimer's disease from dementia with Lewy bodies. Phospho-Tau International Study Group	AD (n=80) DLB (n=43) Controls (n=40).

Data from Blennow K, Hampel H.(2003)

Table 2. CSF Phosphorylaterd tau (p-tau) as a diagnostic marker for AD

Catagory	Reference	Sensitivity (100%) for AD versus controls	Methods	Study Title	Study group
$A\beta_{1-42}$	Galasko et al ., 1998	70-80	ELISA	High cerebrospinal fluid tau and low amyloid beta42 levels in the clinical diagnosis of Alzheimer disease and relation to apolipoprotein E genotype	Probable AD(n=82), control (n=60) ND (n= 74)
$A\beta_{1-42}$	Andreasen et al., 1999	90-100	ELISA	Cerebrospinal fluid -amyloid(1-42) in Alzheimer's disease: differences between early- and late-onset Alzheimer disease and stability during the course of disease	AD (n=53) Control (n=21)
$A\beta_{1-42}$	Andreasen et al.,1999	90-100	ELISA	Sensitivity, specificity and stability of CSF t-tau in AD in a community-based patient sample.	AD (n= 407) Depression(n=28) control (n=65).
$A\beta_{1-42}$	Andreasen et al., 1999C	80-90	ELISA	Cerebrospinal fluid -amyloid(1-42) in Alzheimer's disease: differences between early- and late-onset Alzheimer disease and stability during the course of disease.	AD (n=53) Control (n= 21)
$A\beta_{1-42}$	Hulstaert et al., 1999	70-80	ELISA	Improved discrimination of AD patients using beta-amyloid(1-42) and tau levels in CSF.	AD (n=150) control (n= 100) ND (n=84),
$A\beta_{1-42}$	Otto et al., 2000	90-100	ELISA	Decreased beta-amyloid1-42 in cerebrospinal fluid of patients with Creutzfeldt-Jakob disease	CJD (n=27), AD(n=14), other dementia(n=19), NDC(n=20)
$A\beta_{1-42}$	Kapaki et al., 2001	70-80	ELISA	Highly increased CSF tau protein and decreased beta-amyloid (1-42) in sporadic CJD: a discrimination from Alzheimer's disease?	CJD (n=14), AD(n=38)controls (n=47).
$A\beta_{1-42}$	Sjögren et al., 2002	90-100	ELISA	Decreased CSF -amyloid42 in Alzheimer's disease and amyotrophic lateral sclerosis may reflect mismetabolism of -amyloid induced by separate mechanisms	AD (n = 19), FTD (n = 14), ALS (n = 11) PD (n = 15) controls (n = 17).

Data from Blennow K, Hampel H.(2003)

Table 3. CSF $A\beta_{1-42}$as a diagnostic marker for AD

2.1. Tau protein

One of the major neuropathological hallmarks of AD are neurofibrillary tangles composed of paired helical filaments (PHF). The principal protein subunit of PHF is abnormally phosphorylated tau (p-tau) (Iqbal et al., 1998). Physiologically, tau protein is located in neuronal axons, in components of the cytoskeleton and in the intracellular transport systems. Total-tau (t-tau) and truncated forms of monomeric and p-tau can be traced in the CSF. Using antibodies that detect all isoforms of tau proteins independent of phosphorylation, or specific phosphorylation Core biomarker candidates of Alzheimer's disease 251 sites, ELISA have been developed to measure t-tau and p-tau concentrations (Vandermeeren et al., 1993; Blennow et al., 2002, 1995; Hampel et al., 2003). CSF total tau protein in the differentiation between AD and normal aging. Total tau protein, thought to be a general marker of neuronal destruction, has been intensely studied in more than 2200 AD patients and 1000 age-matched elderly controls over the last 10 years (Sunderland et al., 2003, table 1). The most consistent finding is a statistically significant increase of CSF t-tau protein in AD. The mean level of CSF t-tau protein concentration is about 3 times higher in AD compared to elderly controls. A sensitivity and specificity level varies between studies primarily due to the different control groups used. Specificity levels between 65% and 86% and sensitivity levels between 40% and 86% have been found (Blennow et al., 2001, table 1). In several studies, a significant elevation was also found in patients with early dementia (Galasko et al., 1997; Kurz et al., 1998; Riemenschneider et al., 1997). In these studies of early dementia, the potential of CSF t-tau protein to discriminate between AD and normal aging appeared high, with average 75% sensitivity and 85% specificity. An age-associated increase of t-tau protein has been shown in nondemented subjects (Buerger et al., 2003; Sjogren et al., 2001b). Therefore, the effect of age should be considered when t-tau protein levels are employed diagnostically.

2.2. Phosphorylated tau (p-tau)

Tau protein exists in six isoforms of 352–441 amino acids in length that are subject to a variety of posttranslational modifications (Hanger et al., 2007) and, presumably, function. Of the 79 serine and threonine phosphorylation sites on the longest isoform of tau, 4R/2N, approximately 40 have been verified (Iqbal et al., 2010) of which 25 have been identified as sites of ''abnormal phosphorylation'' (Mazanetz et al., 2007). The phosphorylation state of tau is the net result of a balance of kinase and phosphatase activity. Much of the activity in tau-based drug discovery has been focused on selective finding inhibitors of ''tau kinase'', a combination of the activity of two serine/threonine kinases that can phosphorylate tau – glycogen synthase kinase 3 (GSK3; tau protein kinase I), cyclin-dependent kinase 5 (CDK5; tau protein kinase II) and a third kinase, extracellular signal-regulated kinase 2 (ERK2), from the possible 518 member kinase family, as a possible therapeutic approach to treating AD (Hanger et al.,2009 Mazanetz et al.,2007, Brunden et al., 2009). Other kinases that are possible targets to prevent tau hyperphosphorylation are casein kinase 1 (Hanger et al.,2007), AMP-activated protein kinase (AMPK) (Greco et al.,2009) and DYRK1A and AKAP-13 (Azorsa et al., 2010). From a biomarker perspective, t-tau, a generic measure of cortical axon damage associated with AD, multiple sclerosis (Hernandez et al., 2007, Bartosik-Psujek et al.,2006), stroke and Creuzfeldt-

Jacob disease, and p-tau are increased by three fold in the CSF of confirmed AD patients (Shaw et al.,2009). Of the 40 or so phosphorylation sites on tau, pThr181 (phosphothreonine-181), pSer199, pSer202/pThr205 (AT8, epitopes site), pSer214/pSer212 (AT100, epitopessite), pThr231/ pSer235 (TG3 site) and pSer396/pSer396 (PHF1 site)–have been associated with tau hyperphosphorylation and to screen NCEs for potential ''tau kinase'' inhibitory activity. While pSer199 and pThr231 (p-tau231) have been evaluated as CSF biomarkers (Buerger et al., 2002; Engelborghs et al., 2008., table 2), pThr181 (also designated as p-tau181 or P-Tau181P) is the most widely used CSF biomarker to assess tau hyperphosphorylation (Lewczuk et al., 2002; Hampel et al., 2004) having similar diagnostic accuracy to p-tau231 (Fagan et al., 2009, table 2). Like Ab42, the diagnostic value of both t-tau and p-tau181 has been questioned in terms of their specificity as AD biomarkers (Mattsson et al., 2009).

2.3. β-Amyloid-protein

Extracellular senile plaques consisting of beta-amyloid-protein (Aβ) are one of the histopathological hallmarks of AD (Hyman and Trojanowski., 1997). They are the source of a pathogenic protein with 42 amino acids (Aβ1–42) (Selkoe et al., 1993). Several groups have developed and studied different bioassays specifically designed for Ab1–42 protein (Arai et al., 1997c, Sunderland et al., 2003). The reduction in CSF Ab1–42 found in AD has been hypothesized to indirectly reflect the amyloid deposition in senile plaques (SP), resulting in lower CSF levels in AD. A marked reduction in CSF Ab1–42, however, is also found in CJD, even in cases without Ab-positive plaques (Kapaki et al., 2001; Otto et al., 2000., table 3).

To date, at least 900 patients with clinical AD and 500 healthy individuals have been enrolled in independent research studies (Andreasen et al., 2001; Andreasen et al., 1999; Galasko et al., 1998; Sunderland et al., 2003., table 3). The most consistent finding is a marked decrease in Aβ1–42 protein in AD (to approximately 50% of control levels). Using Ab1–42 protein alone yielded sensitivities varying from 78% to 100% (table 3) and specificities from 47% to 81% when distinguish AD from elderly controls. There is a pronounced overlap, however, between studiesfrom different groups. Based on recent data a cut-off-level of >500 pg=ml has been suggested to discriminate AD best from normal aging (Sjogren et al., 2001a). One study has documented a significant decrease in CSF Aβ1–42 protein in MCI subjects compared to controls, but this study had no follow-up measure (Andreasen et al., 1999a). A second study examined MCI patients who went on to develop AD. However, in this sample Aβ1–42 protein levels did not differ significantly from age-matched normal controls (Maruyama et al., 2001). Blennow et al (2003) found Ab1–42 protein to be an indicator of early identification of AD in MCI subjects taking potential confounding factors into account such as age, severity of cognitive decline, time of observation, apolipoprotein E epsilon (e) 4 (APOE e4) carrier status, and gender (Blennow et al., 2003).Studies correlating CSF Aβ1–42 protein concentrations with cognitive performance in AD have been contradictory. Cross-sectionally, the concentration of Aβ1–42 protein and cognitive measures were either inversely correlated (Kanai et al., 1998; Samuels et al., 1999) or no significant correlation was found (Andreasenet al., 1999b; Hulstaert et al., 1999; Okamura et al., 1999). In a rare longitudinal study, a decrease in CSF Aβ1–42 protein was documented over a three year

follow-up period (Tapiola et al., 2000). A highly significant correlation between low CSF concentrations at baseline and follow up. In a separate study, no correlation was found between CSF levels and duration or severity of AD (Andreasen et al., 1999b).

2.4. Combination of CSF amyloid and tau phosphorylation

The current limitations of the predictive value of Aβ 42, t-tau and p-tau181 as AD biomarkers alone, these have been used together to develop a "CSF AD signature", again, with mixed results (Shaw et al., 2009;Mattsso et al., 2009;Kauwe et al., 2009;Mihaescu et al., 2010;Breno et al.,2008;De Meyer et al., 2010). While some studies indicate that the combination Aβ 42, t-tau and p-tau181 biomarker signature in CSF has high predictivity in identifying cases of prodromal AD in MCI patents (Shaw et al.,2009; Jack et al.,2010; Hansson et al., 2006), there is considerable intersite variability that can confound biomarker accuracy (Kauwe et al., 2009). Reduced CSF Aβ 42 and increased CSF p-tau181 concentrations – were used independently of a clinical diagnosis to stratify patient groups (De Meyer et al., 2010). This AD signature was found in 90%, 72%, and 36% of patients with AD, mild MCI, and cognitively normal groups respectively (De Meyer et al., 2010). The cognitively normal group with an AD signature were enriched in apolipoprotein E4 alleles. Validation of these findings in two further data sets showed that 64/68 (94% sensitivity) of autopsy-confirmed AD patients were classified with an AD signature while 57 MCI patients followed for 5 years had a sensitivity of 100% in progressing to AD based on their biomarker signature. The presence of a CSF AD signature in cognitively normal subjects was interpreted by the authors as an indication of AD pathology being present and detectable far earlier than previously envisioned in disease progression.

2.5. NF proteins

Neurofilaments (NFs) are neuron-specific intermediate filaments and serve as a major cytoskeletal component in neurons. In a mature mammalian neuron, NFs are co-assembled from three subunits, termed NF-H (high), NF-M (medium) and NF-L (low). As NFs are confined to the nervous system, they might be one of the best markers reflecting neuronal pathogenic changes seen in some neurological disorders, such as AD. In AD brain, the levels of phosphorylated NF-H/M (pNF-H/M) have been found to be markedly increased (Wang et al., 2001). Hu et al., (2002) found that, the levels of phosphorylated NF-H/M (pNF-H/M), non-phosphorylated NF-H/M (npNF-H/M) and NF-L were significantly higher (pNF-H/M,,12–24-fold; npNF-H/M,,3–4-fold) in neurologically healthy aged people than young individuals. In AD, the levels of npNF-H/M, and NF-L were similar to vascular dementia (VaD), and higher than in age-matched controls and the levels of pNF-H/M were significantly higher AD and ALS than in aged controls and VaD. Based on these findings, it is suggested that the increased level of total NF, p-NF proteins in CSF could be used as a marker for brain aging and neurodegenerative disorders in general, and the levels of pNF-H/M as a marker to discriminate AD from normal brain aging and as well as neurological conditions including VaD (Hu et al 2002).

Specific antibodies derived from aberrantly and hyperphosphorylated neuronal intermediate filament peptides from AD brain as bio markers for early AD detection

In addition to hyperphosphorylated- tau, recently we have demonstrated the direct evidence of aberrantly and hyperphosphorylated neuronal intermediated proteins (NF-M/H) as integral part of NFTs of AD brain using phosphoproteomics (Rudrabhatla et al., 2011., table 5). Although, NFs have been shown immunohistologically to be part of NFTs, there has been debate that the identity of NF proteins in NFTs is due to the cross-reactivity of phosphorylated NF antibodies with phospho-Tau. This study has provided a direct evidence on the identity of NFs in NFTs by immunochemical and mass spectrometric analysis. For these studies purified NFTs were used and liquid chromatography/tandem mass spectrometry of NFT tryptic digests were analysed (table 4-6). The phosphoproteomics of NFTs clearly identified NF-M phosphopeptides (table 5). Western blotting of purified tangles with SMI31 showed a 150-kDa band corresponding to phospho-NF-M, while RT97 antibodies detected phospho-NF-H. These observations suggest that expression of some of these genes is elevated in AD in addition to their phosphorylation. Apart from phosphor Tau, phosphopeptides corresponding to MAP1B to Ser1270, Ser1274, and Ser1779); and MAP2 (corresponding to Thr350, Ser1702, and Ser1706) were also identified (table 6). These studies independently demonstrate that NF and other microtubule proteins are part of NFTs in AD brains (Rudrabhatla et al., 2011). These promising findings call for further studies on the diagnostic potential of specific antibodies derived from aberrantly and hyperphosphorylated neuronal intermediate filament (NF-M/H) peptides from AD brain as bio markers for early AD detection

Phosphopeptide	Phosphorylation site
TPPAPKT*PPSSGEPPK	Thr181
TPPAPKTPPS*SGEPPK	Ser184
TPPAPKTPPSS*GEPPK	Ser185
VAVVRT*PPKS*PSSAK	Thr231, Ser235
SRT*PSLPT*PPTR	Thr212, Thr217
TPSLPT*PPTR	Thr217
TDHGAEIVYKS*PVVSGDTSPR	Ser396
TDHGAEIVYKSPVVS*GDTSPR	Ser400
TDHGAEIVYKS*PVVSGDT*SPR	Ser396, Thr403

Table 4. Phosphopeptides and phosphorylation sites identified in NFT Tau

Phosphopeptides	Phosphorylation sites
NF-M SPVPKS*PVEEAK	Ser685
NF-M KAES*PVKEEAVAEVVTITK	Ser736
NF-M VSGSPSS*GFRSQSWSR	Ser33
NF-H EPDDAKAKEPS*K	Ser942

Table 5. Phosphopeptides and phosphorylation sites identified in NF-M and NF-H

MAP	Sequence	Phosphorylation site
MAP1B	VLSPLRS*PPLIGSESAYESFLSADDK	Ser1274
MAP1B	VLSPLRS*PPLIGSESAYESFLSADDK	Ser1270
MAP1B	VLS*PLRSPPLIGSESAYESFLSADDK	Ser1270
MAP2	KIDLS*HVTS*KCGS*LK	Ser1702, Ser1706
MAP2	VAIIRT*PPKSPATPK	Thr350

Table 6. Phosphopeptides and phosphorylation sites identified in MAP1 and MAP2

2.6. Microtubule-associated proteins and vimentin

Microtubules are polymers of α- and β-tubulin dimers that mediate many functions in neurons, including organelle transport and cell shape establishment and maintenance as well as axonal elongation and growthcone steering in neurons. The polymerization, stabilization, and dynamic properties of microtubules are influenced by interactions with microtubule-associated proteins (MAPs). Members of this protein family are classified by size: high molecular mass proteins (MAP1A, MAP1B, MAP2a, and MAP2b) and intermediate molecular mass MAPs (MAP2c, MAP2d, and tau) (Gonzalez-Billault,C et al.,2004).

Increasing evidence highlights the critical outcome of MAP modification in cytoskeletal disorganization associated with the early stages of AD development. A decreased content of MAP1B and tau associated with cytoskeletal breakdown was found in the brains of AD patients compared with those of control individuals, suggesting a decreased capacity of microtubule assembly and stability (Nieto,A et.al 1989). These results are consistent with those of Iqbal et al. (1986) describing a decreased capacity in the in vitro microtubule assembly from brain extracts of AD patients. One study has shown an early decrease in MAP2 labeling within dendrites from AD brain (Adlard, P. A., and Vickers, J. C. 2002). Other studies have demonstrated that MAP1B and MAP2 co-localize with NFTs (Kosik et al., 1984; Takahashi, et al., 1991). Alonso et al. (1997) studied the associations of the Alzheimer-hyperphosphorylated tau (AD P-tau) with the high molecular weight MAPs (HMW-MAPs) MAP1 and MAP2. The author found that AD P aggregate with MAP1 and MAP2. The association of AD P-tau to the MAPs resulted in inhibition of MAP-promoted microtubule assembly. These studies suggested that the abnormally phosphorylated tau can sequester both normal tau and HMW-MAPs and disassemble microtubules.

Vimentin is a 57-kDa intermediate filament (IF) protein commonly found in mesodermally derived cells. In the healthy adult brain, vimentin is lacking in neurons and generally restricted to vascular endothelial cells and certain subpopulations of glial cells at specific brain locations. Eli et al (2009) found that Vimentin was localized to neuronal perikarya and dendrites in AD brain, with vimentin-immunopositive neurons prevalent in regions exhibiting intra- and extracellular beta-amyloid1-42 (Aβ42) deposition. Neuronal colocalization of vimentin and Aβ42 was common in the cerebral cortex, cerebellum and hippocampus (Eli et al., 2009). Our lab recently discovered that the protein tangles which are a hallmark of the disease involve at least three different proteins rather than just one (table 4-6). The discovery of these additional

proteins, neurofilaments, MAP2 and Vimentin, should provide better understanding the biology and progression of the disease as well as provide additional biomarker at the early stage of the disease.

2.7. Other CSF biomarkers for AD

As the AD signature approach based on the amyloid and tau causality hypothesis of AD continues to evolve, other CSF biomarkers are also being assessed. These include CSF cytokines (Swardfager et al.,2010; Olson etal.,2010)– specifically TGFβ increases in AD CSF (Swardfager et al.,2010)– CSF proteomic profiles (Papassotiropoulos et al.,2006), clusterin (Thambisetty et al.,2010)and IgG antibodies from the adaptive immune system (Reddy et al.,2011) The latter is a field of intense research, despite the challenges in analyzing proteome profiles, and involves the study of differences in the CSF proteome in AD, MCI and control subject groups (Papassotiropoulos et al.,2006;Zhang et al.,2005; Castano et al 2006; Finehout et al.,2007; Marouf et al.,2009; Choi et al.,2010). One study (Maarouf et al.,2009) reported changes in a variety of CSF proteins including a-2-macroglobulin, α1-antichymotrypsin,a1-antitrypsin, complement and heat shock proteins, cathepsinD, enolase and creatine. The ADNI is also generating CSF proteomic profiles as part of its ''Use of Targeted Multiplex Proteomic Strategies to Identify Plasma-Based Biomarkers in Alzheimer's Disease'' (Miller et al., 2009).

3. Oxidized proteins: Potential candidate biomarkers in AD

Although the pathogenesis of AD is not yet fully known, it is clear that the disease is caused by a combination of risk factors. Among several hypotheses, oxidative stress is considered to play a significant role (Butterfield, 2007). Although CSF represents the most suitable biological fluid to study neurodegenerative diseases since it can reflect the biochemical changes occurring in brain, its analysis is not always easily feasible for a large scale screening, because the costs involved are enormous and procedures are invasive, uncomfortable and not without risk. For a full screening and early diagnosis, biomarkers easily detectable in biological samples, such as plasma, are needed. Up to now, the search for reliable biomarkers for AD in peripheral blood is very challenging because of difficulties with the standardization of the methods of analysis and the low reproducibility of the results. Although a set of plasma markers that differentiated AD from controls have been shown to be useful in predicting conversion from MCI to AD (Song., 2009), the study has not been yet verified by other researchers and the application of these candidate biomarkers have yet to achieve the diagnostic power, sensitivity, and reproducibility necessary for widespread use in a clinical setting. Oxidized proteins may represent potential candidate biomarkers for "oxidative stress diseases", such as AD.

The first report on protein oxidation in CSF samples was from Tohgi et al. (1999) who dem-onstrated that 3-nitrotyrosine moderately but significantly increased with advancing age, and showed a remarkable increase in patients with AD. As the free tyrosine concentration did not decrease, the increase in 3-nitrotyrosine with age or associated with AD did not appear to be directly related to an increase in free-nitrated tyrosines. Rather, the increased 3-nitrotyrosine

was likely due to an increase in nitrated tyrosines in proteins or increased degradation of 3-nitrotyrosin containing proteins, which are highly vulnerable to degradation. The most reliable CSF markers in AD are Aβ42 and tau. Low CSF Aβ 42 is associated with amyloid pathology in the brain and high Tau is linked with neurofibrillary pathology (Frey etal. 2005). Most subjects with decreased CSF Aβ42 and high tau develop AD during the follow-up (Herukka et al., 2007). Therefore, these CSF markers may reflect brain pathology and identify preclinical AD. Interestingly, the levels of CSF Aβ42 showed a tendency to correlate positively with serum oxidative markers in the whole study population and with plasma nitrotyrosines in AD patients. Moreover, a negative correlation between CSF tau and serum nitrotyrosine levels was evidenced in controls (Korolainen et al., 2009). The correlation between CSF AD markers and blood oxidative markers may suggest that oxidative metabolism is changed in AD. This hypothesis is further supported by the finding of decreased CSF protein carbonylation in APOE ε4 carriers, which is considered an important risk factor for developing AD (Raber et al., 2004) and correlates with redox proteomics studies that identified metabolic proteins as oxidatively modified and dysfunctional (Choi et al., 2004).

Subsequently, Ahmed et al. (2005) measured in CSF the levels of protein glycation, oxidation and nitration. The authors found that the concentrations of 3-nitrotyrosine, Nε-carboxymethyl-lysine, 3-deoxyglucosone-derived hydroimidazolone and N-formylkynurenine (as markers of protein glycation) were increased in subjects with AD. The Mini-Mental State Examination (MMSE) score correlated negatively with 3-nitrotyrosine residue concentration. These findings indicated that protein glycation, oxidation and nitration were increased in the CSF of subjects with AD. A combination of nitration and glycation adduct estimates of CSF may conceivably provide an indicator for the diagnosis of AD. Increased levels of protein aggregates in the form of fibrils together with increased lipid peroxidation have been shown, both in AD and MCI brain (Butterfield et al., 2010).

Advanced oxidation end products (AOEs,) during AD, colocalize with neurofibrillary tangles, senile plaques, microglia, and astrocytes and have been also measured in plasma. Advanced oxidation protein products (AOPPs), a relatively novel marker of oxidative damage, are considered as reliable markers to estimate the degree of oxidant-mediated protein damage. A significant increase in protein carbonyls in hippocampus (HP) and inferior parietal lobule (IPL) of AD subjects compared with age-matched controls was observed. Dityrosine and 3-NT total levels were reported to be elevated in the hippocampus, IPL, and neocortical regions of AD brain. Alterations in brain phospholipids pattern, a more specific assessment of lipid peroxidation, have been reported for AD brain (Lovell et al., 1995; Nitsch etal.,1992; Prasad etal., 1998). The levels of phosphatidylinositol (PI) and phosphatidylethanolamine (PE), rich in easily oxidizable PUFA, are decreased in AD brain. The levels of F(2)-isoprostanes [F(2)-IsoP], F(4)-neuroprostane[F(4)-NP], and isoprostane 8,12-iso-iPF2(α)-VI were also found to be increased in AD brain compared to controls (Montine et al.,2002; Mark et al,1999). An increase in free HNE has been demonstrated in amygdala, hippocampus, and parahippocampal gyrus of the AD brain compared with age matched controls (Markesbery.,1998). Several proteins mainly involved in energy metabolism pathways, pH regulation, and mitochondrial functions among others, were found carbonylated, HNE-bound or nitrated in AD brain (Sultana,

2006). Newman et al (2007) also reported that a number of proteins modified by glutathiony-lation in AD IPL.

Previous studies on CSF nitrite and nitrate levels in patients with AD have provided contra-dictory results, with some showing decreased nitrate levels (Kuiper.,1994), others showing unaltered nitrite/nitrate levels (Ikeda.,1995), and still others increased nitrate levels (Tohgi., 1998). However, another study from the same group showed that nitrite/nitrate levels in AD were stage-dependent, being elevated only in the early phase of AD and decreasing to control levels with disease progression (Tohgi., 1998). This finding was interpreted to reflect progres-sive reduction of neurons. In contrast, free 3-nitrotyrosine levels increased significantly in parallel with the severity of AD, suggesting that protein degradation increases with disease progression, resulting in increased release of free 3-nitrotyrosine from tyrosine residues that have been nitrated. 3-nitrotyrosine and the 3-nitrotyrosine/tyrosine ratios in the CSF, both of which are believed to reflect degradation of nitrated tyrosine-containing proteins, increased significantly with age and were remarkably higher in patients with AD than in controls.

A study by Choi et al. (2002) identified uniquely oxidized proteins in AD plasma. These authors applied two-dimensional gel electrophoresis (2DE) coupled with immunological staining of protein carbonyl and the oxidized proteins observed in the plasma of both AD subjects and non-AD controls were determined. However, the level of oxidation of these protein spots was markedly higher in the AD samples. They also found that the increased oxidation was not a generalized phenomenon. In the total protein stain profile, more than 300 spots were detected, but less than 20 spots were positive by immunostain-ing with anti-DNP antibody. Furthermore, of the seven proteins that were most intensive-ly oxidized, their relative levels of oxidation differed. These studies found that fibringen gamma chain precursor and alpha 1 antitrypsinprecursor showed increased levels of carbonyl groups in AD comparedwith controls (Stief et al., 1989).

4. Identification of a new plasma biomarker of AD using metabolomics technology

Current metabolomics research involves the identification and quantification of hundreds to thousands of small-molecular-mass metabolites (<1,500 Daltons) in cells, tissues, or biological fluids. The aims of such studies are typically to understand new diagnosis biomarkers, to understand the mechanism of action of therapeutic compounds, and to uncover the pharma-codynamics and kinetic markers of drugs in patients and in preclinical in vivo and in vitro models (Wilcoxen et al., 2010). Lipidomics is one of the metabolomics approaches used to analyze lipid species in biological systems (Hu et al., 2009; Han et al., 2005; Han and Gross, 2003). Investigating lipid biochemistry using a lipidomics approach will not only provide insights into the specific roles of lipid molecular species in healthy individuals and patients but will also assist in identifying potential biomarkers for establishing preventive or thera-peutic approaches for human health (Hu et al., 2009,Wenk.,2005; Rosenson.,2010). Lipidomics has recently captured attention, owing to the well-recognized roles of lipids in numerous

human diseases such as diabetes, obesity, atherosclerosis, and AD (Wenk et al., 2005; Watson., 2006; Steinberg,. 2005; Sato et al., 2010). In support of the hypothesis that lipid dysfunction plays an important role in AD pathogenesis, previous studies with post-mortem brain tissue samples have demonstrated altered lipidomes at the different stage of AD pathogenesis. For example, multiple classes of sphingolipids are altered not only at the late stage of the disease but also at the earliest clinically recognizable stage of AD. All major classes of phospholipids are ubiquitously decreased at the late stage of AD. Among these, the levels of plasmalogen (a major component in nerve tissue membranes counting for up to 85% of ethanolamine glycerophospholipid, or ~30% of total phospholipids of these membranes) are gradually reduced as progress of AD severity (Han et al., 2011). Sato et al (2011) established a lipidomics method for comprehensive phospholipids evaluation that identified 31 phospholipids as AD biomarker candidates in human plasma using LC/MS (Satoet al., 2010). Moreover, additional studies have suggested that AD associates with other lipid metabolism pathways and lipid carrier proteins such as apoE (Bertram et al., 2008; Corder et al., 1993; Farrer et al., 1997; Strittmatter et al., 1993).

A very recent study by Sato et al (2011) were able to find a biomarker desmosterol that changes in AD compared with plasma from healthy elderly controls. They have shown that desmosterol plasma level and the desmosterol/cholesterol ratio in the same patients was significantly decreased. This study is the first report that plasma desmosterol levels are decreased in AD and MCI. And future studies are needed to confirm whether desmosterol could become an attractive plasma AD biomarker that could perhaps also be utilized for diagnosis and as well as for monitoring noninvasively the effect of future AD drugs on disease progression.

5. MicroRNAs as biomarkers for AD

MicroRNAs (miRNAs) are a class of small, endogenous, noncoding RNA molecules that serve as posttranscriptional regulators of gene expression (Lee etal.,1993;Giannakakis etal.,2007). miRNAs are acquiring important and determinant roles in the regulation of brain gene transcription in health and disease: the fact that approximately 80% of the human brain genome is transcribed into RNA, but only about 2% of the genome is transcribed into protein, underscores the potential of various levels of RNA signaling and epigenetic mechanisms to contribute to physiological gene control (Makeyevetal., 2008). In the last few years, miRNAs have been emerging as important regulators of various aspects of neuronal development and dysfunction (Gao.,2007; Lukiw.,2007). The role of miRNAs in neurodegenerative diseases has been investigated using miRNA microarray profiling in brain tissue samples derived from patients and controls. Using miRNA expression profiling in cortex samples from a well-characterized clinicopathological series of elderly controls, MCI subjects and AD patients, Wang et al (2008) identified miR-107 to be specifically decreased early in the course of AD. Computational analyses predicted BACE1 mRNA as a target of miR-107 and correlative mRNA expression studies confirmed its role in regulating BACE1 expression. An independent miRNA profiling study by Hebert et al (2008) confirmed the importance of BACE1 regulation by miRNAs. The presence of a modulation of miRNA in regions of brain targeted by AD neuropathology was further demonstrated (Lukiw et al., 2008; Lukiw., 2009), thus suggesting

a specific involvement of miRNAs in pathogenetic signaling pathways associated with the AD process. Recent findings suggest that neuronal miRNA deregulation in response to an insult by Aβ may be an important factor contributing to the cascade of events leading to AD (Schonrock, et al., 2010). Of note, the upregulation of peripheral miRNAs in AD could contribute to the diminished plasma proteins reported to be predictive biomarkers for AD (Ray Set al., 2007). In addition, it has recently been reported that miRNAs can be detected in CSF: an altered regulation of miRNA expression in AD brains was paralleled by a modulation of miRNA levels in the CSF (Cogswell et al., 2008). These studies provide an initial hope that miRNAs could represent accessible biomarkers to support clinical diagnosis in the near future.

6. Timing and other influencing factors of biomarker use

Disease modifying drugs are likely to be most effective in the earlier stages of AD, before neurodegeneration is too severe and widespread, so trials for this type of drug will need to include AD cases in the earlier stages of the disease. Validated biomarkers that could enable accurate identification of AD pathology at an early stage would be of great use (Hampel et al., 2011). Alternatively, baseline biomarker measurements can be used for enrichment and stratification in proof-of concept studies, as well as for supporting go/no-go decision making of phase III trials. Biomarkers should be used in all stages of drug development including phase I, phase II and phase III. They can be used to enhance inclusion and exclusion criteria, for stratification. Biomarkers can also be used as outcome markers to detect treatment effects. Particularly, if biomarkers are intended to be used as surrogate endpoints in pivotal studies, they must have been qualified to be a substitute for a clinical standard of truth and as such reasonably predict a clinical meaningful outcome. Finally, biomarkers can be used to identify adverse effects. Nevertheless there are several pitfalls to be faced in the interpretation of biomarker data in AD drug development, such as the fact that biomarkers may be non-specific to AD, it may not be feasible to measure them in the appropriate system (i.e. the central nervous system) and the risk of over-interpreting biomarker data in phase II trials if statistical significance levels are not adjusted for multiple comparisons (Aisen, 2009). Failure to consider these issues could contribute to false conclusions and costly errors (Hampel et al., 2011; Hampel et al., 2004)

7. Conclusion and future directions

Several promising drug candidates with disease-modifying effect, such as Aβ immunotherapy, secretase modulators, and tau aggregation inhibitors, have now reached the stage of being tested in clinical trials. The promise of disease-modifying therapy has created a need for biomarkers to enable the clinical identification of the disease at an early stage. Early diagnosis will be of great importance since disease-modifying drugs are likely to be most effective in the earlier stages of the disease, before neurodegeneration is too severe and widespread. A large number of studies have demonstrated that tests based on

CSF t-tau protein, p-tau and CSF beta-amyloid1–42 have reasonable specificity and sensitivity when differentiating AD from normal aging. A smaller number of studies show similar accuracy when distinguishing AD from major depression. These tests may also be useful in detecting MCI patients who go on to develop AD.

Unfortunately, the value of these biomarkers to clinicians is limited, because they are not specific enough to accurately separate AD from other common forms of dementia, such as VaD and LBD. Sometimes the combination of both CSF t-tau protein and CSF Aβ1–42 markers does not markedly improve on their individual sensitivity. CSF p-tau, based on different phosphorylation epitopes of tau protein, has now been examined in a number of independent studies. Initial results are extremely promising, showing that different p-tau protein epitopes may substantially contribute to improved diagnostic accuracy of AD in comparison with healthy aged controls, elderly depressed patients and those with other types of dementia. Compared with CSF t-tau protein and CSF Ab1–42 markers, CSF p-tau is more specific and less influenced by age or degree of cognitive decline (Hampel et al., 2004). This has an important implication for the value of CSF p-tau to clinicians. If the marker becomes abnormal very early in the course of disease relatively independent from the degree of cognitive decline than the marker may be ideal as a diagnostic test. If, however, the marker is closely linked to current or future cognitive decline, then it may be better suited as a prognostic tool. Studies of all possible biomarkers to date in AD, suggest p-tau comes the closest to the ideal diagnostic marker. However, different epitopes of p-tau may have different strengths and weaknesses. CSF p-tau231 may be most useful in distinguishing AD from frontotemporal dementia (FTD). CSF p-tau181 may improve separation between AD and LBD. In addition, CSF p-tau231 may be the most useful prognostic marker candidates that predicts cognitive decline to AD in MCI subjects.Further studies are needed to decide whether detection of multiple phosphoepitopes may allow a distinct representation of AD related pathology at different stages of the disease (Augustinack et al., 2002).

NFTs contain aberrantly hyperphosphorylated Tau as paired helical filaments. Although NFs have been shown immunohistologically to be part of NFTs, there has been debate that the identity of NF proteins in NFTs is due to the cross-reactivity of phosphorylated NF antibodies with phospho-Tau. Our laboratory recently reported (Rudrabhatla et al., 2010, 2011) the direct evidence of NFs in NFTs. Moreover, neuronal death and degeneration may release fragments of these proteins into body fluids at sufficient levels to be easily detected by specific antibodies at early, preclinical stages of AD. A battery of antibodies to NF-specific phosphoepitopes and Tau in NFTs may offer a unique approach to the design of effective early biomarkers.

The rapidly developing fields of large-scale and massive-scale genomics, proteomics, and metabolomics are now joining functional neuroimaging, structural neuroimaging, and neuropsychometric contenders in the race to establish useful biomarkers of AD and other dementing illnesses. Redox proteomics studies have provided insights into the role of oxidative stress in AD pathology. Posttranslational modifications of brain proteins, induced by oxidative damage, lead to impairment and dysfunction of several cellular functions thus providing clues about important molecular basis of neurodegeneration associated to AD. In addition, these studies have identified specific therapeutic targets in this disorder. In recent

years, growing studies have been focused to establish a direct link between tissue specific oxidation and systemic oxidative damage (Blennow et al., 2010; Korolainen et al 2010; Ahmed et al., 2005; Aksenov et al., 2001). Correlations between total levels of oxidation markers in the brain and in the periphery have been shown. Although some of the reported results in AD are controversial, most of them support the presence of peripheral oxidative damage and of a characteristic panel of systemic oxidation that correlates with the occurrence of the disease. Studies investigating oxidative stress outside of the CNS, particularly in blood, while prove the occurrence of oxidative reactions, are not fully elucidating the complex cascade of events. Thus, one hypothesis is that oxidative stress first develops in the periphery as a result of different causes, and then it will contribute to perturb neuronal homeostasis, either by increasing the production of ROS or by depleting antioxidant defense, which will eventually lead to oxidative damage of the brain and neurodegeneration. The development of new plasma biomarkers could facilitate early detection, risk assessment and therapeutic monitoring in AD. On the other hand, it is also possible to imagine that oxidative stress starts in the CNS where several different metabolic end-products are formed and released into the blood stream. In this context, an important issue is to perform further studies in order to investigate the timing of appearance of oxidative damage signatures at systemic level during the onset of AD early stages and the progression to late stages.

Recently, important steps have been accomplished but there is still a lot of work to be directed towards the discovery, testing and validation of a panel of novel and old assays that could serve all the requirements for ideal biomarkers. However, the emerging trend which results from the collection of multiple data from different source is the wide variability among different studies that led to contrasting results. Thus, there is an urgent need to standardize protocols for replicate experiments on large population, which may allow to better under-standing the effect of systemic oxidative damage in the pathogenesis and progression of AD. Indeed, this is also evident by the lack of redox proteomics and microRNA studies applied to biological fluids. This approach has the power to search for specific microRNA and protein oxidative modification thus allowing the identification of altered miroRNA and protein in complex matrices such as body fluids, which may discriminate AD vs healthy condition.

There are several different reasons to support the development of more sensitive method to detect a biochemical marker in AD: to increase diagnostic accuracy; to identify MCI subjects who will progress to clinical AD; to monitor pharmacological and biological effects of drugs. There is an urgent need to add further peripheral markers of oxidative stress as useful diagnostic biomarker. There is clearly a growing interest among clinicians and basic scientists to tap on each other's expertise in the area of ageing neurobiology research. Such collaborations between geriatricians, neuroimaging specialists, neuropsychiatrists as well as molecular and cellular neurobiologists are being fostered. Further research is necessary to improve especially the early/differential biochemical diagnosis of AD. Some considerations need to be taken into account when designing future studies. These should include high numbers of relevant AD of different origin, a combination of biomarkers and other risk factors, long-term follow- up of patients and if possible neuropathological verification of the diagnosis. Standardization of methods seems critical to reducing inconsistency and increasing reliability. It is necessary to

implement common protocols for sample preparation, experimental design and generation of proteomics data. Thus, global initiatives of standardization are of critical importance and large multicenter studies are needed to further define the added diagnostic value when multiple biomarker modalities are combined.

The essential goal in biomarker discovery studies is the identification of preclinical marker, which facilitates disease diagnosis at earlystages, is hoped that markers of prognosis will enable clinicians to monitor whether new candidate treatments of AD are working, effectively and inexpensively and assesses the response to treatments by the time that disease-modifying treatments become available in clinical practice.

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