Biomarkers of Alzheimer Disease in Plasma

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Summary: Plasma and serum biochemical markers proposed for Alzheimer disease (AD) are based on pathophysiologic processes such as amyloid plaque formation [amyloid β-protein (Aβ), Aβ autoantibodies, platelet amyloid precursor protein (APP) isoforms], inflammation (cytokines), oxidative stress (vitamin E, isoprostanes), lipid metabolism (apolipoprotein E, 24S-hydroxycholesterol), and vascular disease [homocysteine, lipoprotein (a)]. Most proteins or metabolites evaluated in plasma or serum thus far are, at best, biological correlates of AD: levels are statistically different in AD versus controls in some cohorts, but they lack sensitivity or specificity for diagnosis or for tracking response to therapy. Approaches combining panels of existing biomarkers or surveying the range of proteins in plasma (proteomics) show promise for discovering biomarker profiles that are characteristic of AD, yet distinct from nondemented patients or patients with other forms of dementia. **Key Words:** Biomarkers, amyloid β-protein, isoprostanes, homocysteine, 24S-hydroxycholesterol, cytokines, proteomics.

INTRODUCTION

The profound biochemical and pathological alterations in the Alzheimer disease (AD) brain result from cellular processes such as amyloid precursor protein (APP) and amyloid β-protein (Aβ) metabolism, tau phosphorylation, oxidative stress, inflammation, and lipid dysregulation. Biomarkers of AD are intended to detect these features of AD pathophysiology in biological fluids. Cerebrospinal fluid (CSF) closely reflects the composition of the brain extracellular space, and is likely to have the highest yield in biomarker evaluation, as described by Blennow in the present issue of *NeuroRx®* (1:213–225, 2004.) Nonetheless, CSF is not routinely collected in the evaluation of AD, and lumbar puncture is not a widespread procedure in primary care, psychiatric practices, and geriatric practices that often care for AD patients. The identification of biomarker molecules in blood would be more widely applicable, and reduce the need for invasive, expensive or time-consuming testing.

AD is a challenging disease for the development of biomarkers. The assessment of AD biomarkers is complicated by diagnostic imprecision, the long asymptomatic prodromal stages, variability in clinical features and rates of progression, complex disease genetics and multiple molecular etiologies [e.g., presenilin-1 (*PS-1*), presenilin-2 (*PS-2*), and *APP* mutations; apolipoprotein E (*APOE*) allelic polymorphisms; and sporadic AD]. Additional issues arise in the case of serum or plasma biomarkers of AD relative to CSF. The physiology of the blood-brain barrier may limit potential diagnostic biomarkers to small molecules, lipophilic molecules, or molecules with specific transporters. Brain proteins and metabolites that pass into the plasma become markedly diluted into a biochemically complex medium.

This paper reviews proposed serum and plasma biomarkers for AD, focusing on markers of APP processing but also assessing measures of lipid metabolism, oxidation, and inflammation. Because the currently available measures lack high sensitivity and specificity for AD diagnosis, the paper concludes with a discussion of strategies for novel biomarker discovery.

MARKERS RELATED TO APP AND Aβ METABOLISM

Amyloid β-protein

Because amyloid plaques are a defining feature of AD neuropathology, and Aβ can be detected in CSF and plasma, Aβ measures in biological fluids are compelling candidate biomarkers for AD.[1] Aβ occurs in two prominent forms, containing 40 (Aβ40) or 42 (Aβ42) amino

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TABLE 1. *Plasma and Serum Aβ Levels in AD*

First Author [reference]	Assay Antibodies Aβ capture (epitope) Aβ-40/Aβ42	Diagnosis	*N*	Aβ40 (pmol/L ± SEM)	Aβ42 (pmol/L ± SEM)	Results
Scheuner [5]	BAN50 (Aβ1–16) BA27/BC05	AD Control	71 75	190 ± 5* 200 ± 10*	29 ± 2 27 ± 3	No difference by diagnosis
Tamaoka† [8]	BAN50 (Aβ1–16) BA27/BC05	AD Control Neurological control	28 25 40	70.6 ± 7.9 68.6 ± 4.2 63.4 ± 4.0	61.3 ± 7.6 43.1 ± 5.1 57.6 ± 5.7	No difference by diagnosis
Kosaka† [6]	BNT77 (Aβ11–28) BA27/BC05	AD Control Neurological control	44 15 22	68.7 ± 1.7 77.6 ± 2.5 74.1 ± 0.6	9.8 ± 0.5 10.7 ± 0.5 10.3 ± 0.6	No difference by diagnosis No effect of disease stage
Mayeux‡ [9]	6E10 (Aβ1–16) R162/R165	Incipient AD Control	64 105	38.3 ± 1.6§ 31.8 ± 1.2	23.4 ± 2.4§ 14.6 ± 1.2§	Increased Aβ40 and Aβ42 with age and incipient AD No effect of diagnosis on rate of change No effect of sex, *APOE*, family history of AD
Mehta†‡ [10]	6E10 (Aβ1–16) R162/R164	AD Control	78 61	77.2 (28–219)¶ 62.2 (10–139)¶	20.7 (7–250)¶ 23.0 (7–257)¶	Increased Aβ40 in AD Increased Aβ40 with age No effect of sex or MMSE
Vanderstichele†‡ [11]	3D6 (Aβ1–5) 21F12 (Aβ42)	AD Control DLB Nondemented Other dementia	39 12 6 9 10		34.4 ± 2.5 40.9 ± 8.5 36.1 ± 2.9 31.5 ± 1.6 40.0 ± 7.2	No difference by diagnosis No effect of sex or MMSE
Fukumoto [12]	BNT77 (Aβ11–28) BA27/BC05	AD MCI PD Control	146 37 96 92	52.4 ± 1.4 47.8 ± 2.5 48.0 ± 1.8 48.9 ± 1.8	7.4 ± 0.4 6.5 ± 0.5 7.0 ± 0.3 7.0 ± 0.3	No difference by diagnosis Increased Aβ40 and Aβ42 with age 37 No effect of medications, *APOE*, severity or duration of dementia
Mayeux†‡ [13]	6E10 (Aβ1–16) R162/R165	AD at baseline Incident AD Nondemented	79 86 365	43.6 ± 1.5 38.7 ± 1.4 37.9 ± 0.9	19.5 ± 0.8§ 21.7 ± 1.8§ 21.7 ± 1.8§ 16.7 ± 0.5	Increased Aβ42 in AD and incident AD Decline in Aβ42 over 3 years in incident AD Increased Aβ40 and Aβ42 with age Inverse relation between Aβ40 and cholesterol No effect of *APOE*

*Estimated from graph.
†SEM calculated from SD and *N*.
‡Aβ values converted from pg/ml to pmol/L (1 pg/ml = 0.284 pmol/L).
§Statistically significant difference relative to control group.
¶Median (range).
DLB = dementia with Lewy bodies; MCI = mild cognitive impairment; PD = Parkinson disease; MMSE = Mini-Mental State Examination.

acids depending on the C terminus (although various N-terminally truncated forms also occur). Aβ42 is the initial species deposited in brain, and is more toxic and fibrillogenic *in vitro*.[2–4] The development of sensitive ELISAs for Aβ40 and Aβ42 enabled the detection and quantitation of Aβ in human blood. Plasma total Aβ or Aβ42 was increased in familial AD with presenilin or *APP* mutations as well as in Down syndrome with *APP* triplication,[5–7] raising the possibility that sporadic cases of AD might be associated with detectable and diagnostic changes in the plasma levels of Aβ.

Several cross-sectional studies and two longitudinal studies investigated plasma Aβ measures in AD (Table 1).[5,6,8–13] Aβ40 was elevated in a study of 78 AD and 61 control cases[10]; however, most groups have found no significant differences between AD and control cases.[5,6,8,11,12] Aβ40 and sometimes Aβ42 levels correlated strongly with age[9,12–14] and with serum creatinine levels.[15] The broad overlap in plasma Aβ levels between AD and control cases indicates that plasma Aβ cannot reliably differentiate sporadic AD from control cases in a cross-sectional study.

Although not diagnostically useful, plasma Aβ measures can also be evaluated in the context of AD prediction, progression, and therapeutic monitoring. Two longitudinal studies suggested that high plasma Aβ42 levels were a risk factor for developing AD. In a study of 169 nondemented individuals with mean age 74.9 years, those who developed AD during an average follow-up of 3.6 years had higher baseline plasma Aβ42 levels; in individual patients, plasma Aβ42 levels declined by an average of 3% and Aβ40 levels by 12% over 3–4 years, independent of the development of AD.[9] In the Northern Manhattan Aging Study, individuals with AD at baseline or who developed AD within 5 years after plasma collection had higher levels of plasma Aβ42 than individuals who remained nondemented; plasma Aβ42 declined more rapidly over 3 years in individuals who developed AD during the follow-up period.[13] In cross-sectional studies, though, plasma Aβ levels did not correlate with measures of progression or dementia severity.[10,12,16]

Plasma Aβ measures are potentially useful in clinical studies as markers of the pharmacological effects of medications that affect APP processing. For example, reduction in plasma Aβ levels with treatment could confirm the mechanism of action of medications that inhibit the β-secretase or γ-secretase that produces Aβ. Cross-sectional studies found no significant effects of statins, estrogen, non-steroidal anti-inflammatory drugs, antioxidants, or cholinesterase inhibitors on plasma Aβ levels.[12,17] In contrast, in double-blind placebo-controlled studies, lovastatin reduced plasma Aβ levels over 3 months,[18] and transdermal 17β-estradiol was associated with a reduction of plasma Aβ40 over 8 weeks in a small subset of estrogen-naïve patients.[19] As a surrogate marker for therapeutics, medication-related changes in plasma Aβ levels do not necessarily imply clinical benefit, because plasma Aβ levels correlate poorly with severity of dementia.

Thus, plasma Aβ measures are not sensitive or specific markers for the diagnosis of AD. Increasing Aβ species in plasma with aging may be a peripheral reflection of the balance between Aβ production and clearance that in the brain contributes to age-related Aβ deposition and AD risk. Further study is required to clarify the role of plasma Aβ as a biomarker for predicting AD risk, tracking progression, and following the effectiveness of medications.

Brain-plasma Aβ flux

CSF Aβ levels do not correlate with plasma Aβ levels in individual patients[11,16]; even in APP transgenic mice, plasma Aβ levels do not correlate with biochemical or pathological measures of cerebral Aβ deposition.[20] Nonetheless, animal studies indicate that Aβ can pass between the CSF and plasma compartments.[21,22] Peripherally administered compounds with high-affinity binding to Aβ increased the flux of Aβ from the brain and CSF to the plasma in APP transgenic mice.[20,23] The amount of Aβ appearing in the plasma after administration of an anti-Aβ antibody to APP transgenic mice correlated strongly with hippocampal and cingulate amyloid deposition, as well as total brain Aβ by ELISA. If confirmed in humans, measures of brain-to-plasma Aβ efflux could be a peripheral indicator of the extent of cerebral amyloid deposition, even before onset of AD symptoms.[20]

Aβ autoantibodies

Passive and active immunization against Aβ42 reduced cerebral amyloid deposition in APP transgenic mice,[24,25] with suggestive related effects in a human clinical trial of active immunization.[26,27] Based on these results, it was hypothesized that people possessing autoantibodies against Aβ would be protected against AD. Autoantibodies against Aβ were detectable in human plasma and CSF, however titers were similar in AD and non-AD cases.[28,29] These studies focused on autoantibodies against monomeric Aβ; it is possible that autoantibodies against oligomeric or aggregated Aβ are the more clinically relevant species.[30]

Aβ antibody titers were evaluated as a marker of treatment effectiveness in the clinical trial of Aβ42 (AN1792) immunization for AD. Immunogenicity was followed by anti-Aβ antibody titers,[31] as well as by the development of antibodies reactive against amyloid deposits in AD brain tissue.[30] In a subgroup from this clinical trial, patients who developed antibodies reactive to amyloid plaques in AD tissue sections performed better on neuropsychiatric testing than patients who did not develop these antibodies.[30] Anti-Aβ antibody titers were not predictive of which patients developed subacute meningoencephalitis as a side effect of immunization (18 of 298 on active treatment).[31]

Platelet APP isoforms

Alterations in the isoform ratios of APP in platelets in AD were reported by two groups.[32–34] In platelets, 150 kDa intact APP is processed into 120–130 kDa and 110 kDa carboxy-truncated forms; all forms can be resolved by Western blot using the antibody 22C11. The ratio of truncated forms of higher molecular weight (120–130 kDa) to the lower molecular weight form ("APP isoform ratio") in platelets was reduced in AD and mild cognitive impairment,[32–34] but not in other dementias.[33] Sensitivities and specificities for AD diagnosis were in the 80–95% range, based on *post hoc* cutoff scores.[33,34] The reduction in the APP isoform ratio correlated with disease severity and progression.[33,35] Cholesterol reduction, niacin, simvastatin, and cholinesterase inhibitors corrected the abnormally low APP isoform ratios in AD cases.[36–38] APP isoform ratios inversely correlated with age in one study[32] but not another[33]; ratios were inde-

pendent of sex or *APOE* genotype.[32] Quantitation of platelet APP isoforms holds promise for tracking diagnosis, progression, and treatment effects. Limitations of the measurement include technique-related factors (tourniquet, anticoagulant, platelet activation), and the Western-blot-based procedure which precludes high throughput and consistent standardization.[39]

MARKERS RELATED TO CHOLESTEROL METABOLISM AND VASCULAR DISEASE

Cholesterol

Cardiovascular risk factors including hypertension, *APOE* genotype, and cholesterol levels affect AD risk.[40–43] High cholesterol levels were associated with an increased risk of AD or cognitive impairment in several cross-sectional and prospective studies,[41,43–45] although no association was found in the Framingham cohort,[46] and established AD cases had lower cholesterol levels in other studies.[47,48] Cholesterol levels were influenced by *APOE* genotype, sex, age, and stage of AD.[44,49] Blood lipids are modifiable by dietary or pharmacologic intervention, and the lipoprotein cholesterol profile is an established marker of the effects of cholesterol-lowering medications and the associated reduction in cardiac risk. Epidemiological data suggest that statins reduce the risk of developing dementia and AD. The relative risk of dementia in statin users was 0.21–0.29 in case-control studies from the United Kingdom and Canada.[50,51] A protective effect of similar magnitude was found in a review of the databases of three hospitals in the United States.[52] However, no effect on a secondary measure of incident cognitive decline occurred in a placebo-controlled study of simvastatin for cardiac disease involving 20,536 patients, despite a prominent lipid-lowering benefit.[53]

24S-Hydroxycholesterol

Plasma 24S-hydroxycholesterol reflects brain cholesterol homeostasis more closely than plasma total cholesterol. Excess brain cholesterol is converted to 24S-hydroxycholesterol, a brain-specific oxysterol which readily crosses the blood-brain barrier.[54] 24S-hydroxycholesterol levels in plasma represent a balance between production in the brain and metabolism in the liver. Plasma levels show a weak, if any, correlation with CSF levels.[55,56] 24S-hydroxycholesterol was elevated in AD CSF,[56,57] but was only inconsistently increased in AD plasma.[56–58] CSF and plasma 24S-hydroxycholesterol were reduced by statin and niacin treatment.[59–61] Although 24S-hydroxycholesterol is not a diagnostic marker for AD, high levels (similar to high cholesterol levels) may be a modifiable risk factor.

Apolipoprotein E

The *APOE* ϵ4 allele is associated with increased risk of AD, earlier age of AD onset, increased amyloid plaque load, and elevated levels of Aβ40 in the AD brain.[62–64] The apolipoprotein E (apoE) phenotype can be determined from plasma by isoelectric focusing or ELISA,[65,66] although standard *APOE* genotyping of genomic DNA is easier and as informative regarding AD risk.[67] *APOE* genotype influences apoE protein levels, with the *APOE* ϵ4 allele being associated with less apoE protein in plasma.[68] There was no consistent association of serum or plasma apoE protein levels with diagnosis when controlled for *APOE* genotype; studies documented elevated apoE levels in AD,[69] no difference,[70–72] or reduced levels relative to controls.[73,74] The ratio of apoE4 protein to apoE3 protein in the plasma of heterozygous *APOE* ϵ3/ϵ4 individuals did not correlate with AD diagnosis.[66]

Lp(a)

Lipoprotein Lp(a) is a low-density lipoprotein-like particle containing the plasminogen-like apolipoprotein (a) linked by disulfide bridge to apolipoprotein B-100. High Lp(a) levels are associated with atherosclerosis, coronary artery disease, and cerebrovascular disease.[75] Apolipoprotein (a) was detected in primate brain, suggesting that Lp(a) particles (which can also carry apoE) are involved in cerebral lipoprotein metabolism.[76] Serum Lp(a) levels were not associated with cognitive decline over 3 years within an elderly population,[77] although AD patients had higher levels of Lp(a) in a cross-sectional study.[48]

Homocysteine

Homocysteine is a thiol-containing amino acid involved in the methionine cycle as the demethylation product of methionine (which can subsequently be remethylated in vitamin B12-dependent and folate-dependent processes) and in the transulfuration pathway (in which it is irreversibly converted to cystathione in a vitamin B6-dependent process).[78] Elevated homocysteine is a risk factor for cardiovascular disease,[79] and seems to be an independent risk factor for AD.[80] Hyperhomocystenemia is associated with relative deficiencies of folate, B6, and B12, and with age, male sex, estrogen deficit, renal insufficiency, caffeine use, dopamine agonist use, and anticonvulsant use. The pathophysiology of hyperhomocystenemia and AD risk is unknown, but homocysteine levels are modifiable by vitamin supplementation.[78]

MARKERS OF OXIDATION

Antioxidant levels

Increased markers of protein, lipid, and nucleic acid oxidation and reduced activities of antioxidant enzymes in AD brain support a role for oxidative stress in the neurodegeneration of AD.[81,82] Antioxidants in plasma include carotene, lycopene, vitamin A, vitamin C, vita-

min E, urate, and bilirubin. Both vascular dementia and AD were variably associated with reduced serum or plasma levels of vitamins A, C, and E, even when controlled for nutritional status.[83–87] Although vitamin E delayed progression of AD in one clinical study,[88] antioxidant vitamin supplementation did not affect incident cognitive impairment over 5 years, as a secondary outcome of a large placebo-controlled cardiac study involving 20,536 individuals.[89]

Lipid peroxidation

Free radical damage of proteins and polyunsaturated fatty acids results in modified forms that can be measured in fluids as markers of oxidation state. Isoprostanes arise from free-radical-mediated peroxidation of polyunsaturated fatty acids. Isoprostanes were elevated in the brain in a number of diseases, including AD and Huntington disease.[90] F_2-isoprostanes were elevated in AD brain and CSF, with little overlap with control subjects.[91–93] F_2-isoprostanes were elevated in plasma as well,[93,94] although this was not confirmed by another group,[95] perhaps reflecting differences in measurement techniques or cohorts. Plasma isoprostanes are elevated in other illnesses associated with oxidative stress, and can be modified by antioxidant treatment.[96] 4-hydroxynonenal, another product of lipid peroxidation, was also increased in AD plasma.[86]

MARKERS OF INFLAMMATION

Amyloid deposition in the AD brain elicits a range of reactive inflammatory responses including astrocytosis, microgliosis, upregulation of proinflammatory cytokines, complement activation, and acute phase reactions.[97] Whether the accumulation of cytokines and acute phase reactants within brain is reflected in serum or plasma is not straightforward, because many of these proteins do not easily cross the blood-brain barrier. Alternatively, AD may be associated with a more widespread immune dysregulation, detectable in plasma.

Interpretation of measures of immune mediators in AD serum and plasma are limited by discordant results. Inflammatory molecules variably increased in AD include C-reactive protein, interleukin (IL)-1β, tumor necrosis factor-α, IL-6, IL-6 receptor complex, α1-antichymotrypsin, and transforming growth factor-β; these were unchanged in other studies, as were other cytokines including IL-12, interferon-α, and interferon-γ.[98]

IL-6, for example, has been extensively studied in AD. IL-6 is a cytokine implicated in inflammation, acute phase responses, and cell proliferation whose effects are mediated by a receptor complex including the IL-6 receptor α-subunit and glycoprotein 130. Several groups reported increased IL-6 in AD plasma or serum,[99,100–104] although this was not the case in other cohorts.[105–108] Confounding factors in these studies of inflammatory markers include differences in plasma collection protocols, assay methodology, assay sensitivity, small sample sizes, heterogeneous patient populations, effects of disease severity,[103] age,[109] and comorbid inflammatory illness.

BIOMARKER PROFILING

Given the multiplicity of pathophysiological processes implicated in AD, the diagnostic accuracy of biomarkers may be improved by combining several serum or plasma markers,[110] thereby creating a more robust biomarker profile characteristic of AD. Approaches to biomarker profiling can be "knowledge-based," incorporating the range of known putative AD biomarkers, or "unbiased," surveying hundreds or thousands of biomolecules using proteomic or metabolomic methods to discover novel molecular profiles representative of AD. The knowledge-based approach, which attempts to use as much of the known data regarding molecules implicated in AD, is exemplified by a study of 34 AD, 46 Parkinson disease, 47 patients with other cognitive disorders, and 61 controls that examined a panel of 29 serum biomarkers for inflammation, homocysteine metabolism, cholesterol metabolism, and brain-specific proteins. A model incorporating IL-6 receptor, cysteine, protein fraction α1 and cholesterol levels was superior to individual markers in discriminating AD from controls, although specificity relative to other neurological illnesses was weaker.[110] Confirmation in larger populations and incorporation of additional markers of amyloid metabolism and oxidative stress may improve sensitivity and specificity.

Large-scale unbiased approaches evaluate a broad range of proteins ("proteomics") or small molecule metabolites ("metabolomics") in biological fluids. Proteomics methodologies include: separation of proteins by 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) or HPLC[111]; surface chromatography by adsorbing proteins to activated surfaces (surface-enhanced or matrix-assisted laser desorption–ionization protein chip array technology)[112]; and peptide ionization procedures for analysis of proteins from gels or protein chips by mass spectroscopy. A 2D-PAGE study detected 350 silver-stained proteins in the plasma of six control cases, five AD cases, and three non-AD dementias, of which 73 spots were identified by sequencing or immunostaining, including the AD-related proteins apoE, tau, and presenilin-2.[113] The complexity of serum and plasma, imprecision of peak matching in mass spectroscopy and spot matching in 2D-PAGE, and difficulties in assay standardization make these approaches challenging, but advances in technology platforms and bioinformatics will allow broader applicability to diseases such as AD.[114]

CONCLUSIONS

Studies of plasma and serum biomarkers have not yielded a consistent, easily reproducible, sensitive, or specific marker for AD diagnosis, risk, progression, or treatment effects. Measures of APP metabolism, inflammation, cholesterol metabolism, oxidative stress, and homocysteine homeostasis appear to be altered in AD relative to controls, but without sufficient discriminatory power. Several measures are responsive to medications, for instance statins reduce cholesterol and 24S-hydroxycholesterol levels, folate reduces homocysteine levels, and Aβ immunization produces anti-Aβ antibodies. However, a consistent correlation with clinical benefit must be convincingly demonstrated before any measure can be recommended as a surrogate marker in clinical trials (as opposed to being merely markers for the mechanism of drug action.) Omnibus measures combining several biomarkers or incorporating proteomic and metabolomic profiles are promising approaches for the development of plasma or serum-based diagnostic tests for AD.

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