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Developing Novel Blood-Based Biomarkers for Alzheimer's Disease

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1. Introduction

The search for biomarkers of Alzheimer's disease (AD) has yielded numerous expensive and/or invasive candidates including putative disease markers obtained by magnetic

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resonance imaging (MRI) and positron emission tomography (PET), and those that require collection of cerebrospinal fluid (CSF) via lumbar puncture. While considerable progress has been made in demonstrating how these biomarkers relate to the pathophysiology of AD [1], there remains an urgent need for less costly and intrusive, and more widely available blood-based (serum or plasma) biomarkers that can aid in the early diagnosis of AD and to predict disease progression. To assess the state of the science regarding blood-based biomarkers for AD, the Alzheimer's Association and the Alzheimer's Drug Discovery Foundation (ADDF) brought together leading investigators at a meeting in New York, NY on April 12, 2013. The goals of the meeting were to begin outlining the research challenges related to the development of blood based biomarkers, including understanding the context of use from a clinical, research, and regulatory perspective; highlighting the need for standardization and harmonization of protocols; and identifying knowledge gaps and the research efforts needed to fill those gaps. This manuscript summarizes the meeting and the resultant discussion, including identified steps to move the field forward.

2. Challenges in Developing Blood Based Biomarkers

The failure to replicate findings has been the greatest challenge preventing widespread acceptance of any blood-based assay for AD [2]. Between-study variability arises from availability of samples, the variability in the samples themselves, as well as both pre-analytic and analytic components of the assays. Pre-analytic components of the assay include the choice of anticoagulant, needle size, needle components, order of the blood draw, whether the sample undergoes a denaturation/extraction step or the addition of a protease inhibitor [2], the conditions under which the sample is stored, the use of plasma or serum, and the number of freeze/thaw cycles. Blood is complex, comprised of multiple cellular compartments and an ever-changing environment of proteins, lipids, and other biochemical entities [3]. These may be adding to the issues of reproducibility, and require coordinated efforts to align the methodology used across studies.

Analytic aspects that contribute to variability include different assessment platforms, methods of analysis, and analytic performance characteristics such as precision, analytical accuracy, and dilution linearity. Other hurdles to the development of blood-based assays for neurodegenerative diseases include the existence of multiple pathologies and co-morbidities in individuals diagnosed with AD or related dementia (e.g., Lewy bodies, TDP-43, vascular disease), further increasing assay variability, and the relative low number of clinical samples to utilize for standardization and confirmation of study design. In addition, because the peripheral blood has no direct contact with the brain, CSF markers of neurodegeneration may not be present in the blood or at detectable levels, making it difficult to apply correlating CSF biomarker findings to the blood.

3. Blood based markers of amyloid pathophysiology

Amyloid-β (Aβ) is a widely studied plasma biomarker for AD, although the extent to which blood (plasma, serum, cell bound or free) Aβ levels accurately reflect the presence or state of AD is not clear. A meta-analysis by Koyama et al of thirteen studies and 10,303 subjects examining plasma $A\beta_{1-42}$ and the ratio of $A\beta_{1-42}$/ $A\beta_{1-40}$ as predictors of dementia and AD

and concluded that a decrease in the plasma $A\beta_{1-42}$/ $A\beta_{1-40}$ ratio is a statistically significant and clinically meaningful predictor of subsequent cognitive decline [4]. However, significant heterogeneity underlines the need for further investigation of plasma Aβ levels as a preclinical biomarker. Factors that may explain heterogeneity in these studies include the participant's age, the underlying cognitive health of subjects [5], diet of the participants [6], the assay used, and inter-lab variability [4]. In contrast to these results, the plasma Aβ levels from subjects in the Alzheimer's Disease Neuroimaging Initiative (ADNI) showed very little correspondence to clinical state [5].

In addition to $A\beta_{1-42}$ and $A\beta_{1-40}$, blood levels of other Aβ species, particularly $A\beta_{1-17}$ have been proposed to be useful for the diagnosis of AD. In one report, the ratio of free- to cell-bound $A\beta_{1-17}$ in blood discriminated between healthy individuals and individuals with MCI or mild AD with high sensitivity and specificity [7]. Other novel Aβ isoforms may also be useful for monitoring the biochemical effect of treatment. For example in a clinical study of the gamma-secretase inhibitor LY450139 (semagacestat), shorter Aβ isoforms were elevated in the CSF in a dose dependent manner, while there was no effect on plasma Aβ or CSF $A\beta_{1-42}$ or $A\beta_{1-40}$ [8].

A number of platforms have been developed to measure Aβ levels in blood. For example, Araclon Biotech Ltd. has developed an ELISA (enzyme-linked immunosorbent assay) colorimetric tests for $A\beta_{1-40}$ and $A\beta_{1-42}$ in blood to measure direct and calculated biomarkers associated with an increased likelihood of having MCI [9]. Other ELISA-based assays and tests using electrochemiluminescence are also in development [10].

Investigators are also searching for other disease pathology related to proteins in blood (i.e. tau, alpha-synuclein, TDP-43, and FUS), providing insight into not only AD but other neurodegenerative diseases (i.e. Parkinson's disease, Frontotemporal Dementia). Tau is most closely related to neuronal death and is hypothesized to be fragmented in the blood. Henriksen et al described the recent development of a digital ELISA for total tau in serum [11] and suggest that this type of technology may also enable the detection of Aβ peptides. Investigators are examining whether tau cleavage fragments correlate to disease stage [12].

4. Markers of inflammation, oxidative stress, mitochondrial dysfunction, and neuronal and microvascular injury

Neuropathological studies of AD brains reveal not only the presence of amyloid plaques and neurofibrillary tangles, but also neuroinflammmatory changes involving astrocytes and activated microglia, which secrete inflammatory mediators including cytokines, chemokines, components of the complement system, and reactive oxygen species [13]. Systemic inflammation further alters immune function and 'switches' the primed innate immune cells to an aggressive pro-inflammatory phenotype resulting in increased cytokine production and neuronal damage [14]. Because systemic inflammation (e.g., from pneumococcal or urinary tract infection) is rampant in the elderly, this may explain why individuals with AD or other acute inflammatory events (e.g., surgery or brain injury) experience these types of infections and also exhibit increased levels of the proinflammatory cytokine TNFα and a 2-4 fold increase in the rate of cognitive decline [15]. The inflammatory biomarker, serum soluble

TNF receptor 1 (sTNF-R1) has also been suggested to enable discrimination of AD from control samples [16]. Future studies should assess not only cytokines and chemokines, but also immune signatures in both cells and fluids following ex vivo stimulation of peripheral blood mononuclear cells (PBMCs) [2]. Identifying and confirming inflammatory and immune-related biomarkers demands a multi-assay, multi-disciplinary approach, stratified by the presence or absence of systemic infection and other inflammatory comorbidities (e.g. diabetes, obesity).

Neuronal and microvascular injury are two other common neuropathological findings in the AD brain [17]. Sphingolipid metabolism, including ceramides, sphingomyelins, and sulfatides, plays a central role in neuronal function, and results in the synthesis of bioactive metabolites that have been associated with the pathophysiology of AD (reviewed in [18]). Serum levels of ceramides differ between healthy controls, MCI and AD across multiple platforms [19]. High baseline ceramides are associated with an increased risk of cognitive impairment in cognitively normal individuals and cognitive decline and hippocampal volume loss in persons with MCI [19, 20].

Possible markers of microvascular injury include cell adhesion molecules such as intracellular adhesion molecule 1 (ICAM-1), vascular adhesion molecule 1 (VCAM-1) and selectins. Increased plasma levels of VCAM-1 have been found in both late-onset AD and vascular dementia compared with controls, suggesting endothelial dysfunction in both types of dementia [21].

5. Blood-based biomarker panels

Investigators have also assessed whether other proteins in the blood can be used to diagnose AD. O'Bryant et al developed an algorithm using a different set of 30 serum proteins to produce a risk score with 80% sensitivity and 91% specificity for detecting AD [22]. This set of proteins included many inflammatory and vascular markers, and has been shown to provide a high level of diagnostic accuracy in AD comparable to CSF analyses [23] and when combined with demographic and clinical data, correlates with neuropsychological test performance [24]. In a panel of 18 signaling plasma proteins, researchers differentiated AD from control with close to 90% sensitivity and identified individuals with MCI who would progress to AD within 2-years with 81% sensitivity [25]. Another blood-based biomarker panel using multivariate data analysis identified a panel of three blood markers (cortisol, von Willebrand factor, and oxidized LDL antibodies) that distinguished early AD individuals from cognitively normal individuals with over 80% accuracy [26]. Recent work from the AD plasma proteomics project has identified a panel of proteins in plasma, using the Luminex platform, with some overlap to CSF and an association with the ?4 allele of APOE [27]. Quantitative mass spectrometry based Selective Reaction Monitoring (SRM) assays are also in development. As an example, Proteome Sciences has implemented an assay to quantify a panel of 9 candidate proteins within plasma using isotopic Tandem Mass Tag (TMT) technology to measure endogenous levels of distinct peptides against equivalent heavy labeled reference peptides for normalization [28]. The development of assays to quantify particular post-translational modification of proteins is also being pursued.

6. Emerging biomarkers and methods for new discovery

Biomarker signatures are being developed through the use of proteomics, metabolomics, and gene expression studies, with a demonstrated lack of reproducibility across cohorts. A quantitative multiplex proteomic immunoassay panel comprised of 18 analytes was shown to have 70% diagnostic accuracy in early AD [29]. Gel-based proteomics in individuals with AD, MCI or cognitively normal individuals showed a panel of five proteins involved in complement activation and coagulation predicted brain atrophy [30].

AD may be associated with brain gene expression signatures correlating with similar signatures in blood [31]. Skepticism, however, exists concerning whether changes in gene expression in blood white cells would show any relationship to gene expression in brain, in AD or in any CNS disease. Nevertheless, several gene expression-based tests as clinical diagnostics are under development and may be potential tools for an AD diagnostic workup [32, 33]. Similarly to proteins that undergo conformational and post-translational changes (which themselves may provide biomarkers of dementia [34]), gene expression changes in response to environment (i.e. stress, aging, time of day, etc). One gene maker identified to be associated with AD is TOMM40 (translocase of outer mitochondrial membrane 40 homolog), which is thought to play a role in transporting proteins into the mitochondria, and has been shown in several GWAS studies to be down-regulated in AD [35]. Studies of TOMM40 gene expression in blood suggest that a down-regulation of the level of expression may serve not only as a marker for AD, but may also correlate with disease severity or progression [36].

Clusterin, also called ApoJ, is involved in the immune response and is a known amyloid chaperone protein. A genome wide association study (GWAS) identified *CLU*, the gene encoding clusterin, as a risk loci for AD [37]. Although plasma levels of clusterin are not elevated in individuals with AD [38], clusterin is associated with baseline prevalence and severity of AD [39]. Clusterin was also found to be elevated in MCI subjects, negatively associated with cognition [40], and correlated to rate of brain atrophy [41] in some, but not all studies [42]. In a study seeking to identify associations between plasma proteins and hippocampal atrophy and rate of decline, clusterin/ApoJ was the only protein shown to correlate with both of these endophenotypes [43].

Metabolomics technologies provide powerful tools for the diagnosis and exploration of the mechanisms underlying disease. Several different analytic platforms are available to interrogate the metabolome. These technologies have been applied to both animal and human studies and in both plasma and CSF, revealing pathways that are disrupted in AD and MCI. Measures in plasma appear to accurately reflect changes in the CSF and allow discrimination between controls, MCI, and AD. For example, in the plasma of individuals with AD, pathways affected include those related to the metabolism of polyamines, lysine, and tryptophan, and the biosynthesis, from aminoacyl-tRNA in the cytoplasm. Plasma markers related to cortisol biosynthesis from cholesterol, obesity, and type II diabetes mellitus are also affected [44]. For example, desmosterol, a precursor of cholesterol, is decreased in the plasma of individuals with AD compared to controls, and correlates with changes in cognition [45], suggesting that it may be a diagnostic biomarker for AD.

Combining metabolomics signatures with other biomarkers may provide even greater specificity.

Autoantibodies are abundant in human serum and may also represent "signatures" of disease. For example, individuals with AD were distinguished from non-demented controls using 10 autoantibody biomarkers with high specificity and sensitivity; and a panel of only 5 autoantibody biomarkers distinguished individuals with AD from those with PD, with over 86% accuracy [46].

7. Conclusions and future directions

In the current state of biomarker development for AD, blood based biomarkers stand out to the community (research, clinical and individuals affected by AD) as the holy grail of early detection in AD. However, blood based biomarkers do not yet provide valid diagnostic information. This review provides insight into current international efforts by the research community to develop collaborations to address the many challenges that exist as described in Section 2, such as pre-analytical procedures and post-analytical assessments, low sample size availability and the variability among study participants. Single protein assay development has made significant progress in recent time but the need for standardization of efforts from start to finish is still of paramount importance to advance clinical benefit.

This review highlights new developments in international collaborations. The Blood-Based Biomarker Interest Group was established earlier this year as an international working group of leading AD scientists from academia and industry to develop global standards/ best practices for assessing blood based biomarkers; a summary of these initial efforts is summarized in this issue [47]. A professional interest area focused on Blood Based Biomarkers (BBB-PIA), as a part of the Alzheimer’s Association’s International Society to Advance Alzheimer's Research and Treatment (ISTAART), formed to assist in developing a field-wide consensus on the harmonization of pre-analytic and analytic protocols and to address the need for a bio-repository of clinical reference samples to enable not only assay harmonization but also clinical performance assessment. Progress toward clinical utility will require defining the protocol and determining the purpose for which a blood-based biomarker is needed, and accessing samples from defined clinical longitudinal cohorts and other consortia. The development of blood based biomarkers for AD can also model ongoing efforts in the CSF biomarker field, such as the Alzheimer’s Association Global Biomarker Standardization Consortium, which has developed successful strategies for performance assessment across multiple test sites, the development of reference calibrator material and assay harmonization for CSF measures [48]. Building on lessons from the CSF biomarker community, the blood based biomarker research community will need to expand to incorporate validation across sample collection, assay development, standard measures, and study protocol.

Global collaborations to convene researchers are critical to accelerate the development of blood-based biomarkers, to avoiding the pitfalls of the CSF, MRI experiences. Although blood-based biomarkers are not yet ready for research and clinical use in AD, these efforts will accelerate the efforts. To this end, the Alzheimer’s Association ISTAART BBB-PIA

will aims to facilitate international discussion and to support all collaborative efforts of moving forward, with the goal of aligning the efforts to accelerate progress and bring a validated, reliable and inexpensive blood-based method to diagnose, detect and monitor AD progression to fruition.

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