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Recent Progress in the Identification of Non-Invasive Biomarkers to Support the Diagnosis of Alzheimer's Disease in Clinical Practice and to Assist Human Clinical Trials

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Additional information is available at the end of the chapter

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1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder that manifests itself by progressive dementia accompanied by memory deterioration usually in elderlies and is becoming the public health crisis of the 21st century. Currently, there are an estimated 35 Million patients affected by the disease, and this number is expected to burgeon to 115 million by the year 2050 (WHO, 2012). In the United States alone, one patient is diagnosed with AD every 67 seconds according to the Alzheimer's Association website.

This situation is very alarming since Alzheimer's disease has been a graveyard for drug developers with an astonishing 99.6% of trials of potential Alzheimer's treatments aimed at preventing, curing or improving the symptoms of the disease failing or being discontinued from 2002 to 2014 [1]. Although there are FDA approved drugs available including acetylcholine esterase inhibitors (donepezil, rivastigmine, galantamine) and the NMDA receptor antagonist memantine that have been useful in temporarily alleviating short-term memory problems or improving daily functions, they are ineffective in stopping disease progression.

AD is characterized by the presence of amyloid plaques in brain and it is hypothesized that the increase levels of toxic Amyloid beta oligomers and protofibrils leads to Tau neurofibrillary tangles formation, loss of synaptic connections and selective neuronal cell death in the brain (Figure 1) and this sequence of events is referred as the amyloid cascade hypothesis [2]. The amyloid plaques are mostly composed of amyloid-beta peptides (Abeta 40-42) thought to be

toxic once they self-aggregate and subsequently bind to a cell surface to disrupt neuronal signaling and cell viability [3]. It is initially thought that downstream to this event is the formation of neurofibrillary tangles composed of hyperphosphorylated Tau protein. Such hyperphosphorylation is an indicator of neuronal cell death in numerous neurodegenerative disorders or brain injuries [4, 5] indicating that both abnormal processes can take place independently [6]. Two key enzymes necessary for the cleavage of the Amyloid Precursor Protein (APP) to generate Amyloid-beta peptides are the gamma and beta-secretase. According to the amyloid cascade, it is thought that developing Inhibitors of those enzymes would prevent amyloid formation and stop disease progression. Several companies have therefore been testing such inhibitors in human trials. Unfortunately, this has proven to be harder than anticipated. While Bace1 inhibitors trials outcomes are not yet known at the time of this writing, gamma-secretase inhibitors had disappointing results in late-stage trials where worsening of cognition was observed [7]. The reason for this is not totally clear, but the fact that gamma-secretase is responsible for the cleavage of multiple substrates including NOTCH protein may have been a contributing factor.

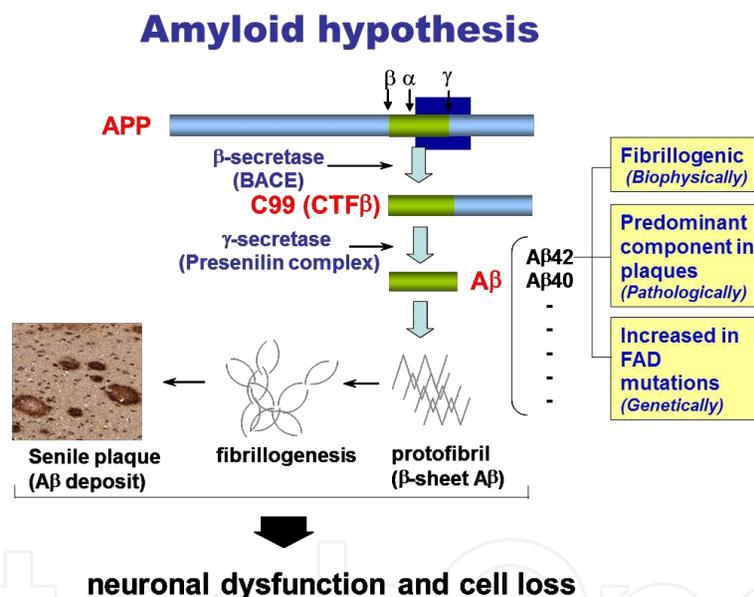


Figure 1. Transmembrane APP protein can be cleaved by three proteases; Beta, Alpha, and Gamma-secretase. Cleavage by B-secretase and G-secretase produces Abeta peptides (mainly 40 and 42). Aggregation of Abeta peptides into toxic oligomers and protofibrils to brain cells is a critical event prior to Abeta plaques formation and disruption of neuronal function and cellular loss.

Other clinical approaches around the amyloid cascade are focusing on passive immunization using administered human monoclonal antibodies against the amyloid-beta peptides, oligomers, protofibrils or plaques [8-10]. Several advanced phase 2 and 3 trials are still ongoing (Table 1) but at least one phase 3 trial outcome, although it did not meet its endpoints has revealed that patients with the mild form of the disease seemed to respond better to treatment [11, 12].

Based on this data, it appears that it might be too late to stop disease progression in patient with mild-to-moderate to severe AD patients with anti-amyloid therapies, so companies are

now focusing their efforts on testing those drugs, including beta-secretase inhibitors, in early Mild Cognitive Impairment patients (MCI) which are known to convert to AD more rapidly, especially if patients test positive for amyloid deposition using Positron Emission Tomography scans (PET) [13, 14]. It also comes as no surprise that companies developing these new therapies are now adding being positive on amyloid PET scan as entry criteria in recent clinical trials [15] (table1). Unfortunately, the cost of amyloid PET imaging is very expensive, and PET centers are not currently available worldwide [16-18]. Even if Amyloid-PET is proven to be useful to identify a target patient population, it is important to also develop a non-invasive biomarker that could either be singly used to identify amyloid positive patients or used as a first-line test before Amyloid PET imaging confirmation.

Drug	Trial phase	Patient population	Enriched Study population	Amyloid PET	CSF Abeta	CSF Tau	FDG-PET	VMRI
Anti-amyloid passive immunotherapies								
Solanezumab	Phase 3	mild AD	yes (Amyloid PET)	yes	yes	yes	no	yes
Gantenerumab	Phase 2/3	autosomal-dominant AD	yes (Amyloid PET)	yes	yes	yes	yes	yes
Crenezumab	Phase 2	mild-moderate AD	no	yes	yes	yes	yes	no
		autosomal-dominant AD	yes (genetics)	yes	no	no	yes	yes
Ban2401	Phase 2	early AD	yes (Amyloid PET)	yes	yes	yes	no	yes
BIIB037	Phase 1	prodromal/mild AD	yes (Amyloid PET)	yes	no	no	no	no
LY3002813	Phase 1	mild-moderate AD	yes (Amyloid PET)	no	no	no	no	no
MEDI1814	Phase 1	mild-moderate AD	no	no	yes	no	no	no
Bace inhibitors								
MK-8931	Phase 2/3	mild-moderate AD	no	yes	no	no	no	yes
	Phase 3	prodromal AD	yes (Amyloid PET)	yes	no	no	no	yes
E2609	Phase 2	prodromal AD/mild AD	yes (Amyloid PET)	yes	yes	yes	no	no
JNJ-54861911	Phase 1	prodromal AD	yes (Amyloid PET)	yes	yes	no	no	no
AZD3293	Phase 1	mild/moderate AD	no	no	yes	no	no	no

Source: Clinicaltrial.Gov and various press releases.

Table 1. Please add caption

In this book chapter, we will review the recent progress in the development of non-invasive AD biomarkers that could be used for such purpose by various research groups with a focus on AD biomarkers our group recently identified in patients' plasma.

2. The diagnosis of Alzheimer' Disease and the need for non-invasive markers

The disease is difficult to diagnose correctly even with the availability of cognitive tests and sophisticated Imaging technologies that include MRI, FDG-PET and Amyloid PET imaging. Currently, a diagnosis of probable AD is made using NINCDS-ADRDA criteria but this is usually possible when the condition has developed and progressed to a point where neuronal cell death and/or irreparable damages have already occurred [19]. While the accuracy of this test was thought to be around 80-90% when it was developed in the early 80's, it's accuracy, especially to diagnose patients at the early stage of the disease, is much lower which further complicates AD biomarker discovery.

The inability to correctly diagnose AD has also probably negatively affected the development of novel therapies aiming at stopping the amyloid cascade via gamma-secretase inhibitors as well as passive immunization therapies using antibodies against Aβ peptides or Aβ plaques [7, 11]. The possible inclusion of patients suffering from non-AD dementia in those trials may have been a contributing factor to those failures.

As a result, research efforts have intensified exponentially in the recent years to identify and develop biomarkers that could be used for diagnosing AD early to support clinical practice and clinical drug development [20].

Much of these efforts have initially focus on looking at pathological changes of amyloid beta peptides, Aβ_{40/42} in CSF as well as P-Tau and T-Tau and has eventually led to the development of a model that define Alzheimer's disease progression [6, 21, 22]. In that original model, gradual reduction in Aβ₄₂ is observed in CSF, presumably due to the aggregation of the peptide in brain and formation of plaques which is followed by gradual elevation of P-TAU and TAU in CSF, indicators of neuronal cell death or injury [23, 24]. The model was initially received with great interest because it described the temporal evolution of AD biomarkers in relation to each other and the onset and progression of clinical symptoms. However, emerging evidence appeared that challenges this model's assumptions. Refinements to the model now include indexing of individuals by the time rather than clinical symptom severity; incorporation of inter-individual cognitive impairment variability in relation to AD pathophysiology progression; modifications to when some biomarkers changes sequentially appear; and acknowledgement that the two major proteinopathies in AD, amyloid beta (Aβ) and tau, might be initiated separately from one another in sporadic AD [6].

Although useful to assist clinical diagnosis of AD with enough sensitivity and specificity [23, 25], stiff barriers exist that prevent the comprehensive utilization of those markers by physicians and especially primary care doctors. Lumbar puncture, for example, that is required to collect CSF is still a delicate medical intervention in several developed countries and is also accompanied by increased frequency of headaches [26]. The nature of the Amyloid peptides itself is also complicating the picture. Recent data have indeed shown that the Aβ₄₂ peptides are prone to stick to collection tubes and their detected concentration is affected by various parameters such as storage temperature, volume and thawing [27-29], probably explaining the frequent lack of correlation between labs using the same immunoassay kits.

Separately to CSF analysis, the research field has also developed a series of imaging approaches to assist clinical diagnosis such as Volumetric Magnetic Resonance Imaging (MRI) (to measure brain areas volume), FDG-PET and Amyloid PET imaging. Those are useful but currently provide only prognostic value to predict the likelihood to convert from MCI to AD [30, 31]. Amyloid PET tracers such as Pittsburgh Compound B and two new tracers, florbetapir-18 and flutemetamol-18, are approved as an *in vitro* diagnostic (IVD) but only to rule out possible AD pathology since a significant % of patients that test positive might never develop the disease [32]. Moreover, Positron Emission Tomography (PET) is very costly, and the scarcity of centers capable to handle this technology is still an issue in many countries. In UK, for example, only ~30 centers can perform this test, and the numbers are even lower in countries such as China [33]. These agents, although not reimbursed in US and other countries, are now proving useful to assist the development of novel drugs aiming to test the amyloid cascade hypothesis and

are being used as enrollment criteria by several companies developing beta-secretase inhibitors as well as passive immunotherapies using anti-amyloid antibodies (Table 1). If these new therapies succeed, the availability of Amyloid-PET imaging as Companion Diagnostic (CDx) will still present the issues mentioned here as well as create additional economic burden on many healthcare systems. It is therefore accepted that having a first-line non-invasive diagnostic blood test comparable to Amyloid PET imaging would be precious in the clinical setting and could be used in tandem to diagnose patients correctly.

3. Recent progress in AD biomarker discoveries

3.1. Amyloid beta peptides and TAU in blood

Given the apparent association between Abeta accumulation and increase of P-TAU and Tau in brain and CSF of AD patients, several studies have looked at the change of Abeta 40/42 ratio in serum and plasma as non-invasive AD marker. At least 14 studies including our own that examined the change in such ratio in AD have been conducted [34] but have produced mixed results. It is not clear why such discrepancy is observed, but several factors not only related to patient's selection but also to assays themselves and how samples were stored and handled are possible explanations. It should be noted that even the Alzheimer's Disease Neuroimaging Initiative study (ADNI) data could not link Abeta40/42 plasma ratios to clinical state [35]. What further complicate the use of plasma Abeta as an AD marker is the fact that it is produced not only centrally but also in the periphery and the nature itself of the peptide which tend to stick to walls and aggregate on itself affect the epitopes available during ELISA assays [34, 36].

Recently, researchers have also looked at Abeta 1-17 as a possible diagnostic marker of AD. One report showed that free-to-cell bound ratio of Abeta 1-17 could discriminate Control, MCI and AD patients with high sensitivity and specificity [37]. Additionally, plasma BACE1 enzyme, one key enzyme essential for the generation of Abeta peptides as well as soluble APP beta (sAPPbeta) have been found to be elevated in one study in AD patients plasma [38]. Despite the challenge of reliably measure Abeta 1-42 in plasma, a group demonstrated that APP669-711 appeared to be an indicator of pathological change of Abeta1-42. Ratio of APP669-711 to Abeta1-42 (APP669-711/Abeta1-42) measured by MALDI-TOF mass spectra showed a very good correlation with PIB+ signal in brain, suggesting that this plasma biomarker could be developed as a surrogate marker of cerebral amyloid deposition[39].

As for Tau and P-Tau detection in blood, demonstrating association with AD has been very challenging [40], especially for P-TAU due to the presence of circulating phosphatases in blood [24, 41] and the fact that TAU/P-TAU is elevated in multiple types of dementia including brain injuries [42]. A recent paper reporting the increase of an enzyme-generated fragment of TAU in serum that is inversely associated with cognitive function [43] seems promising. Another recently developed assay using antibodies reacting to all TAU isoforms could show with greater sensitivity than usual EIA methods the elevation of total Tau in serum of patients suffering from severe brain ischemia [44]. Another group described the finding of oligomeric form of TAU in AD patients platelets [45] providing 76% sensitivity and 80% specificity. Time will tell if these TAU assays will be useful as a screening tool to support AD diagnosis.

3.2. Amyloid beta oligomers in blood

Amyloid beta ($A\beta$), especially $A\beta_{42}$ oligomers play a significant role in early Alzheimer's disease (AD) pathogenesis [46, 47]. In fact, AD-associated inflammation has been thought to be a secondary response to the pathological lesions triggered by $A\beta$ oligomers in the early stage of pathogenesis. Although several studies, including our own (unpublished) have shown an elevation of such oligomers in CSF [48-52], few studies have looked at the correlation between blood oligomers concentration. In one study, levels of plasma $A\beta$ monomers, $A\beta$ oligomers, and soluble tumor necrosis factor α receptors (sTNFRs) were evaluated by ELISA in 120 controls, 32 amnesic mild cognitive impairment (aMCI) patients, and 90 mild AD patients [53]. The study found that levels of $A\beta$ oligomers were significantly increased by ~two fold in mild AD patients compared to levels in aMCI and healthy controls. Interestingly, plasma levels of sTNFR in aMCI and mild AD patients was elevated significantly compared to controls, and both sTNFR1 and sTNFR2 levels were associated with levels of $A\beta$ oligomers in both aMCI and mild AD individuals. Interestingly, changes in $A\beta$ oligomer concentrations and sTNFR levels correctly differentiated mild AD from healthy control subjects.

In a separate study [50], another group have demonstrated that their ELISA system using BAN50 can detect signals in 60% of serum samples and 80% of CSF samples obtained from non-demented subjects.

individual peptide/ protein (plasma, serum)	comments	reference
Abeta 40/42 ratio	mixed results by various group	(34)
Abeta 1-17	free to bound cell ratio discriminate Control, MCI and AD	(37)
BACE1 enzyme, sAPPbeta	elevated in plasma	(38)
APP669-711/Abeta1-42	significant correlation with brain amyloid deposition (PIB+)	(39)
TAU fragment	level in serum inversely correlates with cognition decline	(42)
multiple TAU isoforms combination	elevated in patients suffering from brain ischemia	(43)
Oligomeric TAU	increase levels identified in platelets	(44)
Abeta oligomers (serum)	higher in MCI and AD subjects, not detected in all samples	(49, 52)
sTNFr	higher in MCI and AD subjects	(52)
proteins panels (plasma)		
30 serum proteins combination	set of several inflammatory and vascular related markers combined with clinical data	(54, 55)
18 signalling plasma proteins combination	can differentiate AD and C, predict MCI conversion to AD (1 study could not reproduce this finding)	(56,57)
Cortisol/WWF/oxidized LDL antibodies	can distinguish AD and C with 80% accuracy	(58)
Lipids (plasma)		
10 lipids combination	predicted phenocconversion from MCI to AD within 2-3 y	(61)
Ceramide/sphingomyelin	elevation correlates with MMSE score	(62)
Desmosterol/Cholesterol	decreased in MCI and AD, decrease % change in longitudinal cohorts correlates with rate of cognitive decline	(66,73)
Genes, mRNAs, miRNAs		
96 genes signature (blood)	algorithm correctly predicts AD and discriminate Parkinson's Disease (CE mark test)	(75)
136 genes signature (blood)	algorithm identify AD patients over Controls (CE mark test)	(76)
48 genes signature (blood)	identify AD patients over Controls with even more accuracy when combined with MRI	(77)
TOMM40	expression in blood potentially useful to monitor AD progression/severity	(79)
98-5p,885-5p,483-3p,343-3p,191-5p,7d-5p	Asian population (Serum)	(131)
Let-7g-5p,142-3p,15b-5p,301s-3p,545-3p,191-5p,7d-5p	Caucasian population (Plasma)	(120)
71,1285,107,103a-3p,26b-5p,26a-5p,532-5p,151a-3p,161,7d,112,5010-3p	Caucasian population (Whole blood cells)	(135)
miR-132,128,874,134,323-3p,382	Caucasian population (Plasma) (MCI correlation)	(109)
9,29a,29b,34a,125b,146a	Asian population (Plasma,CSF)	(158)
Others		
Two retinal amyloid depositions scans	detected after oral ingestion of curcumin tracer prior to eye scan using laser found good correlation with PIB amyloid positivity and/AV-45 ointment containing tracer applied to eye before laser scan	AAIC 2014 O2-05-05 O3-12-01
Impairment of smell detection ability	association with brain region atrophy and prediction of conversion from MCI to AD	AAIC 2014

Table 2. Summary of non-invasive AD biomarker candidates

Although the levels of serum Abeta oligomers were reported to be unexpectedly high, the authors made the suggestion that the assay could be detecting non-pathological Abeta complexes associated with serum carrier proteins. Nonetheless, they did show a significant positive correlation with the levels obtained from matched CSF samples, suggesting that this assay system might be useful to support AD diagnosis.

4. Emerging blood-based AD biomarkers: Reproducibility of findings difficult

Novel non-invasive AD biomarkers found in blood are emerging as being a composition of different proteins, metabolites or gene transcripts in blood cells or single analytes. In total, there are as many as 21 literature studies in recent years looking at blood-based proteins association with AD. While the studies varied in size, they all looked at more than 100 proteins and the total number of patients examined ranged from 14 to 961, the 2 largest cohorts being ADNI (566) and AIBL (961). Kiddle et al. have recently published a report where they tried to replicate the findings of those 21 studies that linked a total of 163 proteins to AD using Somalogic's SOMAscan proteomics technology. 94 of those 163 candidate AD biomarkers were assessed in a relatively large cohorts of 677 subjects [54]. Only 9 candidate protein biomarkers were actually found to be related to at least 1 AD-related phenotypes: Pancreatic prohormone, Granulocyte colony-stimulating factor, Clusterin, Complement C3, Complement C6, Insulin-like growth factor-binding protein 2, Alpha-1-antitrypsin, inter-alpha-trypsin inhibitor heavy chain H4 and C-C motif chemokine 18. The outcome of this extensive replication study illustrates well the difficulty the field has been facing when trying to confirm previous findings in different patient cohorts.

5. Protein panel assays in development

Various protein panel assays have been developed by several groups with the use of algorithms to predict AD correctly. This approach is based on the assumption that combining markers together will increase the power of the test to identify patients correctly. One assay, in particular, is looking at 30 serum proteins and has 80% sensitivity and 91% specificity for diagnosing AD [55]. The set of proteins is composed of several inflammatory and vascular related markers and the assay, combined with clinical data, showed a correlation with neuropsychological test performance [56].

Another group identified a panel of 18 signaling plasma proteins that can differentiate AD and control with ~90% sensitivity and identify MCI patients likely to convert to AD within 2 years with 81% sensitivity [57]. However, these results could not be reproduced independently [58]. Combination of 3 blood markers (cortisol, von Willebrand factor and oxidized LDL antibodies) was able to diagnose AD with 80% accuracy [59]. Quantitative mass-spectrometry-based selective reaction monitoring (SRM) is also supporting the development of AD diag-

nostic tests [60] by using isotopic tandem mass tag (TMT) technology to evaluate specific peptides derived from selected AD-related proteins. This approach, although very sound, is more difficult than one would think. In fact, when we tried in-house a similar technique called MRM (multiple reaction monitoring), we could not replicate several AD biomarker protein candidates discovered by other groups. Intriguingly, several peptides from the same protein showed changes in opposite directions (unpublished).

6. Plasma lipids as non-invasive AD biomarkers

The disturbance of several lipid pathways in the brain, in particular in cholesterol biosynthesis has been associated with several brain disorders including AD [61]. So it comes as a little surprise that this category of molecule changes in blood to be another rich source of potential AD biomarkers. In a recent study, 525 community-dwelling healthy participants, aged 70 and older were enrolled as part of a 5 year's observation study. Over the course of the study, 74 patients developed either MCI or mild AD. Using a lipidomic approach, the authors identified and validated a set of 10 lipids from peripheral blood that predicted phenotypic conversion to either MCI or AD within a 2-3 year period with over 90% accuracy [62]. To our knowledge, this study is the first report of a blood-based marker panel that can detect preclinical AD with such accuracy although validation using other cohorts will be required before considering clinical use. As the authors pointed out, alteration of lipids found in the cell membrane may be sensitive markers of neurodegeneration in pre-clinical AD. Another study using shotgun lipidomics, compared AD with control individuals and found a change of ceramide/sphingomyelin ratio in AD [63] and its elevation to correlate with Mini-Mental-State-Examination scores (MMSE). This small study (26 AD and 26 controls) needs to be replicated though. Interestingly, a separate group found that an increase in this ratio was associated with slower disease progression [64]. Analysis of a longitudinal cohort of AD and control samples showed that AD patients had diminished baseline levels of either phospholipids, phosphatidylcholines, sphingomyelin and sterols as opposed to controls although they could not confirm the lipid profile to be a good prognostic panel for estimating the progression to AD [65].

Our group initially discovered plasma desmosterol, the precursor of cholesterol, a metabolite that was recently identified as an LXR and ROR γ agonist [66, 67], as a candidate AD plasma marker [68]. Desmosterol is an essential sterol with hormone-like activity and accounts for as much as 30% of all brain sterols during most species brain development [69, 70]. Multiple activities of desmosterol have also been reported, and it is understood that disturbances of the cholesterol metabolism may contribute to neurodegeneration [71, 72].

In our first study, decreased levels of desmosterol were observed (p value < 0.05, fold change = 0.36) in AD plasma samples versus control plasma as well as in CSF [68]. Other groups also reported a decrease of desmosterol in brain as well as CSF [73] in an independent study but not in plasma. The discrepancy was understood in-house after we determined that this was due to an incomplete separation of cholesterol-desmosterol peaks during Gas Chromatography (AAIC 2012 abstract). Interestingly, we also observed a decrease of desmosterol also in

MCI and in particular, more pronounced in plasma of female AD patients plasma. This change of desmosterol in contrast was not affected by ApoE4 genotype. This finding was further validated and presented recently (AAIC 2013 abstract) using two large cohorts: a commercially available Caucasian sample set and a large Asian cohort. The Caucasian sample set consisted of a total of 109 patients (Control, MCI, and AD) and the large Asian cohort (n=401, 200 C and 201 AD) were both analyzed using LCMS. Our original data showing the association between decreased desmosterol/cholesterol ratio in AD and MCI was replicated in these cohorts. Data analysis showed that desmosterol level in plasma was found to be significantly different from AD and control groups with p-values $2.3E-14$ and comparable AUC of ROC curve as initially found. High correlation between plasma desmosterol level and MMSE score was observed for these two large cohorts. As for novel AD candidate markers, we believe specificity should be investigated in other dementia types in order to understand the clinical usefulness of the marker and this work is currently on-going. In addition, the longitudinal analysis revealed that plasma Desmosterol/Cholesterol ratio (DES/CHO) in AD patients shows a significant decrease at follow-up intervals. The decline in plasma DES/CHO is larger in the AD group with rapid progression than in that with slow progression and the changes in plasma DES/CHO significantly correlated with changes in MMSE score.

Altogether, this data means that plasma DES/CHO decrease in AD patients may serve as a longitudinal surrogate marker associated with cognitive decline. This data, as well as an additional longitudinal cohort data analysis, is now in press at the time of this writing[74].

Very interestingly, a minor allele of an intronic SNP within DHCR24 gene (the gene coding for the protein responsible to convert desmosterol into cholesterol) was identified in a recent ADNI study and was associated with a lower average PiB PET uptake, a first generation imaging amyloid PET agent that is used to understand amyloid deposit load in AD brain [75]. It is tempting to speculate that lower desmosterol levels in the brain (reflected as well in plasma and CSF) could be directly linked to higher amyloid deposition.

In order to further understand the utility of desmosterol as an AD biomarker, we collected patients plasma samples obtained through one of our ongoing AD clinical phase 2 trial, that were either positive or negative on Amyloid Pet scans (Flurbetamol) and data analysis is now ongoing. Possible outcome of this study could help patient stratification in further trials and lead to the development of a first line test prior to conducting more expensive PET imaging scans for patients enrolment in future trials or to the development of a stand-alone *in vitro* diagnostics.

7. Genes, mRNA, and miRNAs

Because gene transcription and translation ultimately determine the production of proteins that regulate cells and tissue functions, several groups have been looking at molecular changes in AD vs Controls in blood components and circulating peripheral cells to identify biomarkers. Among these, one group looked at the expression of 96 different genes in blood. A whole genome analysis was conducted using oligonucleotide microarray and blood from a large

clinical cohort consisting of AD patients and control healthy subjects. a. Gene analysis comparing the gene expression of 94 AD patients and 94 cognitive healthy controls was conducted, and a disease classifier algorithm developed [76].

Validation was conducted on an independent cohort consisting of 63 subjects that included 50% AD patients, 40% aged-matched controls and 10% young healthy controls. The results showed the test to have an accuracy of 87% to predict AD pathology. Additionally, the algorithm also discriminated AD from Parkinson's disease in 24/27 patients (accuracy 89%).

Another group developed an alternate gene AD signature consisting of 136 different genes using 177 blood samples (90 AD patients and 87 controls) [77]. Signature validation was then later performed on a blinded independent cohort of 209 individuals (111 AD and 98 controls). Many of the genes included in the signature are found to be elevated during inflammation processes and apoptosis and have been associated with the amyloid cascade and tau pathology. In a follow-up validation study consisting of 164 patients.. This test performed relatively well and was able to identify AD patients (81.3% sensitivity) correctly and to exclude AD pathology (67.1% specificity). Both of these tests have won approval in Europe (CE Mark) as AD biomarker and are available to physicians but they still haven't been validated in large clinical cohorts such as the Alzheimer's Disease Neuroimaging Initiative (ADNI ½).

At least two other studies showed this transcriptome approach potential. In one study [78], a gene expression signature was discovered in a 156 patients cohorts consisting of AD and controls. The validation study confirmed the performance of the gene signature in a separate cohort composed of 26 AD, 26 healthy age-matched control and 118 mild MCI individuals classified as probable early AD subjects. The 48 genes signature accurately identified 70% of AD patients and when combined with MRI defined criteria, the accuracy went up to 85%.. However, the authors indicated that these results have to be validated in other diseases or dementias.

The same group also looked at changes in gene expression in leukocytes and found alterations in blood seen mild cognitive impairment (MCI) and AD subjects indicating a peripheral response to pathology may occur very early [79]. Noticeably, evidences for mitochondrial dysfunction indicated by a reduce expression of several respiratory complex I-V genes were observed, confirming changes previously seen in AD brain.

One novel single gene marker identified that is associated with AD is TOMM40 (translocase of outer mitochondrial membrane 40 homolog). The protein encoded by TOMM40 seems to transport proteins functionally to mitochondria. Risk Mutation in this gene has been found in several GWAS studies, and one group showed that its expression in blood may serve as an AD marker of disease severity and progression [80].

8. miRNAs

Beside the existing proteomic, metabolomics and nucleic acid based markers, small RNAs (including miRNAs) are an upcoming class of circulating biomarkers that have resulted in

many new findings. miRNAs belong to the class of non-coding regulatory RNA molecules of ~22nt length that regulate gene expression post-transcriptionally by binding (in most cases) to the 3' un-translated region (UTRs) of their targets [81-83]. It is estimated that ~5% genes in the human genome encode for miRNAs and a single miRNA can regulate multiple targets (sometimes in excess of 200) based primarily on the complementarity of the seed region (nt 2-8 of the miRNA) to target mRNA molecules [84]. MicroRNAs play regulatory roles in vital biological processes, including cell proliferation and growth, tissue differentiation, development, and cell death[85]. Interestingly, it has recently been demonstrated, that not only are miRNAs active in their cell of origin, but they can be exported/secreted out, and cause down-regulation of target mRNAs in an alternate target cell [86]. It is this unique property of miRNAs of being present in intact and functional condition in circulating biofluids including CSF, plasma, serum, urine, tears and saliva, which makes them promising biomarker candidates. They are found enclosed in membrane-bound structures (exosomes, microvesicles etc.) [87, 88], and in some cases in "free" form, protected by RNA binding proteins like NPM1, HDL [86, 89] or Argonaute2 [90, 91]. Circulating miRNA signatures have been shown to identify different tumor types [92, 93] indicate staging and progression of the disease [94] and serve as prognostic markers [95, 96]. Recently, five miRNA based diagnostic tests have been made available for clinicians to prescribe (through Rosetta Genomics and Asuragen Inc). Although the first generation of tests requires tumor biopsies, there is now significant work in progress to eliminate the need for getting biopsies, and to be able to get answers from blood, urine or other readily available circulating fluids.

Although the potential of miRNAs as diagnostic markers has been consistently demonstrated in Oncology; recent publications in other areas like neurodegenerative disorders point to their expanding role [99]. In AD, for example, miRNA profiling experiments (in brain tissue) have resulted in the identification of many disease-specific miRNAs that have been confirmed independently in two or more studies [97]. For example, hsa-miR-106, hsa-miR-153 and hsa-miR-101 have been shown to modulate APP [98-101], while BACE1 has been shown to be targeted by hsa-miRNA-29 and hsa-miR-107, linking miRNAs to regulation of amyloid production in AD brains [102]. Based on similar studies, researchers have focused on these disease-specific miRNAs to determine if differential levels are found in more-easily accessible biofluids like blood or CSF. Hsa-miR-29a/b including others was a disease-specific miRNA whose down-regulated levels in the serum of AD patients mimicked the expected down-regulation in the brain tissue [103]. This is a more disease-focused approach, where only those miRNAs that have a known link to the illness is profiled for. However, the nature of circulating fluids, which allows all organs, tissues to be potential sources of biomarkers makes a simple correlation with only diseased focus biomarkers (miRNAs, in this case) hard. There is also now a confirmed presence of a selective gating mechanism that determines a particular profile of miRNAs to be exported out (in exosomes or protein bound). This was recently demonstrated in studies that showed that secreted miRNA profiles (from culture) were not in correlation with intra-cellular profiles. This could explain why higher level of a miRNA in an affected organ is not automatically associated with an increase in its plasma level [104, 105]. Another approach, still under the umbrella of disease-relevant miRNAs looks not at the disease etiology, but broadens the net and looks for all miRNAs known to be expressed in the tissue/

organ of interest. Hence for AD for examples, miRNAs known to be enriched in neurons and synapse destruction were focused on [109-111]. As a result, miR-132 and the miR-134 family of miRNAs were discovered which showed potential for differentiation between MCI and AD, and, in fact, could also predict 1-5 years in advance of a clinical diagnosis. Potential biomarkers like these could be instrumental in identifying the population which would respond best to therapy in the future, or at least identify the correct pool of patients who are MCI for example, but would advance to AD in the absence of any treatment. On the Neurodegeneration side, some focused miRNA analysis has uncovered candidates like miR-146a and miR-155 that were found in higher levels in brain tissue extra-cellular fluid (ECF) in AD patients [112]. Along with the recent report on let-7b that is being investigated as a TLR-7 ligand [113], these recent findings point towards the potential role of inflammation, which ultimately could lead to neurodegenerative disorders.

Without limiting the miRNA profiles to either disease etiology or organ/tissue of focus, unbiased-global profiling is another approach to biomarker research. This is now especially more feasible, considering the significant technological advancements that have allowed researchers to look at thousands of biomarkers using as little as 100 ul of blood, for example. Another reason, why an unbiased approach might be appealing to certain researchers is the potential of finding novel pathways that have so far not been implicated in the disease of interest, and this is especially true for complex, heterogeneous disorders like AD, where there is still a lot of work on going in trying to understand all the biology of the disease. Of course, on the flip-side, it is often difficult to explain the biological significance and connection of the novel biomarker for the illness. The problem is more severe for miRNAs, because it is not a simple miRNA-mRNA relationship, but rather a single miRNA, and hundreds of potential mRNA targets [114-116], which makes it even harder to predict connections to disease. To put this conundrum of multiple miRNA targets into a biological context, this publication proposed [117] that usually biologically meaningful targets of miRNAs were found to be enriched in specific pathways, or a network. The first un-biased miRNA study in blood (PBMCs) was done in 2007 [106] followed by a much-cited study by Cogswell et al.[107] that identified miRNAs differentially expressed in brain and CSF of AD/matched controls. In addition to some related pathways being implicated in neuronal differentiation and actin remodeling (through targets of miR-9 and 132), novel target pathways like brain insulin signaling and oxidative stress were identified. However, the surprise finding was the lack of correlation between CSF and brain profiles, which again hinted at a particular secretion mechanism that regulated the transfer of miRNAs from the cell. In addition, differentially expressed CSF miRNAs like miR-146b (thought to be involved in immune function) were found to be decreased in AD patients, suggesting an activated immune status, potentially offering insights into the role of inflammation in the disease.

Consistent with the global profiling approach, our group had published a novel AD signature that had >95% accuracy in determining AD status from matched controls [108]. It consisted of reduced levels of 7 miRNAs (hsa-let-7d-5p, hsa-let-7g-5p, hsa-miR-15b-5p, hsa-miR-142-3p, hsa-miR-191-5p, hsa-miR-301a-3p and hsa-miR-545-3p) which was further confirmed in an independent sample-set of 20 AD and 17 NC samples, To put a biological context to the

hundreds of potential miRNA target molecules, we enriched for mRNA molecules that were targeted by multiple miRNAs (at least 2). Some neurological canonical pathways identified included axonal guidance signaling, ephrin receptor signaling [109], actin cytoskeleton signaling [110], clathrin-mediated endocytosis signaling [111] and RhoA signaling [112]. These pathways, although diverse, show potential biological relationships with disease etiology [125]. Using an unbiased analysis approach, we removed the filter of neurological pathways in IPA, and got a list of pathways enriched for signature miRNA targets. A type II Diabetes Mellitus signaling canonical pathway was identified. This was interesting because there was also evidence from multiple GWAS studies indicating that SNPs in ApoE [113], Clu [114] and ABCA7 genes [115] were linked to AD biology. This was in addition to another report that linked lipid metabolism to both amyloid and tau pathology [61]. However, due to unclear outcomes after statin treatment in AD clinical trials, the role of lipid metabolism in AD pathogenesis remains to be elucidated [116]. In another global-approach driven study in serum, miRNAs were profiled from 50 AD and 50 matched control samples using next-generation sequencing [117]. This was followed by a validation study using qRT-PCR in an independent cohort of 158 AD and 155 control populations. Amongst other signature miRNAs identified, miR-191-5p and let-7d-5p were identified to be down-regulated in AD patients. This was encouraging because it validated part of our miRNA signature in blood (serum) using a different profiling technology (NGS) as opposed to a hybridization-based technology (nCounter: Nanostring) used by our group. This suggested that the signature had biological relevance and was not likely a profiling or normalization artifact, which often results in little validation rates of miRNA signatures.

Having previously established that the signature miRNAs could reliably differentiate AD from a matched control population NC, we investigated if lower levels of these miRNAs could be observed at earlier stages of dementia. To address this, a new set of samples containing 27 AD, 30 MCI, and 59 NC samples was obtained. All 7 signature miRNAs were confirmed to be differentially expressed between the new cohort of 27 AD and 59 NC samples (internal data, not published). In addition, these miRNAs could reliably differentiate between MCI and NC samples. Meanwhile, no significant difference was observed between MCI and AD samples. To eliminate a potential normalization bias because of our choice of normalization strategy (geometric mean of ath-159a and hsa-miR-106), the data was normalized in two additional ways. The geometric mean of hsa-miR-16-5p and ath-miR-159a was used for normalization, given previous use of hsa-miR-16 as the miRNA of choice for normalization for plasma-based miRNA profiles [118, 119]. In addition, spike-in ath-159a was used in isolation to account for the possibility that normalization with endogenous control miRNAs might prevent detection of valid and meaningful biological variation. We observed no significant change in fold-changes or p-values for AD/NC and MCI/NC confirming that the signature was robust and not sensitive to different normalization strategies. Inter-site reproducibility was also investigated and an aliquot of total RNA was tested at another site by a different operator following the described protocol. Excellent correlations were observed for all 7-miRNA signatures, demonstrating the robustness of the entire assay workflow.

It was encouraging, that the same signature set of miRNAs that could differentiate AD from NC individuals was also downregulated in MCI patients. This suggested that these signature miRNAs were potentially related to early events in the disease and could be valuable for the early identification of AD/MCI patients for potential stratification in clinical studies. However, care should be exercised in how one interprets these findings. Patients that have been diagnosed as MCI are a heterogeneous population, which can have very diverse outcomes as a result of their MCI diagnosis. Some patients continue in the MCI phase or advance to more severe MCI states while, for others, conditions might deteriorate towards dementia. While some MCI patients go on to develop dementia linked to Frontal Temporal Lobe Dementia (FTLD) or Dementia with Lewy Bodies (DLB), a majority of patients develop dementia driven by pathophysiological processes attributed to Alzheimer's with a conversion rate of ~15-20% per year [120]. So it is important to follow up with more studies to understand the course of progression for the MCI population tested, and evaluate if we can predict conversion of MCI to AD (for example) using this signature set of miRNAs. In addition to the specificity of any diagnostic signature for Alzheimer's disease, determining how early in disease etiology the biomarker in question changes is also critical. Archived samples are going back years before the actual diagnosis of MCI or AD would need to be accessed and processed to understand the timing of the biomarker aberration. For a biomarker signature to be valuable for a longitudinal evaluation, it is helpful to comprehend the variation and stability of the proposed biomarker across time. We observed an average coefficient of variation between 15 and 25% for 6 out of 7 miRNAs for eight healthy individuals across samples taken from multiple 6-month visits spread over 3-4 years (unpublished). This set of data indicates that the signature miRNAs are indeed stable across time in individuals, and are therefore promising candidates to evaluate in longitudinal samples from individual patients to understand at what point, these biomarkers start to change (in Alzheimer's progression, for example).

In another global, unbiased study, researchers looked at whole blood cells from Alzheimer's and age-matched control samples to discover diagnostic miRNA signatures. They utilized a next-generation-sequencing platform for profiling the miRNAs in a discovery cohort of 48 AD and 22 unaffected control samples, while the validation was done using qRT-PCR in a larger cohort of over 200 patients comprising not only of AD patients but also patients suffering from other CNS illnesses. They achieved a 12-miRNA signature, which had an accuracy of 93% to differentiate AD from matched control samples [121]. The accuracy was significantly lower (74-78%) to distinguish AD from other CNS disorders. In another study, researchers looked at profiling serum samples from 22 AD and control samples, which comprised of 18 non-inflammatory neurological disease controls (NINDCs) and eight inflammatory neurological disease controls (INDCs). Although they used an unbiased approach and did not restrict the number of miRNAs to disease-associated candidates, they only profiled the most abundantly expressed miRNAs (a panel of 192 miRNAs), and then followed up with qRT-PCR validation. MicroRNA-125b and miR-26b were found to be down-regulated in AD, and confirmed in CSF from the same patient population [122]. Accuracy was determined to be 82% for differentiating between AD and NINDC cohorts. Although they had an FTD cohort (Frontotemporal Lobe Dementia), the number of patients was too small [10] to make significant conclusions about the specificity of the signature.

While there is a lot of activities, excitement, and hope for a non-invasive, specific, cost effective and quantitative biomarker for early detection of Alzheimer's, there has also been a concerning lack of concordance reported amongst individual studies trying to reproduce previous signatures for Alzheimer's (Fig. 2). This is true for miRNA signatures for other diseases as well. A number of unique, independent signatures, especially for Oncology have been reported previously [123] but most of them remained un-validated or never progressed to the clinic stage. There are several reasons for this. Throughout the process of miRNA profiling and subsequent validation, there are steps in which individual biases get introduced, which are unique to each profiling method. The choice of starting material, be it plasma, serum, whole blood cells, PBMC's, or even exosomes from the blood impact signature profiles. The extraction method is another source of variation, as evidenced by the recent retraction[124], where it was reported that Trizol based preparations were susceptible to non-uniform extraction biases depending on initial concentration of certain miRNAs (with particular GC content profiles) in the sample. Gender, ethnicity, age [125] are some other factors that are known to affect miRNA profiles. Hence if one study utilized a defined cohort of patients that were Caucasian in ethnicity, while another group tried to replicate the signature in a cohort that was mixed with Hispanic or African American patient samples, concordance between the two studies could be compromised. There has also been considerable concern about presence of blood-cell derived miRNAs that are found in the plasma fraction, occurring because of hemolysis of blood cells during plasma preparation [126, 127]. Hence, subtle differences in plasma preparation methods could impact plasma signatures significantly. Platelet contamination during plasma preparation is another source of potential discordance [128]. Even in our study, we have observed center to center variation and now more work needs to be done in identifying the source of variation, be it plasma handling leading to platelet contamination from some centers, or the effect of platelet activation leading to microparticle shedding, which also could impact the miRNA signature performance.

Post sample preparation, the choice of profiling platform utilized for discovery and validation has a significant effect on miRNA levels. A study compared biases in miRNA profiling across hybridization-based array platforms and a Next Generation Sequencing (NGS) platform [129]. AU-rich miRNAs were detected with higher sensitivity using NGS based platforms; while GC-rich miRNAs were preferably detected using Hybridization based array platforms. Within a NGS platform itself, biases for certain miRNAs exist, that are driven by sequence (3' nt) and secondary structure at the ligation site [130] of individual miRNAs and adapters, affecting ligase enzyme efficiency during the library construction step. What further compounds the issue is that typically after discovery using a high-throughput platform, miRNA signatures are usually validated using qPCR based methods, which adds their bias to the analysis. Stem loop RT-PCR primers, that are often the "gold standard" for miRNA detection only bind to the 3' 8-10 nt of the miRNA in question, and hence are susceptible to stable secondary structures at the 3' end of miRNAs that inhibit efficient primer initiation in the typical temperature range of reverse transcription (37° to 42°C). Alternatively, with the LNA (modified primers using Locked Nucleic Acid modifications to increase the T_m of smaller sized primers to accommodate size limitations of miRNAs method, polyadenylation is used to elongate the short miRNA

sequence, followed by RT-qPCR detection. Due to substrate preferences and secondary structures at the 3' end of the miRNA, certain miRNAs are better substrates than others for this first step, causing a bias. A recent analysis published in *Nature* captured the variability in miRNA profiling platforms [131]. They evaluated up to 12 profiling platforms using standardized sample sets. The platforms were PCR, hybridization or sequencing based. As expected, the PCR-based platforms resulted in higher sensitivity, although sometimes at a cost of accuracy and specificity. Metrics tested included reproducibility, dynamic range performance, accuracy, accuracy at lower RNA input, sensitivity, sensitivity at a lower RNA input specificity and cross assay reactivity. The lower volume metrics were designed to address applications like detection of circulating miRNAs from body fluids, where the concentrations of miRNAs are typically very small. Although differences were expected between platforms, what was surprising was the extent of discordance observed between platforms. The average validation rate between any two platforms was as low as 54%. This labors the point, that it is paramount to profile and validate using two different platforms to confirm potential signature miRNAs in order to eliminate platform artifacts. Moreover, lastly, the multiple normalization strategies that are used for circulating miRNA analysis further reduce concordance between independent studies. Because of a lack of a well-established and accepted normalization miRNA candidate (a GAPDH equivalent for miRNAs), there have been a variety of strategies utilized and have been previously reviewed [132, 133]. Each approach makes certain assumptions, and it is important to consider those when comparing different miRNA profiling studies. Given these above mentioned sources of variation, it is not surprising that multiple studies which started in 2007, where miRNAs were profiled using microarray technologies (that modified mRNA based strategies to work with the much shorter miRNAs) to today, where you have the next generation of technologies that have been built keeping miRNAs in mind, the miRNA profiles are significantly different. Furthermore, there have been constant additions/subtractions and even sequence edits to the miRBASE registry over the years, which have an impact on profiling platforms, since they have to modify probes in order to accommodate these changes. With the recent discovery and advancement in technologies to look at exosomes, another dimension of complexity has been added, where one can distinguish exosome encapsulated fractions and truly cell-free fractions, further reducing concordance between studies. Hence it is important to maintain very standard protocols, and then follow through with them till the end of the study, including multiple validations with many independent cohorts of samples taken from different centers, ethnicities and ages.

The last 3-4 years have seen the beginning of the utilization of circulating miRNAs in the neurodegenerative disorders domain, and it is still a maturing field. The potential of miRNAs to provide a cost effective, non-invasive, accurate and sensitive diagnostic assay resulting in a positive impact on patient health is undeniable, but care needs to be exercised in interpretation of these signatures in the absence of thorough validation. Furthermore, significant work detailing how these novel biomarkers tie into disease etiology is a must to increase confidence and understand the reason behind biomarker modulation due to illness or subsequent treatment.

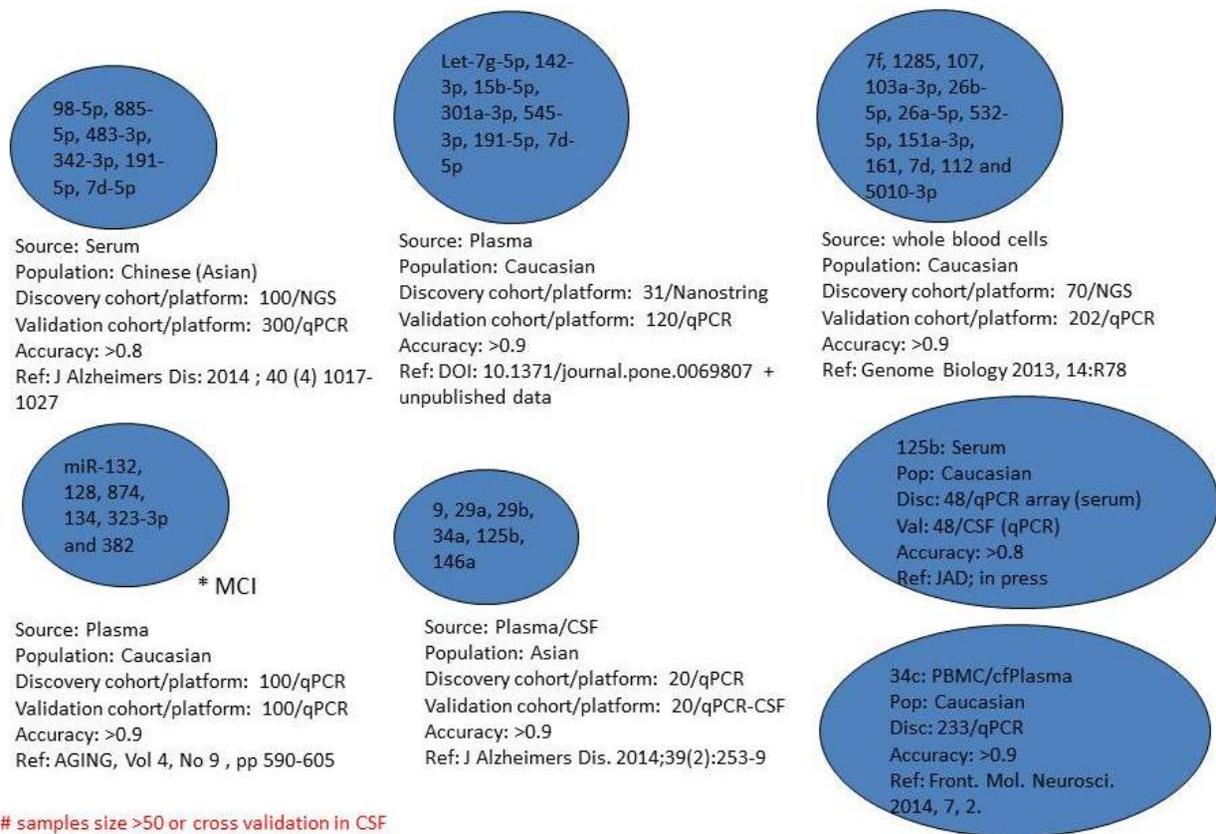


Figure 2. Snapshot of current circulating miRNA signatures[#] for Alzheimer's Disease

9. New non-invasive biomarkers on the horizon

Despite the promising collection of novel blood-based biomarkers as we just described, there is still a possibility to unravel additional novel non-invasive biomarkers for AD and MCI in other accessible body matrices. The human retina shares many features with the brain, including embryological origin, anatomical (ex. Microvasculature bed) and important physiological characteristics such as blood-tissue barrier [134] So researchers have looked at the possibility that the retina may offer an easily accessible and non-invasive way of examining human brain pathology. As it turned out, it is becoming evident that amyloid is also accumulating in the eyes and that this landmark event could be detected by relatively straightforward eye examinations according to data derived from multiple research trials data presented during the summer of 2014 at the Alzheimer's Association International Conference held in Copenhagen.. Data from independent studies showed that the level of beta-amyloid detected in the eye was significantly correlated with the level of beta-amyloid deposition in the brain and allowed researchers to accurately identify patients with Alzheimer's in the studies.

In the first study looking at healthy patients from the Australian Imaging, Biomarker and Lifestyle Flagship Stud Preliminary data from the first 40 participants showed that amyloid levels detected in the retina using an orally administered curcumin supplement were signifi-

cantly correlated with brain amyloid levels, as shown by PiB PET imaging. In addition, Retinal Amyloid Imaging (RAI) differentiated participants with AD from those without AD with 100% sensitivity and 80.6% specificity. Furthermore, longitudinal data showed a 3.5% elevation on average in retinal amyloid signal during a 3.5-month period, suggesting that the technique may be used as a means of monitoring response to therapy.

The second separate phase 2 studies included 20 individuals with probable mild to moderate AD and 20 healthy, age-matched control participants. In this study, participants had a small proprietary molecule applied to the eye in the form of a sterile ophthalmic ointment. The compound was left to diffuse into the eye overnight and the next day the eye was scanned with the laser and results computed. As in the first study, all 40 participants also underwent PET amyloid brain imaging but this time with Amyvid PET agent. The test was capable to distinguish individuals with Alzheimer's from healthy control participants with 85% sensitivity and 95% specificity significantly and as in the first study, amyloid levels in the lens significantly correlated with PET imaging results.

Given the rapidity and simplicity of the diagnostic test (5 minutes) it is easy to understand how revolutionary this would be for the medical field as it could be used by general practitioners and specialists at point-of-care in hospitals and offices. Time will tell if it could also be used to monitor disease progression and monitor efficacy of new anti-Alzheimer's drug in development (Alzheimer's Association International Conference (AAIC) 2014. Abstracts O2-05-05 and O3-13-01).

10. Impairment of odor detection as early AD diagnostic

It is becoming evident that as AD sets in, impairment of the olfaction system in its ability to correctly distinguish odors appears to be an early phenomenon that could predict cognitive impairment at an early stage (AAIC 2014, in Copenhagen). In two studies, the decreased ability to identify various defined odors was significantly associated with loss of neuronal cells function and progression to Alzheimer's disease as measured by a variety of cognitive tests.

Imaging data from one study revealed that a smaller hippocampus and a thinner entorhinal cortex accompanied by higher levels of brain amyloid were linked to worsening of smell identification abilities and memory after adjusting for parameters that includes age, gender, and an estimate of cognitive reserve(AAIC 2014).

A separate study conducted at Columbia University Medical Center looked at odor Identification deficits association with Transition from Mild Cognitive Impairment to Alzheimer's. Researchers investigated a multi-ethnic sample of 1037 non-demented elderly people in New York City, (average age of 80.7) and assessed their olfaction abilities in a variety of ways at three time periods. 109 people developed dementia (101=Alzheimer's and eight non-AD dementia) a significant incapacity to correctly identify odors was found to be associated with the early development of dementia in those patients.

Although further large-scale studies will be required to confirm these results it is encouraging for the medical practice field that such relatively inexpensive test may be used one day to detect early stage of AD and those at risk of cognitive decline.

11. Urine

There are very few reports describing the discovery of any AD-related urine biomarker, and the few that are reported and published have met the AD research field with controversy. One of them is called NTP (Neural Thread Protein), a membrane-associated phosphoprotein that made the headlines in 2007 when a company called Nymox Corporation got an EIA kit CE approved in Europe for the diagnosis of AD using urine samples. Although Nymox claimed the utility of the test, one blinded study conducted in the Czech Republic by a reference lab using the Nymox test found that when compared to the diagnosis established by NINCDS-ADRDA for AD, the test appeared to have low sensitivity and specificity. Very recently, two studies evaluating the levels of NTP in urine were published [135, 136]. In the first study, levels of NTP in AD, PD, and Healthy participants were evaluated (AD (49), PD (20), HC (22)) using Nymox AlzhemAlert test. AD patients had significantly higher levels of NTP than HC and that those of PD. Although the authors concluded that urine NTP could be used as a promising biomarker of AD, it should be noted that there was an age difference among participants that could potentially affect this interpretation. Average age for each group was as follows: AD (72.2+/-7.5), PD (66.4 +/- 8.8), Control (64.1+/-6.8).

In a second separate recent study [137], NTP levels were compared in relation to age in HC volunteers divided into 5 groups (20-29, 30-39, 40-49, 50-59 and ≥ 60) using a different test called 7c Gold. It is not clear as to why the levels detected in this study are in ng/ml range as opposed to the ug/ml range for the Nymox Kit since both studies were conducted on Asian patients. The authors concluded though that urine levels of NTP increase with age significantly which might explain the controversy around NTP as an AD biomarker.

12. Conclusion

12.1. Hurdles to blood-based AD biomarker development

Replicating candidate blood biomarker findings has been the biggest challenge of the research field [138]. Several pre-analytical components factors are likely to make discoveries very challenging: choice of anticoagulant (EDTA, Heparin), addition of protease inhibitors, needle size, order of blood draw, processing time, storage condition, freeze/thaw cycles and centrifugation procedures are just a few parameters that can affect drastically the detection of several analytes [128, 138]. Longitudinal analysis from the same patient is essential to find early markers, but the success of this approach highly depends on analyte stability during >10 years storage. Blood is also a constantly changing matrix where components levels are affected by multiple factors such as diet, lifestyle, circadian changes and other co-morbidities, especially

in an elderly population such as Alzheimer's Disease patients which quite frequently suffers from other diseases where inflammation is implicated such as diabetes, rheumatoid arthritis and cardiovascular diseases [139]. Further complicating discoveries is the fact that several patients have mixed dementias such as Vascular Dementia, Fronto Temporal Lobe Dementia (FTLD) and Dementia with Lewy-body (DLB) making it difficult to identify a particular marker. Importantly, the integrity of the blood-brain barrier and its impact on AD-related biomarkers might differ from patient to patient based on genetics and other factors [140]. Another less discussed parameter that we found that is crucial for the discovery of true AD blood biomarkers is the definition of healthy controls. We realized talking to clinical physicians in Japan, US, and EU that in many instances, healthy elderly control samples are obtained from caregivers or spouses living with the patients. This is a particular concern since epidemiological studies have demonstrated that living with an AD patient increases the risk to develop the disease by six-fold [141]. The reason is not exactly known but contributing factors such as exposure to pollutants (air, water, contaminants in food, etc) and pathogens [142, 143] by the patient and the spouse for several years living in the same environment may play a role. We also heard through interviews with clinicians that several healthy control samples are obtained from patients who went to a clinic after complaining of some abnormalities that were later ruled out as not being related to dementia. The inclusion of such patient samples in the control group category may also contribute to the difficulty of identifying a true AD biomarker.

While co-morbidities cannot be avoided when comparing AD and control groups, it is important to the research field to agree on standard practices to ensure reproducibility of data and that careful selection of healthy controls be conducted before doing any comparisons. At least, initiatives like the Blood-Based Biomarker Interest Group and the release of FDA guidelines for the analytical validation of assays that meet GCP/GLP will hopefully lead to the adoption of robust standards for the research field that applies to the analysis of proteins, metabolites, lipids and miRNAs in serum and plasma to control for precision, analytical accuracy and dilution linearity.

One more important point for the successful development and adoption of blood-based AD biomarkers is to understand the relationship with the disease as AD is essentially a brain disorder with little manifestation of illness in the peripheral system. Such understanding of the biomarker, its function, and its contribution to the illness state is essential to promote the confirmation by peers. Moreover, such clarification could lead to the discovery of even better biomarkers that could be used to detect the disease at an even earlier state or lead to the identification of novel drug targets. Such functional identification as much as the validation is critical to promote the verification of novel candidate biomarker in exploratory studies that are part of sponsored clinical trials.

In conclusion, blood tests or other non-invasive tests as biomarkers for AD are appealing as they could be applied to many uses such as patient screening, disease prognosis, diagnosis and aid to support clinical trial development. The development of such markers will be greatly facilitated once we fully understand what is causing sporadic AD in the first place and after more comprehensive studies will be able to look at correlation between endophenotypic changes in the brain using imaging technologies and the candidate biomarkers.

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