Alzheimer’s Disease: A Journey from Amyloid Peptides and Oxidative Stress, to Biomarker Technologies and Disease Prevention Strategies—Gains from AIBL and DIAN Cohort Studies


aCentre of Excellence for Alzheimer’s Disease Research and Care, School of Medical and Health Sciences, Edith Cowan University, Joondalup, WA, Australia
bAustralian Alzheimer’s Research Foundation, Ralph and Patricia Sarich Neuroscience Research Institute, Nedlands, WA, Australia
cDepartment of Biomedical Sciences, Macquarie University, Sydney, NSW, Australia
dSchool of Psychiatry and Clinical Neurosciences, University of Western Australia, Perth WA, Australia
eKaRa Institute of Neurological Diseases, Sydney NSW, Australia
fSchool of Biomedical Sciences, Faculty of Health Sciences, Curtin Health Innovation Research Institute, Curtin University of Technology, Bentley, WA, Australia
gSchool of Psychology and Exercise Science, Murdoch University, Perth, WA, Australia
hCSIRO Australian e-Health Research Centre/Health and Biosecurity, Perth, WA, Australia
iAnglicare, Sydney, NSW, Australia
jCSIRO Health and Biosecurity, Australian E-Health Research Centre, Brisbane, Australia
kDepartment of Neurology, Icahn School of Medicine at Mount Sinai, New York, NY, USA
lUniversity of North Texas Health Science Centre, Fort Worth, TX, USA
mDepartment of Nuclear Medicine and Centre for PET, Austin Health, Heidelberg, Australia
nFlorey Institute of Neuroscience and Mental Health, Parkville, VIC, Australia
oCooperative Research Centre for Mental Health, Carlton, VIC, Australia

*Correspondence to: Professor Ralph N. Martins, School of Medical and Health Sciences, Edith Cowan University, 270 Joondalup Drive, Joondalup, Western Australia, 6027 Australia.
Tel.: +61 8 9347 4200; Fax: +61 8 9347 4299; E-mail: ralph.n.martins@gmail.com.

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INTRODUCTION

Alzheimer’s disease (AD) is a devastating neurodegenerative disorder and is the most common cause of dementia in the elderly population. AD is characterized clinically by a progressive loss of function of various cognitive domains, usually starting with short-term memory dysfunction, impaired judgement and reasoning, disorientation, anxiety, and minor personality changes, but eventually leading to a total loss of memory and personality. A patient in late stages will take to bed, cease to communicate, and will need 24-hour help with eating and self-care.

The characteristic neuropathological hallmarks of the disease are intracellular neurofibrillary tangles (NFT) and extracellular aggregated amyloid fibrils and plaques. NFT are mostly composed of hyper-phosphorylated forms of the microtubule-associated protein tau [1–3], while amyloid plaques are mainly built up of aggregated and fibrillized amyloid-β (Aβ) peptides. The prevalence of this AD brain pathology increases dramatically with aging [4].

Some of our group’s earliest studies, carried out in collaboration with the Konrad Beyreuther laboratory in Heidelberg, Germany, involved the characterization of Aβ peptides, the major component of amyloid plaque cores. Aβ peptides are hydrophobic 39–43 amino acid long products generated by the sequential proteolytic processing of the amyloid-β protein precursor (AβPP) [5, 6].

Since the sequencing of the gene for AβPP, many major discoveries have been made. It is known that AβPP is a transmembrane protein found in almost all tissues, and is cleaved by one of two pathways, either a non-amyloidogenic pathway or amyloidogenic pathway, with the latter producing Aβ via trans-membrane proteolysis, to produce Aβ peptides, of which the most common are Aβ40 and Aβ42. Mutations in AβPP, or in components of the γ-secretase enzyme that carries out the final step of Aβ production (presenilin-1 or presenilin-2), have been detected in rare families (<1% of all AD) that develop AD well below the age of 65 [7]. These mutations have helped underscore the key role of abnormally high levels of Aβ, particularly the longer more amyloidogenic Aβ42 form, in AD pathogenesis.

The Aβ peptide is a normal proteolytic product produced in most body tissues, but it is thought that the AD pathology that develops in the brain is due to an overproduction or lack of clearance of the peptide (or both). This build-up of Aβ is thought to be initiated by various factors including oxidative stress and chronic inflammation [8, 9]. This results in...
abnormally high Aβ levels, which then aggregate into toxic oligomers (particularly the longer Aβ42 forms), and as these two factors themselves increase Aβ production, and as Aβ peptides exacerbate oxidative stress, a toxic cycle develops [10, 11]. The pathology develops slowly, and it may take over two decades before clinical symptoms appear [12, 13]. By the time a clinical diagnosis is made, there is widespread synaptic loss and neuronal death, microglial infiltration, and brain shrinkage.

Early studies looking for other proteins associated with amyloid plaques soon found that apolipoprotein E (apoE, protein; APOE, gene) was also present in plaques and NFTs [14]. Subsequently, work led by Professor Allen Roses and his colleagues from Duke University identified the e4 allele of the APOE gene as an important genetic risk factor for AD. This seminal finding was confirmed in cohorts from US, Europe, and Australia [15–19] and APOE e4 is now recognized as the major genetic risk factor for AD in all populations. Geneticists believe that APOE e4 is the most common and most potent AD risk factor that will ever be discovered. Our understanding of how APOE e4 predisposes to AD is limited, though several studies have shown that apoE is involved in Aβ clearance and others have indicated it modulates Aβ aggregation, with the e4 isotype being linked to worse clearance and greater aggregation [16–18, 20–22]. The link to apoE also led to the finding that differences in lipid metabolism occur in AD, and most likely predispose to AD, as well as other conditions that themselves increase the risk of AD. These conditions include obesity, cardiovascular disease, hypertension, insulin resistance, and type 2 diabetes (T2D) [23–26]. APOE e4 and T2D also appear to act together to drive cognitive dysfunction and increase the risk of both AD and vascular dementia. For example, glucose hypometabolism is a key feature of both mild cognitive impairment (MCI) and AD, and apoE e4 has also been linked to lower levels of glucose metabolism. The brains of patients with MCI and AD are functionally insulin resistant [27], and amyloid clearance rate, neuroinflammation, and synaptic dysfunction, which have all been linked to APOE e4 alleles, are also influenced by increased brain insulin levels [28].

**EARLY STEPS**

Early studies on AD concentrated on the immuno-histochemical findings in the brain, the distribution of amyloid in plaques, the filamentous NFT detectable in neurons, and the loss of neurons and brain shrinkage. In the mid-1980s, our group concentrated on the neuropathological changes that could be seen in AD brains. Postmortem pathology and studies of other neurodegenerative diseases in the 1980s had led people to suggest a variety of causes for AD, including aluminum toxicity, viruses, accelerated aging, defects in the immune system, and even a late-onset form of Down syndrome [29].

**Oxidative stress: First signs**

It was already well-known that head trauma could lead to AD many years later, and that brain injury involved oxygen radical generation, lipid peroxidation, and cell death [30]. It had also been shown in 1976 that levels of choline acetyltransferase (necessary for the synthesis of the neurotransmitter acetylcholine) were lower in AD, particularly in regions most affected such as the cortex and hippocampus [31], thus it was logical to suggest perhaps the pathology in AD involved oxidative stress, leading to lower neuron numbers, lower cholinergic neurotransmission, and thus reduced brain function. Researchers had also recently found that Parkinson’s disease patients had lower levels of glutathione peroxidase, again suggesting oxidative stress might be contributing to neurodegeneration—although in a different condition [32]. In our own studies, we applied specific assays for certain enzymes of the hexose monophosphate pathway to try to determine whether oxidative stress was increased in postmortem AD compared to age-matched control brain tissue. We found that, compared to controls, levels of the enzymes glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase were both nearly doubled in the AD brains [33]. One major role for G6PD is to reduce NADP+ to NADPH, thus providing a source of reducing power for glutathione, a potent protective reducing agent in the body. Many studies have since shown that levels of reduced glutathione (as well as the ratio of reduced:oxidized glutathione and levels of the enzyme glutathione-S-transferase) are lower in AD as well as in MCI [34–36], and in fact a plethora of pathological changes have been linked to oxidative damage, from the earliest stages of detectable AD neuropathology [9, 37, 38]. Other early studies which used fibroblasts from familial AD subjects showed evidence of oxidative stress in AD, including abnormalities in calcium signaling, mitochondrial conditions such as Down syndrome [29].
oxidation and ion channel function [39]. Recent studies have reignited interest in the particular pathway that involves G6PD—the pentose phosphate pathway (PPP)—as it was demonstrated that due to the rapid degradation of phosphofructokinase B3 (a rate limiting enzyme in glycolysis), neurons preferentially metabolize glucose via the PPP, as opposed to glycolysis, which contrasts with most other cell types [40]. Furthermore, in investigations using APOE e3 and APOE e4 mice fed high fat diets to induce insulin resistance: it was found that the combination of genome-wide measures of DNA hydroxymethylation with comprehensive untargeted metabolomics, novel alterations in purine metabolism, glutamate metabolism, and the pentose phosphate pathway were identified [41]. Worse effects were detected in the e4 mice, yet these could be reduced by switching to a low-fat diet, demonstrating both the greater susceptibility of e4 carriers to metabolic impairments, and the value of healthy diets in preventing AD-associated pathology.

Our laboratory’s strong interest in lifestyle factors, diet and physical activity in particular, are discussed later in the section concentrating on studies carried out through the Australian Imaging, Biomarker and Lifestyle study (AIBL) of aging. Directly below, we describe our (and some others’) subsequent studies aimed at understanding APOE allele effects on AD pathogenesis.

Apolipoprotein E

After the discovery that APOE allelic differences were linked to AD risk and the aggregation of Aβ in the early 1990s, we investigated APOE genotypes in AD in the Australian population, and as in other populations we found the e4 allele frequency was higher in Australians with both early-onset sporadic AD (p < 0.002) and late-onset sporadic AD (p < 0.0001), and APOE e2 allele frequency was found to be lower in the late-onset sporadic AD group (p < 0.01) [19]. There was also some evidence that APOE e4 alleles can negatively influence age of onset in Down syndrome subjects, as in AD. In later studies we investigated whether plasma apoE levels were altered in AD, and found significantly higher plasma apoE levels in both late-onset and early-onset AD patients who had not fasted [42]. This is quite different to pre-clinical findings, with the most recent prospective study agreeing with other studies, and showing that genetic and hence lifelong low apoE is associated with a high risk of dementia in the population [43], although this does not necessarily reflect a causal relationship.

We investigated several other aspects of apoE function and its influence on Aβ metabolism. Cell culture studies had shown that apoE e4 was associated with poorer clearance of Aβ compared to the other isoforms, yet the relevance of apoE in Aβ clearance had not been investigated in vivo. In early mouse studies we showed that the clearance of peripheral Aβ was dependent on the presence of apoE. Additionally, we found that apoE-deficient mice did not transfer the Aβ efficiently to the liver or kidneys compared with normal C57Bl/6J mice [20]. With the availability of APOE KO mice bred to carry human APOE alleles, we then found that brain Aβ levels rose in KO mice and in mice carrying only the APOE e4 allele. In contrast, this age-related Aβ increase was prevented in mice carrying the e3 allele [44]. As part of that study we also measured brain levels of the oxidized lipid F2 isoprostane (F2IP) and found that levels rose with age in the KO mice and the KO mice carrying the APOE e4 allele, but not those with the APOE e3 allele. This reflected the changes in brain Aβ levels, and thus supported the theory that oxidized lipids are associated with AD pathogenesis.

In cell culture, we demonstrated that the Aβ could influence the binding of apoE isoforms to cultured fibroblasts, such that Aβ improved the normally poor binding of apoE e2, yet reduced the binding of apoE e3 and e4 [45]. Although the dynamics of apoE binding to Aβ and the LDL receptor family was not well understood at this stage, this and other similar studies were adding to the evidence of apoE isoform-specific effects on Aβ and lipid metabolism. We carried out further studies on the APOE KO mice carrying human APOE e2, e3, or e4, as well as APOE KO mice treated with lipidated recombinant apoE isoforms. When the APOE KO animals were treated with lipidated recombinant apoE e4, injected Aβ42 was retained in the plasma significantly longer, similar to the APOE e4 knock-in mice, when compared to the equivalent APOE e3 or e2 mice [21]. In other studies of the same knock-in mice, we used electrospray ionization mass spectrometry to measure levels of lipids including glycerophospholipids, sphingolipids, cholesterol, and triacylglycerols in the mouse brains. The findings were that variations in apoE isoforms did not significantly affect bulk lipid homeostasis in the brain [46]. However, when the same knock-in mice were subjected to a high-fat, high-cholesterol diet, our findings demonstrated changes in lipid metabolism especially in APOE e4 mice [47]. Overall, these studies support
the current concept that the pathogenicity of the APOE ε4 allele is at least in part due to lower Aβ metabolism by apoE ε4 at the blood-brain barrier [48], and lower cellular uptake and lysosomal trafficking in brain cells [49], properties that would reduce brain clearance of Aβ.

APOE allele status was first shown to influence brain glucose metabolism in people at risk of AD over 20 years ago [50, 51]. Many studies have since improved methodology and provided supportive evidence that reduced glucose usage occurs in brain areas susceptible to AD pathology well before symptom onset [52, 53]. These were all conducted on cohorts in the USA. We wished to determine if these reported findings could be replicated in an Australian population. Fluoro-2-deoxy glucose-PET was carried out in cognitively healthy (Australian) individuals as well as subjective memory complainers, all aged 50–80. We found that APOE ε4 carriers again demonstrate mild glucose hypometabolism in brain areas associated with AD when compared to the normative NeuroStat database; furthermore, subjective memory complainers (but not non-complainers) also showed a pattern of glucose hypometabolism [54]. Furthermore, it has been suggested that plasma apoE levels are age- and sex-dependent, and that brain regional glucose usage and grey matter volume correlate with peripheral apoE levels, as well as cognitive performance [55]. This is further evidence of AD being a systemic condition, and that a pre-clinical peripheral biomarker panel is an achievable objective.

In other studies of community-dwelling volunteers, we found that neurological soft signs (abnormalities in sensory and motor performance) were associated with APOE genotype, age, and Mini-Mental State Examination (MMSE) determined cognitive performance, suggesting that such neurological soft signs may be useful in determining people at greater risk of cognitive decline [56], and again showing that APOE genotype influences the risk of cognitive decline.

INTRODUCING AIBL

From its start in 2006, the collaborative project known as the Australian Imaging Biomarkers and Lifestyle study (AIBL) of aging [57] has been particularly productive with respect to increasing our knowledge of how much lifestyle choices such as diet, physical activity, and sleep can affect the risk and development of AD [58, 59]. This study is an ongoing collaboration between the two major AD research groups in Australia based in Melbourne and Perth (led by Colin Masters, Chris Rowe, and David Ames in Melbourne, and Ralph Martins in Perth), in partnership with the CSIRO throughout Australia. AIBL is a flagship study of aging which recruited 1,112 individuals over the age of 60 to do prospective research into AD. Early publications described the screening, diagnoses, collection of medical history and current medications, comprehensive baseline cognitive testing, blood collection, as well as extensive health and lifestyle questionnaires with the aim of using this data to help predict potential AD risk factors and protective factors [60]. The AIBL cohort therefore comprises highly-characterized individuals keen to be part of this long-term research program. Participants were assessed at 18-month intervals for over a decade, a quarter of the participants also underwent amyloid PET imaging using 11C-Pittsburgh compound B (PiB-PET)—a specific in vivo amyloid marker [61], and MRI brain imaging. A subgroup of 10% also underwent ActiGraph activity monitoring and body composition scanning. For AIBL, the MRI parameters of the Alzheimer’s Disease Neuroimaging Initiative (ADNI) were adopted, and the PiB-PET acquisition and neuropsychological tests were designed to permit comparison and pooling with ADNI data.

Early cross-sectional analysis of the baseline data revealed links between cognition, brain amyloid burden, structural brain changes, biomarkers and lifestyle factors [62]. It was also evident early on that there was a strong relationship between Aβ deposition and brain atrophy very early in the disease process [63].

Examining APOE in AIBL

With such a highly characterized cohort, it was possible to extend the research on apoE, for example by doing longitudinal investigations into the influence of APOE allele status on various indices of memory decline. In our initial studies of the influence of the ε4 allele on cognitive function in the AIBL older adults, we found relatively little evidence of a role for the ε4 allele [64]. However, using a subset cohort of 84 cognitively normal people with high Aβ burden (as assessed by PiB-PET), cognitive tests such as the MMSE, Clinical Dementia Rating...
scales, and the Cogstate Brief Battery carried out every 18 months for up to 54 months demonstrated that possession of APOE e4 alleles is associated with a faster decline on memory tasks [65]. We carried out similar studies with a larger group (317 subjects) from the AIBL cohort, with assessments done using the Cogstate Brief Battery and the California Verbal Learning Test, Second Edition. The cognitively normal adults with high Aβ burden (as assessed by PiB-PET) who were APOE e4 carriers had the most pronounced decline in learning and working memory over 18 months, whereas in non-carriers high Aβ burden was unrelated to such cognitive decline [66]. Cognitively normal APOE e4+ve older adults with low Aβ levels also showed a significantly increased rate of decline in learning, yet an improved cognitive performance on measures of verbal episodic memory after 18 months. When the majority of the AIBL cohort was used to investigate whether possession of APOE e4 alleles alone influenced cognitive decline (irrespective of brain Aβ levels) in a study spanning 3 years, healthy APOE e4 +ve control subjects showed a slightly greater decline in verbal episodic memory, yet APOE e4 +ve MCI individuals showed a greater decline in several cognitive tasks compared to APOE e4-ve MCI individuals, possibly reflecting imminent AD [67]. Overall these results indicate APOE e4 alleles increase the rate of cognitive decline in older adults and add to the evidence that brain Aβ levels and possession of APOE e4 alleles are strong indicators of AD risk, particularly in the preclinical stages of the disease.

Our more recent study of correlations between APOE e4 carriage and cognitive decline in a cognitively normal subset of the AIBL cohort has shown that the presence of subjective memory complaints, APOE e4 genotype or advancing age all help identify elderly participants who have high Aβ burden, and who may benefit from prevention trials [68]; and suggests that subjective memory complaints may be the first clinical expression of AD pathology. We have also extended some longitudinal studies to 72 months in a cognitively normal subset of the AIBL cohort (n = 423) who have undergone Aβ PiB-PET imaging [69]. Some previous studies had not detected Aβ-related memory decline in APOE e4 non-carriers, however in our extended study, we found that compared to Aβ-ve APOE e4 non-carriers, both Aβ+ve APOE e4 carriers and non-carriers showed significantly increased declines in measures of memory, language, and executive function as well as higher rates of progression towards a clinical classification of MCI. The rate of decline was slower in the Aβ+ve APOE e4 non-carriers, yet these results show a correlation between pre-clinical Aβ accumulation and cognitive decline, regardless of APOE e4 status.

The large number of Aβ-imaged people (n = 423) in the AIBL cohort and the longitudinal nature of the study has made it possible to determine the extent and nature to which carriage of APOE e4 alleles increases the risk for clinical disease progression from cognitively normal status. Analysis of data without taking into account Aβ status, being APOE e4 +ve (compared to APOE e4 -ve) increased the risk over a 72-month period by 2.66 times [70], yet if Aβ levels are taken into account, carriage of APOE e4 is no longer predictive of progression. These results support the theory that apoE e4 is less efficient at facilitating Aβ clearance from the brain, resulting in greater Aβ deposition, thus aiding AD pathogenesis. This theory is further supported by a recent cross-sectional AIBL study which involved most of the AIBL cohort, including a subset all of whom had undergone Aβ PiB-PET imaging as well as MRI hippocampal volume measurement [71]. The aim was to investigate the relationship between APOE e4 allele status and Aβ levels, hippocampal volume, as well as memory [71]. It was found that APOE e4 alleles influence Aβ levels, episodic memory and hippocampal volume in a dose-dependent fashion, again underscoring the influence of apoE e4 on AD pathogenesis.

Although the majority of research into apoE’s isoforms modulating AD risk involves Aβ clearance and Aβ aggregation, there are other apoE roles that may influence AD pathogenesis. These include apoE effects on vascular function, neuroinflammation, metabolism, synaptic plasticity, and transcriptional regulation [72, 73]. For example, recent studies have found that apoE binds a microglial receptor, triggering receptor expressed on myeloid cells 2 (TREM2), a member of the Ig superfamily of receptors. Certain TREM2 mutations influence the risk of AD, and TREM2 is thought to be involved in Aβ clearance; however, the exact relationship between these proteins is currently still being researched [74]. Perhaps more importantly, APOE e4 alleles are a well-known factor for cardiovascular disease (CVD) [75], thought to be due to APOE e4 allele-associated higher levels of total serum cholesterol, particularly LDL, which are themselves known risk factors for CVD. Since CVD and associated conditions such as obesity, dyslipidemia, and hypertension have all been linked to an increased risk of AD, this underscores the theory that apoE e4 is a protein that carries out apoE
functions less effectively than the more common \( \text{apoE} \ v3 \), and due to this deficiency, can lead to a greater risk for many conditions. The link to CVD, obesity and other conditions consolidates the theory that improved diet and exercise could reduce risk of all these conditions, by reducing the pressure on the lipid metabolic pathways, as discussed further below.

**DIAGNOSIS**

It is now well-accepted that AD pathogenesis starts 2-3 decades before the onset of symptoms. However, to date, there is still no simple, inexpensive and minimally invasive test to diagnose AD prior to the onset of symptoms. Imaging techniques using PiB-PET that show gradual A\( \beta \) accumulation in the brain, as well as the measurement of CSF levels of A\( \beta \)\(_{42} \), and phosphorylated tau are proving to be relatively reliable indicators of imminent AD [76]; however these are relatively expensive and invasive diagnostic techniques that, while serving as a gold standard for investigative work and clinical trials, would be difficult to apply in general population screening. Cures and effective treatments for AD have not eventuated despite several decades of research. There are several potential reasons for this: by the time AD manifests, there is already widespread damage to the brain, including considerable loss of synapses, neurons, and brain tissue, and there has been no success in trying to slow or prevent this gradually increasing pathology, after symptoms have begun [77]. Effective treatment needs to be implemented at pre-clinical stages when damage is minimal, cognition is relatively intact, and the pathogenesis can be slowed or prevented from progressing. For potential disease-preventing or disease-delaying treatments to be tested, the tests need to be carried out on populations at these pre-clinical stages—people who are in the very early stages of disease development. For this to occur, clinicians need to be able to identify at-risk populations which requires a very good understanding of the early stages of AD pathogenesis. Some of the major outcomes of the AIBL studies, which have involved considerable collaboration with many research groups, has been the significant increase in understanding of AD early pathology (particularly A\( \beta \) accumulation), risk factors, the discovery of many potential peripheral biomarkers, and the discovery of the importance of physical activity and diet in AD risk as well as disease management.

The outcomes of these studies that have had significant input from our research group are discussed below.

**A\( \beta \) imaging: PiB-PET**

Both the ADNI and AIBL cohorts have been investigated extensively as part of biomarker and brain imaging research. Yet while AIBL and ADNI adopted very similar approaches to neuropsychological assessments, blood biomarkers, and structural MRI, the approaches to disease-specific biomarkers differed, with AIBL concentrating from the very beginning on A\( \beta \) imaging, while ADNI initially focused on 18(F) fluorodeoxyglucose-PET imaging and CSF biomarkers.

About one third of AIBL participants underwent structural MRI and A\( \beta \) imaging scans with PiB-PET [60, 78]. Initial results showed that the prevalence of high A\( \beta \) burden (A\( \beta \)+) in cognitively unimpaired individuals increased with age, and that it was higher in individuals carrying at least one \( APOE \ v4 \) allele [78]. Furthermore, while memory in the cognitively unimpaired adults with low A\( \beta \) burden (A\( \beta \)−) remained stable over 18-months, all aspects of episodic memory were observed to deteriorate substantially in A\( \beta \)+ non-demented participants [79, 80].

From a clinical perspective, some biomarkers have been shown to serve as predictors of disease progression. For example, A\( \beta \) imaging data demonstrated that A\( \beta \)+ amnestic MCI were much more likely to progress to AD over 18–36 months than A\( \beta \)− MCI [81, 82]. It was also observed that subtle memory impairment in A\( \beta \)+ healthy individuals indicated a high risk for progressing to MCI or AD within three years. Furthermore, A\( \beta \) deposition was found to be strongly related to grey matter atrophy, where the rates of atrophy were significantly higher in A\( \beta \)+ cognitively unimpaired individuals [83, 84]. Moreover, hippocampal volume and temporal A\( \beta \) deposition provided independent contributions to memory deficits, suggesting that both factors should be independently targeted in therapeutic trials aimed at reducing cognitive decline [85]. These associations were not observed at the MCI and AD stages, suggesting that other pathological, probably downstream, events might be responsible for the progressive atrophy and cognitive decline [63].

The prospective longitudinal design of the AIBL study allowed the examination of changes in A\( \beta \) burden over time, where small but significant increases in neocortical A\( \beta \) burden were observed in the AD
and MCI groups, and in Aβ+ve controls, confirming the notion that Aβ deposition precedes cognitive impairment [81]. Furthermore, higher rates of Aβ deposition were associated with higher Aβ burden and identified the existence of Aβ ‘accumulators’ and ‘non-accumulators’, with Aβ ‘accumulators’ even found among Aβ- controls [86]. Consequently, Aβ imaging data from the 3-year follow-ups were then used to calculate the rates of Aβ deposition over time, showing that Aβ deposition is a slow protracted process that takes about two decades to go from the threshold of abnormal Aβ burden to the levels usually observed in AD, and that Aβ deposition precedes hippocampal atrophy and memory impairment by more than a decade [12]. Interestingly, when comparing the rates of Aβ deposition, memory decline and hippocampal atrophy between the sporadic cases in AIBL and the autosomal cases in the Dominantly Inherited Alzheimer’s Network (DIAN) [87, 88], the rates of annual change in those three variables were almost identical [12].

In AIBL studies, we have used Aβ imaging as the gold standard for the validation of CSF assessments [89] and for the determination of the different biochemical pools of Aβ in the brain [90]. Aβ imaging has also been used to assess the accuracy of a panel of blood-based biomarkers in predicting brain Aβ burden [91, 92], as well as disease progression [93]. These blood-panels used 6 plasma biomarkers as well as age, APOE genotype and Clinical Dementia Rating (CDR)-Sum of Boxes (CDR-SOB), and were able to predict brain Aβ burden with an accuracy >80%; accuracy that was further validated using independent biomarker data from ADNI (more on ADNI below) [92]. The CDR involves interviews with a patient as well as a reliable informant, and rates the severity of AD using a 5-point scale that categorizes a patient’s ability to function in the six cognitive categories of memory, orientation, judgment and problem solving, community affairs/involvement, home-life and hobbies, as well as personal care. The CDR can either provide a global score by using an algorithm that weights memory more heavily than the other categories, or it can be scored using the SOB method in which all categories are weighted equally [94]. Either way, the higher the score, the greater the severity of dementia.

The implementation of the new biomarker criteria for the AD spectrum found that about 70% of healthy elderly controls did not fit the three categories [95], where 43% had no positive marker of amyloidosis or neurodegeneration, and 23% were classified with neurodegeneration without evidence of amyloidosis. On the basis of this observation, Jack and colleagues introduced two new categories: a Stage 0 which comprised those healthy elderly controls with no evidence of amyloidosis or neurodegeneration, and a group termed “suspected non-AD pathophysiology” (SNAP) consisting of older adults with AD-like neurodegeneration but no evidence of amyloidosis [96]. As a consequence of this extended classification, several studies tried to elucidate the short and long term clinical, cognitive and volumetric trajectories of these four groups, the overwhelming majority showing that, in contrast with those with amyloidosis/AD pathway, those classified as SNAP did not decline over time and were indistinguishable from those elderly controls with no evidence of amyloidosis or neurodegeneration, suggesting a different, non-AD, underlying pathophysiological mechanism [97]. The lack of a strong association between Aβ deposition and measures of cognition, synaptic activity, and neurodegeneration in AD, in addition to the evidence of Aβ deposition in a high percentage of MCI and asymptomatic healthy controls, suggests that Aβ deposition is an early and necessary, though not sufficient, cause for cognitive decline in AD [81, 98, 99], indicating the involvement of other downstream mechanisms, triggered or not by Aβ, such as NFT formation, synaptic failure, and eventually neuronal loss.

The detection of Aβ pathology at the presymptomatic stages is of crucial importance because it is precisely the group that may benefit the most from therapies aimed at reducing or eliminating Aβ from the brain before irreversible neuronal or synaptic loss occurs [100]. Aβ imaging with PET is therefore contributing to the development of more effective therapies by allowing better selection of patients for anti-Aβ therapy trials and providing a means to measure their effectiveness in removing Aβ from the brain [101, 102]. However, different pharmacological and pharmacokinetics properties from separate Aβ tracers have presented small issues for multicenter studies wishing to compare results. Therefore, a method has recently been developed to produce a single common quantitative output value, called the Centiloid, for Aβ imaging across tracers and imaging analysis approaches, to improve clinical and research use of these Aβ tracers [103]. All F-18 labelled Aβ tracers are being cross-calibrated against PiB. Among them, 18F-NAV4694 and 18F-florbetaben have been the first two Aβ tracers validated using the Centiloid approach [104, 105].
Benefits of studying dominantly inherited AD

The establishment of DIAN by Professor John Morris from Washington University, of which there are three sites in Australia (Perth, Melbourne, and Sydney) has enabled access to mutation carrier and non-carrier members of autosomal dominant AD families. This global partnership has helped determine many changes that occur very early in the disease process in familial AD. Although it cannot always be assumed that early (pre-clinical) pathogenic changes that occur in the mutation carriers of these families will apply to sporadic AD, the study of these families has been invaluable in revealing how early pathogenic changes do start. For example, our studies of dominantly inherited AD have shown that CSF Aβ42 appears to decline 25 years before expected symptom onset, and Aβ deposition is detectable 15 years before expected symptom onset [87], as discussed below. We have also detected elevated levels of CSF tau, phosphorylated tau (181), and visinin-like-1, all markers of NFT and neuronal injury, in asymptomatic mutation carriers 10–20 years before their estimated age of symptom onset, and before the detection of cognitive deficits [106]. Longitudinal studies of asymptomatic AD mutation carriers have shown that amyloid burden (as determined by PiB-PET) predicts future decline in episodic memory, whereas in symptomatic carriers, cerebral amyloidosis correlates with worse baseline performance, and predicts greater decline in global cognition, working memory and MMSE results [107]. More recently, we have found that white matter hyperintensities, as measured by T2-weighted MRI scans, are increased approximately 6 years before expected symptom onset in autosomal dominant AD, suggesting these changes are a core feature of AD [108]; we have also shown low body mass index appears to correlate with preclinical stages in autosomal dominant AD, with signs of weight loss occurring 10–20 years before expected symptom onset [109].

BIOMARKERS: PREFERABLY PERIPHERAL

As we have mentioned previously, it is estimated that AD takes 2-3 decades to develop in the brain, before clinical symptoms are apparent. Brain functions decline as consequence of synaptic loss and neuronal death. A cure at this late stage is unlikely, since such widespread brain damage has already occurred by the time symptoms appear, and current treatments mostly reduce symptoms and temporarily reduce the rate of decline. The emphasis is currently on treatments that may address the underlying pathogenesis at the earliest stage possible, so that people in preclinical stages can have therapy to delay, or even prevent disease progression. To investigate and monitor such treatments, an understanding of disease progression is essential, to help determine an individual’s risk or pre-clinical disease stage, if pathology is already present. The search for pre-clinical biomarkers has occupied many laboratories worldwide, and many advances have been made. As described above, the study of brain Aβ accumulation is helping to determine disease pathogenic stages, yet this is clearly a method that is too expensive and technically complicated to use for routine diagnosis. Below we describe other developing technologies for AD diagnosis.

Cerebrospinal fluid (CSF)

In AD subjects, the CSF concentration of Aβ42 decreases over time, while 181-phospho-tau and total tau concentrations increase, when compared to healthy controls (including patients with psychiatric disorders such as depression) [110]. CSF studies have shown that the combined measurement of CSF Aβ42, total tau, and 181-phospho-tau levels can diagnose AD [111] with a sensitivity and specificity reaching 92 and 89%, respectively [112]. Other studies have suggested an assay using Aβ42 and T-tau levels can accurately discriminate AD from controls by means of a discrimination line, which has been validated in clinical practice [113] and in autopsy-confirmed patients, with sensitivity levels of 100% and specificity of 91% [114]. Changes in these three CSF biomarkers allow the diagnosis of AD already in its prodromal stage—people with MCI [115].

A collaboration with the DIAN study group, involving PiB-PET data, CSF biomarker measurement, and cognitive assessments, has helped investigate changes in AD mutation-carrying individuals long before their estimated time of symptom onset. The study suggests that CSF Aβ levels decline 25 years before disease onset; that Aβ deposition (detected by PiB-PET), increases in tau protein levels and greater than normal brain atrophy are all first detected about 15 years prior to expected disease onset, and that cerebral hypometabolism and impaired episodic memory can be observed about 10
years prior to symptom onset [87]. Cognitive impairment, as measured by the MMSE and the Clinical Dementia Rating Scale, was detected 5 years prior to expected symptom onset. These results are important as they underscore the long time-frame and stages of AD pathogenesis, and the early disruption in Aβ metabolism; and although the results may not all be applicable to sporadic AD cases, there will undoubtedly be many similarities.

However, the use of CSF for diagnosis is not ideal, as CSF collection is a relatively invasive and expensive procedure. Ideally, a blood-based test for AD using serum or plasma would be a better choice, as it would be inexpensive, relatively non-invasive, and widely accessible.

Blood biomarkers

Several groups have attempted to create biomarker panels to differentiate between AD and other forms of dementia, and to detect early (preclinical) stages of AD. In 2007, Ray and collaborators devised a plasma biomarker panel of 18 proteins that was able to predict the conversion to AD 2–6 years later [116]. This panel was considered a breakthrough in the field, other groups have since attempted to confirm these results in different cohorts. In 2008, one study suggested that a biomarker panel of only 5 proteins from the former 18-protein panel was sufficient to distinguish controls from AD with the same accuracy [117], yet in one study of the original 18-protein panel, only 3 proteins (epidermal growth factor (EGF), platelet-derived growth factor-homodimer (PDGF-BB), and the inflammatory chemokine MIP-1β) [118] were found to be associated with AD, whereas another study which investigated 16/18 of the proteins found 5 proteins [EGF, MIP-1β, the macrophage inflammatory protein MIP4, glial-derived neurotrophic factor (GDNF), and chemokine ligand 5 (also known as RANTES)] were found to be associated with AD, whereas another study which investigated 16/18 of the proteins found 5 proteins, the same accuracy [117], yet in one study of the original 18-protein panel, only 3 proteins (epidermal growth factor (EGF), platelet-derived growth factor-homodimer (PDGF-BB), and the inflammatory chemokine MIP-1β) [118] were found to be associated with AD, whereas another study which investigated 16/18 of the proteins found 5 proteins [EGF, MIP-1β, the macrophage inflammatory protein MIP4, glial-derived neurotrophic factor (GDNF), and chemokine ligand 5 (also known as RANTES)] were found to be associated with AD, whereas another study which investigated 16/18 of the proteins found 5 proteins [EGF, MIP-1β, the macrophage inflammatory protein MIP4, glial-derived neurotrophic factor (GDNF), and chemokine ligand 5 (also known as RANTES)] were found to be associated with AD, whereas another study which investigated 16/18 of the proteins found 5 proteins [EGF, MIP-1β, the macrophage inflammatory protein MIP4, glial-derived neurotrophic factor (GDNF), and chemokine ligand 5 (also known as RANTES)] were found to be associated with AD, whereas another study which investigated 16/18 of the proteins found 5 proteins [EGF, MIP-1β, the macrophage inflammatory protein MIP4, glial-derived neurotrophic factor (GDNF), and chemokine ligand 5 (also known as RANTES)] were found to be associated with AD, whereas another study which investigated 16/18 of the proteins found 5 proteins [EGF, MIP-1β, the macrophage inflammatory protein MIP4, glial-derived neurotrophic factor (GDNF), and chemokine ligand 5 (also known as RANTES)] were found to be associated with AD, whereas another study which investigated 16/18 of the proteins found 5 proteins [EGF, MIP-1β, the macrophage inflammatory protein MIP4, glial-derived neurotrophic factor (GDNF), and chemokine ligand 5 (also known as RANTES)] were found to be associated with AD, whereas another study which investigated 16/18 of the proteins found 5 proteins [EGF, MIP-1β, the macrophage inflammatory protein MIP4, glial-derived neurotrophic factor (GDNF), and chemokine ligand 5 (also known as RANTES)] were found to be associated with AD, whereas another study which investigated 16/18 of the proteins found 5 proteins [EGF, MIP-1β, the macrophage inflammatory protein MIP4, glial-derived neurotrophic factor (GDNF), and chemokine ligand 5 (also known as RANTES)] were found to be associated with AD, whereas another study which investigated 16/18 of the proteins found 5 proteins.

Some that significantly increased (cortisol, pancreatic polypeptide, insulin-like growth factor binding protein 2, β2 microglobulin, vascular cell adhesion molecule-1, carcinoembryonic antigen, matrix metalloprotein 2, CD40, MIP1α, superoxide dismutase, and homocysteine) and decreased (apoE, EGF receptor, hemoglobin, calcium, zinc, interleukin 17, and albumin) in AD. Other researchers have detailed similar analyses in their cohorts, using different biomarker panels [120–123].

The biomarkers found to distinguish between healthy controls, MCI, and AD are often quite different between studies, and this will have occurred for many reasons. Some of these reasons include the differences in cohort ages, disease severity, diagnostic methods, assay platform, blood collection and processing methods, populations being compared, and whether assays are cross-sectional or allow for longitudinal data to be analyzed too. A further complication came to light recently as a study of serum samples from Mexican Americans (AD and healthy controls) found that the biomarker profile from this population was different to that found in prior studies of non-Hispanic populations [124], again complicating the interpretation and comparison of studies, and suggesting that further studies are needed to characterize racial/ethnic differences in biomarker profiles. It is most likely that assays dependent on APOE e4 allele status will be necessary, due to the widespread influence of the apoE protein on AD-related biomarkers—an issue highlighted in one biomarker study [121], which found increases in pancreatic polypeptide, N-terminal protein B-type brain natriuretic peptide and tenascin C levels, and decreases in IgM and apoE in patients with AD and mild cognitive impairment. The study also found that the APOE genotype was associated with a unique biochemical profile irrespective of diagnosis, as APOE e4 carriers (e3/e4 and e4/e4) were characterized by low C-reactive protein and apoE levels and by high cortisol, interleukin 13, apolipoprotein B, and gamma interferon levels [121]. Our most recent set of results, which again emphasize the effect of the APOE e4 allele, showed that high levels of IL-10 and IL-12/23p40 were significantly associated with amyloid deposition in healthy controls, suggesting that these two biomarkers might detect at risk individuals. Additionally, other biomarkers (Eotaxin-3, Leptin, Peptide YY) exhibited altered levels in AD participants possessing the APOE e4 allele [125]. One of our more recent AIBL cohort studies is a 54-month follow-up investigation of a blood-based
signature which had already shown promise at stratifying individuals into high and low neocortical Aβ burden [98]. Results included the finding that 40% of the participants with MCI who had estimated high NAB progressed in comparison to 5% of the participants with MCI who had estimated low NAB (odds ratio = 12.3) [93]. These results indicate that a simple blood-based signature not only provides estimates of amyloid deposition, but also predicts cognitive decline and disease progression, which is essential for the testing and monitoring of potential interventions or therapies. Nevertheless, the advances made in the last decade in blood biomarker studies, together with advances in other potential diagnostic avenues (such as those listed below) suggest that preclinical diagnosis, and even disease “staging” may be available in the next few years.

A drop in levels of Aβ42 in the CSF is considered one of the gold standard biomarkers for AD pathogenesis. It can be argued that changes in CSF Aβ levels would be more likely than changes in plasma Aβ levels to reflect pathological changes in the brain, however the need for more accessible biomarkers prompted us, and others, to investigate plasma Aβ levels.

Disruptions to lipid metabolism, obesity, hypertension, and cardiovascular disease are all linked to increased AD risk, and our group has investigated several aspects of lipid metabolism, for example the effects of APOE allele status on Aβ clearance, and the links between dyslipidemia and AD. We extended these studies to determine whether plasma Aβ levels correlate with plasma lipid profiles. In cognitively normal people as well as people with subjective memory complaints (may indicate very cognitively normal people as well as people with AD), we found that Aβ40 levels correlated negatively with HDL levels. [126]. Cause and effect has not been established here, but if so, the results support the concept that lifestyle interventions or novel therapeutics could help slow disease pathogenesis. Some lipid changes are more likely part of AD pathogenesis, as suggested by studies of autosomal dominant AD mutations: in subjects from the DIAN Australian cohort, carriers (symptomatic and asymptomatic) and non-carriers of PSEN1 mutations, we investigated plasma phospholipid and sphingolipid profiles. Of the 139 plasma lipid species measured, significantly altered species belonged mostly to choline and ethanolamine-containing classes, and ceramides. Within the mutation carrier group, three phosphatidylcholine species correlated with CSF tau, and two plasmalogen ethanolamine species correlated with CSF tau and brain NAB [127]. These statistically relevant differences were found in this pilot study of only 26 people, and further studies should be carried out in the larger DIAN cohort as well as in sporadic AD populations. Other recent studies involving cognitively normal individuals with preclinical AD demonstrated alterations in the erythrocyte fatty acid composition, wherein increased arachidonic acid and decreased docosa-pentaenoic acid were observed in high NAB individuals (compared to those with low NAB) [128]. Further lipid studies will provide greater characterization of these pathogenic changes, and also determine if these changes can be used as preclinical diagnostic markers.

Investigations in the AIBL cohort enabled comparisons between PiB-PET-determined Aβ load and levels of potential blood biomarkers. In one study, plasma Aβ40, Aβ42, and N-terminal cleaved fragments were measured using both a commercial multiplex assay and a well-documented ELISA [129]. We found that lower plasma Aβ42 levels and Aβ42: Aβ40 ratios were observed in patients with AD, and were inversely correlated with PiB-PET derived brain Aβ load. In another cross-sectional study of the AIBL cohort, we investigated whether plasma apoE levels correlated with AD pathogenesis (determined by PiB-PET brain Aβ load), as previous studies had produced conflicting results, yet few studies had had the opportunity to correlate with AD pathology premortem. We found that total apoE and apoE e4 levels were significantly lower in patients with AD in the entire cohort, and within the subset that had brain Aβ load assessed by PiB-PET, ApoE levels decreased with increasing Aβ load. ApoE levels were also significantly lower amongst the e4 homozygous individuals [130]. Similar links between low apoE levels and AD risk, as well as APOE allele-related differences in plasma apoE levels, have been found by other researchers, supporting these results [43, 55, 121]. Continuing these studies, we have shown that apoE is decreased in individuals with AD compared with healthy controls at an 18-month follow-up, consistent with our results published at baseline. The results also showed lowest apoE levels in e4/e4 individuals [131].

Our later studies, which provided both baseline and 18-month follow-up Aβ measurements, demonstrated a decrease in the Aβ42/Aβ40 ratio in patients with AD, which was inversely correlated with NAB [132]. Furthermore, over the 18 months, plasma Aβ42 decreased in subjects with MCI, and in those transitioning from healthy to MCI. Thus we first suggested
that baseline plasma Aβ42 and the Aβ42/Aβ40 ratio could be putative biomarkers indicative of cognitive decline, and then provided validation for these suggestions using 18-month data. Our published results to date indicate that plasma Aβ levels may be useful as part of a panel of peripheral biomarkers [132, 133].

In support of these results, a recent study of 41 subjects (23 brain Aβ+ve and 18 Aβ–ve) has also suggested plasma Aβ may be useful as a brain amyloidosis biomarker, though in a different way. A stable isotope labeling kinetics protocol was used to investigate the turnover of Aβ38, Aβ40, and Aβ42 in human plasma. The study found faster fractional turnover of Aβ42 relative to Aβ40, as well as lower Aβ42 and Aβ42/Aβ40 concentrations in amyloid-positive participants, suggesting blood Aβ42 shows similar concentration changes to those seen in CSF [134].

Our interest in oxidative stress and inflammation in the pathogenesis of AD led us to investigate levels of homocysteine, vitamin B12, and folic acid levels in the AIBL cohort. Homocysteine is needed for methionine biosynthesis, which requires both folate and vitamin B12, and diet (a modifiable factor) influences vitamin B12 and folate levels. Plasma homocysteine levels are known to increase with age, and correlate inversely with vitamin B12 and folate levels in the blood. A relationship between plasma homocysteine levels, cognitive performance, and the risk of AD has previously been reported, particularly in longitudinal studies with 5–9 year follow-up, showing increases in homocysteine correlating with cognitive decline [135], though some studies have not found this correlation [136], possibly due to shorter follow-up time (2.7 years). There has also been disagreement on blood vitamin B12 and folate levels, possibly as many study cohorts were small. With access to the large AIBL cohort, we investigated whether levels of these three blood components correlated with cognitive decline. We found homocysteine levels were significantly higher in female AD patients compared to female healthy controls, but this association was not present in the male population. Nevertheless, episodic memory and global cognition correlated negatively with homocysteine in all clinical categories. Red cell folate has a U-shaped association with homocysteine, such that high red cell folate levels were associated with worse long-term episodic memory total episodic memory and global cognition [137]. Thus, we have added to the evidence of an association between homocysteine levels and cognitive decline (although this is not unique to AD), and our red cell folate results may reflect low conversion of homocysteine to methionine, though this requires further investigation. In a later investigation of cognitive impairment in a cohort of over 1,300 elderly subjects, participants with low serum vitamin B12 (<250 pmol/L) and high red cell folate (>1594 nmol/L) levels were more likely to have impaired cognitive function, when compared to participants with normal range levels [138], suggesting supplements providing high levels of folic acid may be detrimental to the elderly if they have low vitamin B12 levels.

OTHER DIAGNOSTIC AVENUES

Eye tests

The diagnostic potential of the eye has been investigated widely, as it shares many neural and vascular similarities to the brain and potentially reflects the brain pathology [139, 140]. The eye is also accessible and easily imaged. The first studies to find changes in AD versus healthy controls discovered abnormal patterns in electroretinograms; later studies found enhanced pupil response to cholinergic drops, retinal nerve fiber layer (RNFL, ganglion cell axons) thinning as well as optic nerve degeneration [141, 142], which indicated widespread ganglion cell loss. In our own studies, we have shown that pupillary reactions such as pupil flash response, can distinguish autosomal dominant AD mutation carriers from non-carriers prior to symptom appearance [143]. AD patients have also been found to be more sensitive to tropicamide eye drops (muscarinic cholinoceptor antagonist), thought to be due to AD-associated loss of noradrenergic neurons in the locus coeruleus [144].

Over the years, higher resolution imaging technology has allowed for better sensitivity in measuring RNFL thickness, thus it is possible to distinguish MCI and AD from healthy controls. However, the specificity of the RNFL thickness and other eye biomarkers is low due to confounding factors such as age and comorbid eye disorders including glaucoma [142, 145]. Polarization-sensitive optical coherence tomography, which has been shown to detect AD-associated birefringence due to microtubule damage, is a sensitive new technique [146]. The birefringence is thought to precede RNFL thinning and thus this method appears promising to detect AD at an earlier stage.

Various other eye abnormalities have been linked to AD, such as changes in choroidal thickness [147], though this could be present in other eye conditions.
Changes to retinal blood flow on the other hand have been shown to distinguish MCI and AD from controls [148]. In our own studies of the AIBL cohort, we have been able to detect vascular abnormalities such as venular branching asymmetry and higher arteriolar length-to-diameter ratios in healthy individuals with high levels of brain Aβ load [149]. Thus, retinal blood flow may have good preclinical diagnostic potential. Our recent studies of retinal vasculature, RNFL, and retinal ganglion cell layer thickness in AD subjects and individuals with subjective memory complaints showed significant association with NAB [150], adding evidence for the diagnostic potential of retinal measures in AD. We have also investigated the correlation between AD and early signs of age-related macular degeneration in a subset of participants of the AIBL cohort, and found a highly significant association with AD diagnosis (p < 0.0001); the reason for this association is unclear [151] and warrants further investigation. Furthermore, we have investigated the association between retinal arteriolar central reflex and retinal vessel width, and found that the central reflex:vessel width ratio (CRR) is higher in APOE e4 carriers, and there is also a trend toward higher levels in AD patients compared to controls [152]. This may prove to be useful for monitoring apoE isotype effects on cerebrovascular disease.

The eye itself has been shown to produce AβPP and accumulate Aβ, and novel Aβ-binding agents such as curcumin are showing promise as detection agents [140, 141, 153]. Preliminary results (n = 40) from our laboratory using in vivo curcumin fluorescence retinal Aβ imaging method in the AIBL cohort showed high correlation (r = 0.762, p = 0.0001; calculating the retinal amyloid index) with brain Aβ plaques (Frost S et al., unpublished results). Besides the high correlation, the test could also differentiate between AD and non-AD with 100% sensitivity and 80.6% specificity, respectively. However, not all studies have had the same outcome; some have found no Aβ deposits in the eye, and others indicate retinal hyperphosphorylated tau may be a better marker [142]. In a recent animal study, retinal Aβ was identified using a novel hyperspectral imaging method in live mouse retina, without any extraneous agent [154]. Aβ deposition has also been reported in the postmortem crystalline lens of AD individuals [155]. To conclude, the ability to identify changes occurring in the eye which reflects the build-up of brain Aβ could be an excellent candidate or surrogate marker in AD diagnostic process and for monitoring therapeutic response.

**Buccal tests**

Buccal cells were initially collected from AD and control subjects mostly to carry out APOE genotyping. However, a CSIRO study of such cells revealed that the frequency of basal cells, condensed chromatin cells, and karyorrhectic cells were significantly lower in AD patients [156]. Collaborating with these CSIRO researchers, we then found abnormal numbers of chromosome 17 and 21 (aneuploidy) in AD compared to age-matched control buccal cell samples, significantly greater amounts of DNA/cell and greater numbers of abnormal nuclear shapes were found in both MCI and AD compared to controls [157], yet similar significant differences were not found in hippocampal tissue when comparing AD and controls. Another group carried 3D quantitative imaging of telomeres in buccal cells, and were able to distinguish between mild, moderate and severe AD patients, based on five 3D parameters: 1) telomere length, 2) telomere number, 3) telomere aggregation, 4) nuclear volume, and 5) a/c ratio, a measure of spatial telomere distribution [158]. The most recent study by the same group used a different cohort with participant information blinded to the analysis. The 3D telomere profiles can distinguish between AD and control subjects [159], and further studies must aim to improve the technology for this promising biomarker, as well as investigate earlier (preclinical) stages of the disease. In our own AIBL studies, we have shown that numbers of buccal cell (intermediate filament) expressing cytokeratin 14 are significantly lower in MCI as well as AD. We also found in this pilot study that APOE e4 carriers trended toward lower CK14 expression [160]. Following up on this in a larger AIBL study, we put together a biomarker panel which included CK14 expression, plasma vitamin B12, Mg2+, LDL, and homocysteine. We again found that CK14 levels were significantly lower in the MCI and AD groups compared with controls, and that this correlated with changes in plasma Mg2+ and LDL levels, as well as red blood cell volume, hematocrit, and basophil cell count [161]. When combined in the biomarker panel, the level of significance was enhanced (particularly when incorporating vitamin B12 and homocysteine: MCI (p = 0.003) and AD (p = 0.0001) groups compared with controls).

More recent studies have investigated Aβ and tau content of these cells, and found little difference in tau levels, yet buccal cell Aβ levels correlated with MMSE scores (r = –0.436, p = 0.001) and several blood-based biomarkers [162]. The automated
assay used in these studies, which was developed using laser scanning cytometry, also demonstrated higher levels of Aβ in AD compared to control cells [162]. More recent findings by other groups of telomere changes [159] and DNA structural changes [163] in buccal cells in AD compared to controls add to the evidence that AD is a systemic pathology. The evidence also supports the potential of an AD combined peripheral biomarker panel, which would aid in early diagnosis and the testing and monitoring of potential therapies.

OXIDATIVE STRESS, INFLAMMATION, AND AD

Oxidative stress, chronic inflammation, mitochondrial dysfunction, and dyslipidemia are all early events in AD [164]. These early pathological changes are also seen in conditions which are becoming increasingly common in middle age; such as insulin resistance, obesity, T2D, and cardiovascular disease and these conditions in turn have all been linked to an increased risk of AD [8].

Insulin and diabetes

Changes in glucose use in AD brains compared to elderly controls had been detected as far back as 1980 [165]. A link between AD and diabetes was confirmed in 1996 in the Rotterdam study, which indicated a positive association between the two conditions [166]. Many studies having been carried out since then; it is now clear that abnormalities in glucose metabolism, and changes linked to insulin resistance and T2D, may be some of the earliest pathogenic changes in AD [167, 168]. Further, in AIBL, we have shown that increased insulin resistance in the cognitively normal older adults is associated with poorer performance across several cognitive domains, including episodic memory and executive function [169]. There are many metabolic changes common to both conditions, and it is hard to determine which changes or steps initiate neuronal dysfunction and neurodegeneration. For example, hyperglycemia, dyslipidemia, and hyperinsulinemia are all known to promote Aβ accumulation, and these occur in both conditions [8]. Oxidative stress and inflammation are common to both conditions, and signs of these include higher levels of reactive oxygen species [170], an increase in advanced glycation end products [171], increases in inflammatory cytokines TNF-α, IL-1β, and IL-6 [172], and higher Ca²⁺ levels [173]. In addition, aging, hypertension, insulin resistance, diabetes, hypoxia/obstructive sleep apnea, obesity, and vitamin B12/folate deficiency (among others), also synergistically promote cerebral hypoperfusion as well as low glucose usage in the brain, adding to the sources of inflammation and oxidative-nitrosative stress in the brain [174].

It is known that the reduced glucose utilization and energy metabolism seen in AD are associated with brain Aβ and hyperphosphorylated tau accumulation, increased oxidative stress, and the accumulation of unfolded/misfolded proteins [175, 176]. As insulin had also been shown to influence AβPP processing, we investigated whether Aβ binding to the insulin receptor could influence AβPP processing. Using an in vitro model, we showed that insulin could facilitate the release of AβPP from cells transfected with insulin receptors, and that the addition of Aβ could block this release [177].

Another early link between diabetes and AD was the finding that a major insulin breakdown enzyme, the insulin degrading enzyme (IDE), also degraded Aβ peptides. For these reasons, and with the knowledge that both insulin and Aβ are amyloidogenic peptides, we investigated whether Aβ could bind to the insulin receptor. We found reduced insulin binding and receptor autophosphorylation, with the reduction in binding caused by a decrease in the affinity of insulin binding to the insulin receptor [178], suggesting that Aβ could compete directly with insulin binding.

It has been shown that cardiovascular disease, obesity, and dyslipidemia are also all associated with AD, which is not that surprising since there is considerable overlap in the underlying changes that lead to these conditions (oxidative stress, disruptions to glucose and lipid metabolism, and chronic inflammation) as in diabetes and AD [8, 179]. Proteomics studies, and recent lipidomics studies in particular, are beginning to reveal common pathological pathways that link these conditions [168].

Evidence is also mounting for the intriguing concept that AD pathology could contribute to insulin resistance and T2D [180]. The Aβ peptide and AβPP have been suggested to regulate systemic metabolism, as reviewed in [181, 182], and plasma levels of the more pathogenic Aβ₄₂ are increased in T2D compared to aged matched controls [183]. Tau has roles in insulin transport and secretion by the pancreatic β-cells [184, 185], and can also mod-
ulate insulin-dependent translocation of the glucose transporter, GLUT4 [186, 187], which is critical for glucose uptake by tissues. The deposition of both Aβ and phospho-tau can be found in postmortem pancreatic tissue from T2D [188], in animal models of AD [189], and in a novel mouse model with overlapping T2D and AD developed by Professor Paul Fraser (University of Toronto) [190]. Studies in AD mouse models also have indicated that Aβ impairs insulin signaling in liver and muscle tissue, contributing to insulin resistance in these mice when fed a high fat diet [190, 191]. Aβ active immunization has been reported to improve insulin sensitivity and glucose tolerance in the mice [190]. Together, these findings provide strong evidence for a contribution of Aβ in moderating peripheral insulin sensitivity and glucose metabolism. Whether the accumulation of phospho-tau contributes similarly remains to be determined.

Diet

There is considerable evidence that all these conditions linked to AD can be ameliorated by dietary changes. High calorie diets, which contain significant amounts of processed carbohydrates, simple sugars, processed fats, and which are low in fiber, vitamins, minerals, antioxidants, and healthy fats, are strongly linked to obesity, T2D, hypertension, insulin resistance, and cardiovascular disease. In contrast, adherence to a traditional Mediterranean diet (MeDi) [8] is known to be associated with longevity and good health [192]; similarly, the traditional Okinawa diet, along with an active and social lifestyle, is also associated with longevity and good health [193]. The MeDi is characterized by a high intake of vegetables, legumes, fruits, cereals, fish and unsaturated fatty acids (mostly in the form of olive oil), low intake of saturated fatty acids, meat, and poultry, low-to-moderate intake of dairy products (mostly cheese and yogurt), and a regular but moderate amount of alcohol (mostly wine and generally with meals).

As part of the longitudinal AIBL studies, we investigated the dietary patterns of participants via questionnaires. In one study, adherence to the MeDi (based on a score of 0–9 for adherence) was greater in the healthy control participants compared to the MCI and AD subjects, with a greater difference observed between AD and healthy control subjects (p < 0.001) [194]. In a subsequent analysis only looking at healthy control participants, MeDi, western and prudent dietary patterns were investigated in relation to cognitive change using a global cognitive score, as well as six cognitive domains, over 36 months. The western and prudent dietary patterns reflect actual dietary intakes observed in a given population, independent of any assumption on their beneficial or harmful effect. Our western dietary pattern was heavily loaded with red and processed meats, chips, refined grains, potatoes, sweets, and condiments, while our prudent dietary pattern was loaded heavily with vegetables, fruits, and nuts. The cohort of 527 cognitively healthy older adults completed the Cancer Council of Victoria food frequency questionnaire at baseline, and underwent a comprehensive neuropsychological battery at baseline and two follow-ups. Higher adherence to the MeDi was associated with less decline in the executive function cognitive domain in APOE ε4 allele carriers (β = 0.077; p < 0.001), and a higher adherence to the western diet was associated with increased decline in the visuospatial functioning domain in APOE ε4 allele non-carriers (β = −0.0006; p < 0.01). No significant relationships were observed between prudent diet score and cognitive decline. We hypothesized that the oily fish component of the MeDi (the n-3 fatty acids eicosapentaenoic acid and docosahexaenoic acid found in oily fish) may be mediating the effects observed via a mechanism involving inflammation.

Various other studies carried out in our laboratories have shown links between dyslipidemia and AD. For example, in a small study, plasma Aβ42 levels were found to correlate with body mass index in healthy people [196]. The study also found (non-significant) associations with insulin levels, HDL, and the inflammatory marker C-reactive protein, thus larger longitudinal studies are required to determine the significance of the results. We have also shown that plasma Aβ40 and Aβ42 were lower in individuals with T2D compared to others from the same community-based cohort without diabetes [183]. The Aβ42: Aβ40 ratio was also significantly higher in those with diabetes. Apart from showing an association between plasma Aβ levels and T2D, such variation needs to be considered when assessing plasma Aβ peptides as AD biomarkers.

Most recently, we have found that serum HDL is associated with better cognitive function, in particular short and long delay-free recalls, in older women (average age 62.5) [197]. This positive effect of HDL on verbal memory warrants further investigation in longitudinal studies, and since lipid intake is a major factor influencing HDL levels, this is further evidence of the importance of diet on AD and cognitive decline.
As oxidative stress and inflammation are thought to be central phenomena in the early pathogenesis of AD (as well as other conditions linked to AD), dietary supplements, or dietary changes that may increase antioxidant or anti-inflammatory compound intake, and reduce the intake of oxidized and processed lipids and proteins, have been recommended. We have reviewed a number of these recommended dietary supplements and changes. For example, we have reviewed tea as it has been suggested to contain potent antioxidants: it is rich in phytochemicals including flavonoids, tannins, caffeine, polyphenols, boheic acid, theophylline, theobromine, anthocyanins, gallic acid, and in particular epigallocatechin-3-gallate. Studies have shown that catechins (flavonoid phytochemicals) may inhibit Aβ plaque formation, and enhance cognitive function [198]. Further studies of tea are warranted, to determine more clearly any benefits of the components in reducing AD risk.

Other antioxidant and anti-inflammatory food sources that have also been investigated by us and others include curcumin (a component of the spice turmeric), cinnamon, ginger, and the pepper family [25, 199]. We have investigated the ability of a curcumin formulation (BiocurcumaxTM) in a 12-month study involving community-dwelling older adults. The main finding was a significant time × treatment group interaction observed for the Montreal Cognitive Assessment, which was subsequently revealed to be driven by a decline in function of the placebo group at 6 months that was not observed in the curcumin treatment group. Further longitudinal assessment is required to investigate changes in cognitive outcome [200]. Other clinical studies have also failed to find significant improvement following supplementation with curcumin, thought to be partly due to low solubility and bioavailability, and also due to cohorts already having AD, which is likely to be at a stage too late to produce significant positive effects, due to considerable neuronal loss already being present. Nevertheless, as described in our review [201], in vitro studies have indicated that Aβ metabolism is altered by curcumin, and animal studies report that curcumin may influence brain function and dementia development, most likely due to antioxidant and anti-inflammatory properties.

High carbohydrate diets are thought to contribute to insulin resistance, which is associated with a host of peripheral changes that can all impact on AD pathogenesis, including hyperglycemia, hyperinsulinemia, dyslipidemia, and inflammation [168]. In addition, we have recently shown greater carbohydrate intake to be associated with poorer performance in verbal memory in APOE ε4 allele non-carriers, and poorer performance in attention in APOE ε4 allele carriers [202]. These findings suggest that lowering carbohydrate intake may offer neurocognitive benefits, with our study suggesting specific cognitive domains are affected in an APOE genotype-dependent manner; however, all these findings need validation in longitudinal studies.

Due to the low glucose usage in the brain in the very early stages of AD, we are investigating the potential benefits of adding a modified version of coconut oil to the diet, or more likely replacing some other dietary fat with some coconut fat. Unlike the fats in most other dietary fat sources, a significant amount of lipids in coconut oil consists of medium chain fatty acids, which may be converted to ketone bodies, which in turn can provide an alternative energy source to the brain. There is already mounting evidence that coconut oil may be beneficial in the treatment of obesity, dyslipidemia, elevated LDL, insulin resistance, and hypertension (all risk factors for AD), and certain phenolic compounds and hormones (cytokinins) found in coconut may help prevent the aggregation of Aβ [203]. However, some studies question the cardiovascular benefits of coconut oil, and in fact argue that it is detrimental to cardiovascular health. More definite conclusions as to its clinical significance particularly with respect to brain health must await findings from randomized controlled trials.

Physical activity

Numerous studies have reported positive impacts of physical activity on cognitive function [204, 205]. However, the majority of previous studies have relied on self-report questionnaires, which by nature may introduce reporting biases. To remove this source of potential bias, we investigated habitual physical activity levels (quantified from actigraphy units worn for seven days) undertaken by 217 cognitively healthy participants from the AIBL cohort, aged 60–89. Actigraphy units measure total physical activity and intensity of physical activity, and the cohort was split into tertiles based on physical activity intensity. Comprehensive neuropsychological assessment was also carried out, and participants in the highest tertiles of intensity were found to be performing significantly better on the Digit Symbol, Rey Complex Figure Test copy, and verbal fluency tests, compared with the lowest tertile [206]. Nevertheless, when the cohort was split into tertiles based on total amount of
physical activity, no differences in cognitive performance were observed, indicating that intensity may be more relevant in the association between physical activity and cognitive function.

We have also examined the relationship between habitual physical activity levels and neuroimaging biomarkers. In particular, we investigated the relationship between self-reported physical activity levels and hippocampal volume in a sub-cohort of AIBL study cognitively healthy participants. We observed that participants reporting the highest levels of habitual physical activity had the largest hippocampal volume [207]. In this study, we also examined the effect of the brain-derived neurotrophic factor polymorphism (BDNF Val66Met) on this relationship: We observed that only Val/Val homozygotes (i.e., those we assume not to have impaired function of BDNF on hippocampal neurons) received the benefit of physical activity in terms of larger hippocampal volume, whereas Met carriers (i.e., those more likely to have impaired action of BDNF on hippocampal neurons) did not have an association between physical activity levels and hippocampal volume.

We have also used questionnaires to investigate exercise levels in a subset of the DIAN cohort. In 139 pre-symptomatic mutation carriers, the relationship between self-reported physical activity levels and hippocampal volume in a sub-cohort of AIBL study cognitively healthy participants. We observed that participants reporting the highest levels of habitual physical activity had the largest hippocampal volume [207]. In this study, we also examined the effect of the brain-derived neurotrophic factor polymorphism (BDNF Val66Met) on this relationship: We observed that only Val/Val homozygotes (i.e., those we assume not to have impaired function of BDNF on hippocampal neurons) received the benefit of physical activity in terms of larger hippocampal volume, whereas Met carriers (i.e., those more likely to have impaired action of BDNF on hippocampal neurons) did not have an association between physical activity levels and hippocampal volume.

Sleep

Another aspect of lifestyle which is gaining interest in the field of AD research is sleep. Importantly, it is becoming apparent that rather than simply manifesting as a comorbidity of AD, suboptimal sleep actually appears to contribute both to cognitive decline and AD pathology, as discussed in our review which details the proposed bidirectional relationship between suboptimal sleep and AD pathology [213]. Numerous studies have linked suboptimal sleep to faster cognitive decline and increased AD and dementia risk [214, 215]. A recent systematic review and meta-analysis of 18 longitudinal studies indicates that insomnia, in particular, is linked to an increased risk of AD [216]. Furthermore, as part of the AIBL study, we investigated the relationship between sleep quality and PET-determined brain Aβ burden in cognitively normal individuals. We found longer sleep latency to be associated with higher brain Aβ burden, with a 30-minute longer sleep latency potentially translating to an equivalent of 2 years of brain Aβ accumulation [217]. Interestingly, in our cohort, APOE ε4 allele status had no effect on this relationship. However, our additional investigations using the AIBL study cohort suggest that genetic variation in the cerebally expressed water-channel protein, Aquaporin-4, does moderate the relationship between sleep and brain Aβ burden (Rainey-Smith SR et al., Translational Psychiatry, in press), an intriguing finding given that Aquaporin-4 is an astrocytic end-feet expressed water channel protein postulated to be involved in glial system-mediated clearance of Aβ from the brain [218]. Further studies, particularly longitudinal follow-up studies, are needed to gain greater insight into the extent sleep deprivation can influence cognitive decline.

Some other sleep investigations have involved the analysis of electroencephalograms (EEG) for both wakefulness and rapid eye movement (REM) sleep, performed over the temporal regions of AD patients and age-matched control subjects. Analysis of the spectra indicated that AD patients had much slower EEG readings during REM sleep when compared to being awake, and asymmetry on the awake EEG of AD patients was found to be even more prominent than on the REM sleep EEG [219].

HORMONE STATUS AND AD

Although many factors may influence the incidence of AD, most studies agree that about twice
as many women as men develop AD. An obvious gender difference is the sudden drop in sex hormones in women around menopause, and therefore the relationship between menopause and cognitive decline has been the subject of many research studies. Hormone studies have since found that estrogen can protect neurons from oxidative stress, aid neuroplasticity, help regulate learning and memory, shift A\betaPP metabolism toward the non-amyloidogenic pathway, and attenuate A\beta-induced apoptosis and inflammation [220, 221]. Treatment of women with hormone replacement therapy (HRT) has produced mixed findings, with most studies showing benefits when treatment is given around the time or just after menopause. HRT treatments given at a much later age, or once AD symptoms have appeared, have rarely been found to be beneficial; some have even been found to be detrimental [222]. Experimental evidence from animal models suggests the formulation and regimen of HRT is also of critical importance, with the best neuroprotective outcomes observed when estrogen is combined with cyclic rather than constant progesterone regimens. In our own studies of healthy post-menopausal women given estrogen replacement therapy, we found some improvements in memory functioning and only in women who did not carry APOE \epsilon4 alleles [223], indicating yet another potential link between apoE and AD.

In contrast to women and menopause, men experience a gradual decline in testosterone levels with age, known as andropause. However, there can be considerable variation, with some men experiencing much more severe declines in testosterone levels, and this age-related decline has also been linked to cognitive decline and AD risk [224]. Animal studies have shown benefits from testosterone supplementation in improving cognition and reducing AD pathology [225]. Observations of hypogonadal men and men on androgen deprivation therapy have shown that lower androgen levels can impair cognitive function, particularly verbal memory, visuospatial ability, and executive functions [226, 227]. Therefore, several research groups, including ours, have investigated the benefits of testosterone treatment on cognitive function in men who have low testosterone levels. Using the AIBL cohort, we investigated associations between gonadotropins, testosterone, and brain and plasma A\beta in men at risk of AD. We found that luteinizing hormone (LH) levels influenced plasma A\beta_{40} and A\beta_{42} levels, whereas brain A\beta load as assessed by PiB-PET was associated negatively with calculated free testosterone levels [228], supporting the concept of these hormones influencing preclinical stages of AD. In randomized, placebo-controlled, crossover studies of men with subjective memory complaint and low testosterone levels, we investigated the effect of testosterone supplementation, and found firstly that the treatment was well-tolerated and did not raise hormone levels above a healthy range [229]. We also found that such treatment provided modest improvements on global cognition [230]; however, future clinical trials with longer follow-up, and the measurement of blood and brain biomarkers, would provide more conclusive results.

Some of the research interest in sex hormones has now shifted to LH. This hormone is produced by the gonadotropic cells in the anterior pituitary gland, and it controls the release of both testosterone in men and estrogens and progesterone in women. Evidence is mounting that age-related increases in LH may influence AD pathogenesis as it has been implicated in inflammation, changes to cholesterol homeostasis, altered metabolism of A\beta and A\betaPP, and insulin metabolism [231]. In some of our own studies we have shown that LH levels (and not testosterone) correlate with plasma A\beta levels in elderly men [232], whereas in women, we have found high endogenous LH is associated with a lower cognitive score, particularly in women who are depressed [233]. In addition, well-preserved cognitive functioning was found in the oldest women in the community-dwelling cohort who had high endogenous follicle stimulating hormone levels. Using a transgenic mouse model of AD, we found the potent analogue of LH, human chorionic gonadotropin, to impair working memory and modestly increase brain A\beta levels. Others have since shown that LH can influence hippocampal-related spatial memory [234], and that downregulating LH (but not estrogen therapy) can improve cognitive dysfunction and spine density loss induced by ovariectomy [235]; however, the underlying mechanisms by which these hormones influence A\beta accumulation, metabolism, inflammation, or contribute to neurodegeneration, are not completely understood [236], and further research is needed to determine if some form of hormone therapy may reduce the risk of AD.

**SUMMARY**

Current available treatments for AD, at best, only target amelioration of symptoms. Preventative
strategies will need to be implemented, and therefore tested, within the early stages of the disease pathogenesis trajectory, preferably at a preclinical stage prior to cognitive decline and irreversible damage. Exactly “how early is early enough?” to prevent clinical changes remains a major unknown. There is a reasonable chance that—especially for APOE ε4 women—intervention might need to begin in the fourth or fifth decade of life. A major challenge involves the development of an intervention that is sufficiently safe to be administered for decades to asymptomatic subjects.

The current gold standard biomarkers of AD, namely brain Aβ load and CSF tau and Aβ1-42 concentrations, have enabled identification of individuals within the preclinical stage of AD. However, these diagnostic modalities are not easily accessible or economically viable for population wide screening. Therefore, laboratories worldwide including our own teams, are focusing on identifying less invasive economical markers to meet the dire need of early diagnosis. As such, given that the blood is an easily accessible medium, emphasis has been placed on identifying blood biomarkers reflecting preclinical AD, wherein particular protein, lipid and metabolite profiles have been observed to reflect the gold standards, with particular marker panels showing considerable accuracy; establishing the highly characterized AIBL longitudinal study of aging has enabled us to make major strides towards these objectives not only with blood biomarkers identification but buccal cell biomarkers and more recently with retinal imaging biomarkers. However, although promising, these studies require further replication across all diverse ethnic groups. More importantly, further research is required to reduce the number of analytes within biomarker panels for commercialization and clinical setting usage purposes, as current panels within the existing literature while showing considerable accuracy require a panel of several analytes (~20). Interestingly, these biochemical alterations manifesting in the blood in AD pathogenesis also exhibit the systemic nature of the disease and provide insight into AD pathomechanisms. Finally, while effective treatments for AD are yet to be established other approaches in addition to drug therapy need to be considered and investigated. Clinical trials targeting healthy lifestyle approaches provide hope to reduce risk of AD and should augment the effectiveness of drug action to combat AD.

DISCLOSURE STATEMENT

Authors’ disclosures available online (https://www.j-alz.com/manuscript-disclosures/17-1145r1).

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