Validation of a priori candidate Alzheimer’s disease SNPs with brain amyloid-beta deposition

Michael Vacher1*, Tenielle Porter2,3, Victor L. Villemagne4,5,6, Lidija Milicic2, Madeline Peretti2, Christopher Fowler5, Ralph Martins7, Stephanie Rainey-Smith7, David Ames8, Colin L. Masters5, Christopher C. Rowe4,6, James D. Doecke5 & Simon M. Laws2,3,9

The accumulation of brain amyloid β (Aβ) is one of the main pathological hallmarks of Alzheimer’s disease (AD). However, the role of brain amyloid deposition in the development of AD and the genetic variants associated with this process remain unclear. In this study, we sought to identify associations between Aβ deposition and an a priori evidence based set of 1610 genetic markers, genotyped from 505 unrelated individuals (258 Aβ+ and 247 Aβ−) enrolled in the Australian Imaging, Biomarker & Lifestyle (AIBL) study. We found statistically significant associations for 6 markers located within intronic regions of 6 genes, including AC103796.1-BDNF, PPP3R1, NGFR, KL, ABCA7 & CALHM1. Although functional studies are required to elucidate the role of these genes in the accumulation of Aβ and their potential implication in AD pathophysiology, our findings are consistent with results obtained in previous GWAS efforts.

Alzheimer’s disease is the most prevalent cause of dementia in elderly populations (age > 65 years). Currently affecting more than 40 million people worldwide, this number is projected to increase at least three-fold by 2050, with the continuing growth and ageing of the population. Hallmarks of disease pathology generally appear several years prior to the onset of clinical symptoms. Although the slow progression provides opportunities for preclinical therapeutic interventions, our ability to accurately detect the disease remains limited.

The accumulation of Aβ occurs at a variable rate early in the development of AD, starting over 20 years before the onset of cognitive decline and structural brain atrophy. The process is a well-recognised histopathological hallmark of AD, and Aβ deposition is necessary for the pathologic diagnosis of the disease. However, the formation of Aβ plaques alone is not sufficient to cause cognitive dysfunction. Individuals with high Aβ accumulation but no or minimal cognitive deficits have been observed in several studies. In addition, recent studies have shown that the presence of substantial Aβ deposition had low specificity for predicting the development of AD. These observations reflect the intricate contribution of Aβ formation in the development of AD, and the need for more research in the developmental processes of the disease.

This complex pathogenesis of AD involves multiple external risk factors and comorbidities with varying susceptibilities based upon genetic backgrounds. In recent years, genome-wide association studies (GWAS) have identified more than 20 genetic risk loci robustly associated with the disease. Large meta-analyses such as the one conducted by the International Genomics of Alzheimer’s Project (IGAP), have played a key role in...
AC103796 previously established AD-specific loci include including the presence/absence of the nominal evidence for association (p rs4147911, rs4147910, rs76348507) that reached genome-wide significance in the IGAP meta-analysis. Figure 1 per region of linkage disequilibrium, resulting in 6 independent SNPs (Table 2). Evidence of associations with TOMM40, 12.4 kb) genes.

region containing the apolipoprotein E (APOE, 3.6 kb) and the translocase of outer mitochondrial membrane 40 tus corresponded to a set of 5 SNPs (rs429358, rs769449, rs6857, rs157581, rs2075650) located within a 20 kb with rationale to identify possible Aβ specific variants, from a large list of AD-related candidate genes. were genotyped for an a priori evidence based targeted selection of Single Nucleotide Polymorphisms (SNPs), with rationale to identify possible Aβ specific variants, from a large list of AD-related candidate genes.

Results
We conducted an association analysis using 1610 genetic markers from 505 unrelated participants of the AIBL study (258 cases and 247 controls). Comparing demographic and clinical characteristics between Aβ groups (p <0.05), and MCI and AD groups were more likely to be Aβ+ than Aβ− (p <0.0001).

In preliminary analysis containing all the markers, the strongest associations (p <1e−8) with amyloid status corresponded to a set of 5 SNPs (rs429358, rs769449, rs6857, rs157581, rs2075650) located within a 20 kb region containing the apolipoprotein E (APOE, 3.6 kb) and the translocase of outer mitochondrial membrane 40 (TOMM40, 12.4 kb) genes.

To enhance the identification of additional Aβ specific variants, we conducted the same association analysis including the presence/absence of the ε4 allele as a covariate. A total of 10 SNPs, located within 6 loci, showed nominal evidence for association (p < 5e−4). Results were clumped to keep only the most representative SNP per region of linkage disequilibrium, resulting in 6 independent SNPs (Table 2). Evidence of associations with previously established AD-specific loci include AC103796.1-BDNF (rs2049048; p = 3.62e−04; OR, 0.45 [95% CI, 0.29–0.70]). A total of 10 SNPs, located within 6 loci, showed nominal evidence for association (p <5e−4). Results were clumped to keep only the most representative SNP per region of linkage disequilibrium, resulting in 6 independent SNPs (Table 2).

Table 1. Population characteristics. P values determined by Fisher’s test (APOE ε4 and Gender), t-test (age), and Chi square analyses (diagnosis). N number, HC healthy control, MCI mild cognitive impairment, AD Alzheimer's disease, APOE ε4 apolipoprotein ε4 allele.

Table 2. Significant SNPs and associated loci.
shows a Manhattan plot of all SNPs tested in the current study, with the -log10 of the p-value on the y-axis, chromosome on the x-axis, and dot colour representing the presence/absence of markers in the IGAP study.

Lastly, we assessed the gene ontologies and functional interactions amongst the genes that reached nominal significance using the GeneMANIA resource. Through the gene-gene interaction network, we were able to demonstrate the presence of physical and genetic interactions between the identified genes and several other genes with similar biological functions (Fig. 2).

Discussion
As with most complex traits, multiple genetic variants with small and cumulative effects are likely to explain the heritability of AD. Consistent with this assumption, we identified robust associations with several previously established loci in a sample of cognitively normal and AD subjects. First, by identifying several markers located in the TOMM40-APOE region, our analysis supports the hypothesis that APOE on chromosome 19 is a major susceptibility gene for AD. The APOE ε4 allele has been associated with an increased risk of developing AD in a number of independent studies. Therefore, these results were expected but can be considered as a validation of our dataset. Aside from SNPs within the TOMM40-APOE locus, we identified a set of 6 variants at the nominal significance level showing evidence of association with Aβ status. A gene-gene interaction network revealed direct and indirect interactions amongst the 6 genes in which the genetic markers are located, suggesting a collective influence of genetic polymorphisms (Fig. 2).

The top-ranked SNP, rs7593613, was located in the regulatory subunit of the protein phosphatase B gene (PPP3R1), also known as calcineurin B. This marker was in high linkage disequilibrium (LD) with 2 other SNPs located on the same locus and showing significant associations with AD (rs28694054, \( p = 3.4 \times 10^{-3} \); rs11692815, \( p = 4.3 \times 10^{-3} \)). Variants in PPP3R1 have been previously reported as potential modulators of tau and phosphorylated tau levels in the presence of amyloid deposition. These changes are suspected to result in an accelerated progression of AD. Calcineurin is involved in a number of pathways that regulate synaptic activity and neuronal excitability. Therefore, any impairment in this complex could have substantial effects and lead to pathological synaptic loss.

The second top-ranked SNP identified, rs2049048, is located in the AC103796.1-BDNF region. Although the role of AC103796.1 remains unclear, the gene overlaps BDNF over 20 kb and therefore, may contribute to its function. BDNF is a neurotrophin involved in synaptic plasticity, neurogenesis, neuronal survival, and cognitive health. Changes in BDNF levels are not specific to AD and have been reported in a number of neuropsychiatric disorders. It remains a key target for therapeutic treatment due to its pivotal role in the central nervous system. Increasing evidence suggests that BDNF could modulate Aβ accumulation by decreasing Aβ formation, limiting Aβ-mediated cell death and repairing Aβ-related damages. Our findings indicate that a specific polymorphism in the AC103796.1-BDNF gene region (rs2049048; \( p = 3.62 \times 10^{-4} \)) is indeed over represented in those who were Aβ+. Another variant, rs6265, has been more widely investigated and found to be associated with reduced hippocampal volume and cognitive decline. However, contradictory results have also been reported, suggesting a more complex relationship between AC103796.1-BDNF and cognition. In our analyses, rs6265, did not show a significant association with Aβ accumulation (\( p = 0.7 \)).

Another notable association was found with the variant rs9908234 (\( p = 2.45 \times 10^{-3} \)), located in the nerve growth factor receptor (NGFR) gene which encodes for a cell surface receptor for neurotrophins. A gene-gene interaction network indicated that NGFR has multiple indirect interaction with other genes identified in this study, including...
AC103796.1-BNDF, ABCA7 and CALHM1 (Fig. 2). In a recent meta-analysis of genome-wide association for migraine, rs9908234, was the most significantly associated marker with the disorder. Although the link between the two conditions remains unclear, migraines are known to cause micro brain lesions which are promoting the development of MCI and AD. In addition to the interaction of NGFR with the aforementioned genes, it also binds one of the major receptors for NGF and has also been reported to bind directly to APP. These studies and others postulate a relationship between APP processing/Aβ accumulation and NGF/NGF receptor mediated signaling pathways that warrants further investigation. This relationship is further supported by the association of genetic variation in NGFR with Aβ accumulation in the current study.

We also report an association between the rs648202 marker and Aβ accumulation (p = 2.90e-03). The marker is located in the klotho (KL) gene, which codes for a single-pass transmembrane protein involved in cellular metabolism and has been associated with several age-related diseases. Recent studies have shown that mutations of KL caused systemic aging and reduced longevity in mice. Conversely, overexpression of the gene resulted in healthier aging and prolongation of life. Thus, as a key modulator of the aging process, klotho has become a candidate of interest for the development of novel therapeutic treatment for AD. However, the role of KL in the development of AD remains to be defined, as a recent study showed that a functional variant in KL, namely KL-VS, had no influence on cognitive decline in preclinical AD. The rs648202 variant, associated with Aβ in this study, is in linkage disequilibrium (D' = 1.0) with the KL-VS variants (rs9527025/rs9536314), however they are not highly correlated (r² = 0.03).

The SNP, rs3764650, located in an intron of the ABCA7 gene showed a moderate association with Aβ accumulation (p = 3.07e-03). This specific variant was identified as one of the main susceptibility loci for late-onset AD in two independent cohorts. Furthermore, recent studies have shown that rs3764650 was associated with cortical and hippocampal atrophy in cognitively normal and mild cognitive impairment (MCI) subjects as well as with memory decline in MCI and late-onset AD patients. In addition, ABCA7 has been identified as a major mediator of phagocytic clearance of Aβ, which supports the reported association.

In CALHM1, the polymorphism rs2986018 showed marginal evidence for association (p = 4.16e-03). Several genetic epidemiological studies have suggested that rs2986017, a marker located in CALHM1 and within the same LD block as rs2986018, could influence age at onset of AD. Although the underlying mechanisms by which CALHM1, which codes for a calcium channel, modulates AD's pathogenesis remain unclear, it has recently been identified as a repressor of Aβ accumulation, in cell lines and in vivo. These findings indicate that CALHM1 is potentially involved in Aβ degradation in the brain, a molecular mechanism highly relevant to AD's pathogenesis.

In summary, although the present study was subject to a lack of power due to the limited number of cases and controls available, it provides suggestive evidence for the implication of several genes previously hypothesised to have a role in the development of AD, through the a priori evidence based approach employed for marker selection. Therefore, replication analyses in independent samples is warranted to confirm our findings and increase the significance of true associations. Whilst the nature of the marker selection employed in this study is a potential strength, the biased nature of this selection may have resulted in the exclusion of, as yet unknown, Aβ-associated genes.
and PET A\textsuperscript{β}-classification as PET A\textsuperscript{β}+\β. Biosystems™ QuantStudio™ 12 K Flex Real-Time PCR system using the manufacturer’s instructions.

Of the 1572 participants enrolled into the AIBL study 1416 of these underwent genetic analysis using the methodologies described below. Participants were classified as MCI\textsuperscript{52} or AD\textsuperscript{53} when the clinical criteria for diagnosis of was met. In the absence of these diagnoses a classification of cognitively normal (CN) was given by a clinical review panel, blinded to Amyloid-β status. Ethics approval for the AIBL study and all experimental protocols was provided by the ethics committees of Austin Health, St Vincent’s Health, Hollywood Private Hospital and Edith Cowan University. All experiments and methods were carried out in accordance with the approved guidelines and regulations and all volunteers gave written informed consent before participating in the study.

Methods

Participants. Data from the AIBL study, a prospective longitudinal study of ageing, is presented here. The AIBL study design, enrolment process, neuropsychological assessments and diagnostic criteria have been previously described\textsuperscript{39}. Of the 1572 participants enrolled into the AIBL study 1416 of these underwent genetic analysis using the methodologies described below. Participants were classified as MCI\textsuperscript{52} or AD\textsuperscript{53} when the clinical criteria for diagnosis of was met. In the absence of these diagnoses a classification of cognitively normal (CN) was given by a clinical review panel, blinded to Amyloid-β status. Ethics approval for the AIBL study and all experimental protocols was provided by the ethics committees of Austin Health, St Vincent’s Health, Hollywood Private Hospital and Edith Cowan University. All experiments and methods were carried out in accordance with the approved guidelines and regulations and all volunteers gave written informed consent before participating in the study.

SNP selection, genotyping and quality control. A thorough literature review was conducted in PubMed to identify genes with an a priori evidence of association with AD risk, cognitive performance, pathological characteristics (i.e. Aβ/tau, atrophy), candidate peripheral/CSF biomarkers, hypothesised pathomechanisms (e.g. Aβ clearance/metabolism) and other AD related biological pathways or comorbidities (e.g. endocytosis, cholesterol metabolism, steroidogenesis, diabetes/insulin resistance, cardiovascular disease). This resulted in the selection of an a priori candidate list of 270 genes. The final selection of 2088 genetic markers across these loci was based on prior phenotypic association and/or extended coverage of each loci. The list of genetic variants is available in Supplementary Table 2.

Genotype data was obtained from 1416 samples from the AIBL cohort using using a combination of an Illumina GoldenGate array containing 1536 markers and multiple TaqMan® OpenArray™ assays.

The GoldenGate array was performed by the Beijing Genomics Institute (BGI, Shenzhen, China) as per manufacturer’s protocols. OpenArray™ assays were developed using inventoried or custom designed TaqMan® genotyping assays, whilst TaqMan® assays were used for APOE genotyping (rs7412, assay ID: C____904973_10; rs429358, assay ID: C__3084793_20; Life Technologies, Carlsbad, CA) using the TaqMan® GTXpress® Master Mix (Life Technologies). All TaqMan® and OpenArray™ assays were performed on an Applied Biosystems™ QuantStudio™ 12 K Flex Real-Time PCR system using the manufacturer’s instructions.

Genotype data was prepared by removing markers with a genotyping call rate below 95% and a minor allele frequency (MAF) of at least 0.05. In addition, markers not in Hardy-Weinberg equilibrium ($p < 10^{-6}$) were removed. Samples identified with discordant sex information and samples with a call rate below 95% were discarded. Approximately 65% of samples (N = 918) and over 77% of genetic markers assayed (N = 1610) reached QC procedures.

This study limited its analyses to a subset of 505 participants who had previously undergone positron emission tomography (PET) to assess neocortical Aβ burden. PET imaging was performed with three different Aβ-imaging radiotracers 11C-Pittsburgh Compound B (PiB), 18F-florbetapir (FLUTE) or 18F-flutemetamol (FBP). Methodology for each tracer has been previously described\textsuperscript{54}. Briefly, standardised uptake values (SUVs) were calculated via summing spatially normalised PET images sampled using a narrow cortical regions of interest template using CapAIBL\textsuperscript{8}, a web-based, freely available software\textsuperscript{55,56}. The SUVs were then scaled to each tracer’s recommended reference regions to define the SUV ratio (SUVR). Reference region for PiB was the cerebellar cortex\textsuperscript{37,58}, for FLUTE the pons\textsuperscript{59} and for FBP the whole cerebellum\textsuperscript{60}. All participants were then classified to a dichotomous Aβ deposition phenotype, being either high (Aβ+; n = 258) or low (Aβ−; n = 247), based on each tracer-specific neuropathology established thresholds (PiB: 1.4 SUVR, FLUTE: 0.62 SUVR and FBP: 1.05 SUVR)\textsuperscript{38–40}. Despite displaying different dynamic ranges and subtle differences in the uptake and selectivity of the probes\textsuperscript{41,62}, head to head comparisons of the three radiotracers have previously shown >98% concordance in their classification as PET Aβ+ and PET Aβ−\textsuperscript{63–65}.

Association analyses. To identify associations between genetic marker and the dichotomous Aβ status, we performed a logistic regression using PLINK\textsuperscript{266}. The analyses incorporated age, gender and the presence/absence of the ε4 allele as covariates. Considering the limited sample size of the data set and the targeted nature of the study, we used the following approach to identify an adequate threshold for significance. To estimate an empirical threshold for significance, we measured the distributions of the P-values of the variants and defined a threshold, \( \text{Psig} \), as the 99th percentile (1 - \( \alpha \)) at a significance level of \( \alpha = 0.01 \). We calculated \( \text{Psig} \) using the Harrell–Davis distribution-free quantile estimator\textsuperscript{57}.

With the increasing numbers of traits examined through genetic association analyses, it has become increasingly clear that individual genetic components are insufficient to explain complex phenotypes such as Alzheimer’s disease. Instead, such traits are most likely modulated by the collective influence of tens or even hundreds of genetic loci with small individual effects. In this study we identified 6 variants associated with the accumulation of Aβ, a key process in the pathogenesis of AD. The identified variants are located in the intronic regions of 6 distinct loci that are involved in major neurological and neurocognitive functions. Further studies are needed to fully understand the role of these variants in the AD’s pathogenesis. However, this study opens doors to the investigation of novel biological targets for AD treatment to be considered in future studies.
Data availability

All data and samples used in this study are derived from the Australian Imaging, Biomarkers and Lifestyle (AIBL) Study. All AIBL data, and that specific to this study, is publicly accessible to all interested parties through an Expression of Interest procedure and is governed by the AIBL Data Use Agreement, for more information please see https://aibl.csiro.au/awd/.

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Author Contributions
M.V. developed the computational pipeline, performed the data analysis and wrote the initial manuscript. T.P., L.M. and M.P. contributed to the acquisition of genetic data, V.V. contributed to acquisition of imaging data and revising the manuscript. J.D. and M.V. verified the analytical methods. S.L. devised the project and the main conceptual ideas, supervised the findings of this work and obtained funding. M.V., T.P., J.D. and S.L. discussed the results and contributed to the manuscript. D.A., C.M., R.M. and C.R. contributed to AIBL study design and obtaining funding. All authors reviewed the manuscript.

Competing Interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to M.V.

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