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Plasma biomarkers and genetics in the diagnosis and prediction of Alzheimer's disease

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Plasma biomarkers for Alzheimer's disease-related pathologies have undergone rapid developments during the past few years, and there are now well-validated blood tests for amyloid and tau pathology, as well as neurodegeneration and astrocytic activation. To define Alzheimer's disease with biomarkers rather than clinical assessment, we assessed prediction of research-diagnosed disease status using these biomarkers and tested genetic variants associated with the biomarkers that may reflect more accurately the risk of biochemically defined Alzheimer's disease instead of the risk of dementia.

In a cohort of Alzheimer's disease cases [$n = 1439$, mean age 68 years (standard deviation = 8.2)] and screened controls [$n = 508$, mean age 82 years (standard deviation = 6.8)], we measured plasma concentrations of the 40 and 42 amino acid-long amyloid- β (A β) fragments (A β_{40} and A β_{42} , respectively), tau phosphorylated at amino acid 181 (P-tau181), neurofilament light (NfL) and glial fibrillary acidic protein (GFAP) using state-of-the-art Single molecule array (Simoa) technology. We tested the relationships between the biomarkers and Alzheimer's disease genetic risk, age at onset and disease duration. We also conducted a genome-wide association study for association of disease risk genes with these biomarkers.

The prediction accuracy of Alzheimer's disease clinical diagnosis by the combination of all biomarkers, APOE and polygenic risk score reached area under receiver operating characteristic curve (AUC) = 0.81, with the most significant contributors being $\epsilon 4$, A β_{40} or A β_{42} , GFAP and NfL. All biomarkers were significantly associated with age in cases and controls ($P < 4.3 \times 10^{-5}$). Concentrations of the A β -related biomarkers in plasma were significantly lower in cases compared with controls, whereas other biomarker levels were significantly higher in cases.

In the case-control genome-wide analyses, APOE- $\epsilon 4$ was associated with all biomarkers ($P = 0.011$ – 4.78×10^{-8}), except NfL. No novel genome-wide significant single nucleotide polymorphisms were found in the case-control design; however, in a case-only analysis, we found two independent genome-wide significant associations between the A β_{42} /A β_{40} ratio and *WWOX* and *COPG2* genes.

Disease prediction modelling by the combination of all biomarkers indicates that the variance attributed to P-tau181 is mostly captured by APOE- $\epsilon 4$, whereas A β_{40} , A β_{42} , GFAP and NfL biomarkers explain additional variation over and above APOE. We identified novel plausible genome wide-significant genes associated with A β_{42} /A β_{40} ratio in a sample which is 50 times smaller than current genome-wide association studies in Alzheimer's disease.

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Introduction

Alzheimer's disease (AD) is one of the greatest health challenges, affecting tens of millions of people worldwide. The clinical diagnosis of this disease is, however, often inaccurate; around 25% of people with clinical AD do not have underlying pathology at autopsy, and many people who have not yet developed AD-type dementia have incipient pathology, the prevalence of which increases with age.¹ Detecting AD at the earliest possible stage remains essential to combating its effects and to further our understanding of this devastating illness. By diagnosing early, we can better understand how the disease progresses, plan and implement treatments earlier and monitor response to drugs currently being trialled.

Amyloid- β ($A\beta$) and tau pathology are the defining pathological features of AD.² For many years, it has been possible to detect AD pathology (amyloid aggregation, tau tangles and neurodegeneration) using imaging and CSF biomarkers. Although CSF and PET biomarkers of $A\beta$ and tau are highly accurate for detecting disease pathology,³ the costs, invasive nature and low availability of the tools needed to detect these biomarkers hamper their feasibility for use in clinical diagnostic practice and for screening in clinical trials.

Assays for plasma $A\beta$ fragments [ratio of $A\beta_{1-42}$ ($A\beta_{42}$) to $A\beta_{1-40}$ ($A\beta_{40}$)] reflect brain amyloidosis⁴⁻⁷; however, these assays have limitations, including the impact of substantial peripheral $A\beta$ production.⁸ By contrast, CSF and plasma tau phosphorylated at threonine 181 (P-tau181) is a highly specific pathological marker of AD that remains normal in other dementias.^{9,10} Glial fibrillary acidic protein (GFAP) and neurofilament light chain (NfL) are putative non-amyloid plasma-based biomarkers indicative of ongoing neuroinflammatory and neurodegenerative disease processes. Increased GFAP suggests abnormal activation and proliferation of astrocytes, for instance secondary to neuronal damage. It has been shown that GFAP levels in plasma and CSF are higher in AD and correlate with cognitive impairment.¹¹⁻¹³ Plasma NfL is a marker of neuronal injury, increased in AD,¹⁴ but this biomarker has low specificity, because increases are also reported in several other neurodegenerative disorders.^{13,15,16} Thus, while NfL has potential as a monitoring biomarker, GFAP might be a valuable prognostic biomarker, predicting incident dementia.¹³ Recent reports show that plasma P-tau181 concentration starts to increase around 15 years prior to clinical disease onset in familial AD¹⁷ and that plasma P-tau181 predicts disease neuropathology at least 8 years prior to autopsy in sporadic disease.¹⁰

Early disease prediction can be helped with genetic data as an individual's genetic makeup does not change over time and genetic data are precise and inexpensive to measure; however, the prediction accuracy using genetics is limited.¹⁸ Biomarkers, in contrast to genetics, can only indicate the presence of AD pathology after the disease has already been triggered, i.e. a biomarker change marks the onset of a pathological process. Nevertheless, the prediction accuracy of e.g. P-tau181 and P-tau217 for discriminating AD from other neurodegenerative diseases,¹⁹⁻²¹ when combined with APOE genotype, memory and executive function phenotypes, was reported to reach area under receiver operating characteristic curve (AUC) > 90% in predicting the progression from mild cognitive impairment (MCI) to AD in two relatively small samples of participants ($n = 340$ and 543).²²

Identifying genetic loci associated with biomarkers could aid understanding of the specific pathophysiological components underpinning these biomarkers. Genome-wide association studies (GWAS) of CSF biomarkers in AD case/control samples have found loci in genes *GEMC1* and *OSTN23* as well as more commonly reported loci such as the *TREM* cluster, *APOE*, *APOC* and *TOMM40*.²⁴ However, these have also only focused on small sets of biomarkers, typically P-tau181 and $A\beta_{42}$. GWAS of blood plasma P-tau181 and NfL levels^{25,26} have identified only loci within the *APOE* genomic region, and only for P-tau181. Investigation of the relationship between AD polygenic risk score (PRS) and plasma P-tau181²⁷ has revealed highly significant associations with PRS containing the *APOE* region ($P = 3 \times 10^{-18} - 7 \times 10^{-15}$) and moderate association when *APOE* was excluded. GWAS studies for plasma $A\beta_{40}$, $A\beta_{42}$ and $A\beta_{42/40}$ ratio in non-demented participants from population-based studies have identified GWAS significant variants in *APOE* and *BACE1* genes, and *APP*, *PSEN2*, *CCK* and *ZNF397* genes in gene-based analysis.²⁸

The aims of this study were (i) to test the prediction ability of the biomarkers for clinical AD diagnosis in our cohort (over and above commonly used predictors such as *APOE*, age and AD PRS); and (ii) to identify genetic loci associated with these plasma biomarkers. The latter may shed light on which single nucleotide polymorphisms (SNPs) associated with clinical AD are also associated with plasma biomarkers. This could help to further refine the relevance of the AD GWAS genes to different biological processes, which the biomarkers represent. To that end, we measured plasma biomarkers in a sample of 1439 early and late onset AD cases (mean age

68 years [standard deviation (SD) = 8.0] and 508 elderly screened controls [mean age 82 years (SD = 6.7)]. We used ultrasensitive Single molecule array (Simoa) assays to measure P-tau181, NfL, GFAP, A β ₄₀ and A β ₄₂, and calculated the ratio of A β _{42/40}. We then tested these biomarkers for association with the clinical diagnosis of AD and, in case samples, the relationship of the biomarkers with age at sample collection, age at onset and disease duration. To identify genetic loci associated with these biomarkers, we undertook a GWAS for P-tau181, NfL, A β ₄₀, A β ₄₂, ratio of A β _{42/40} and GFAP biomarkers in the largest case-control sample set to date.

Materials and methods

Alzheimer's Disease Cardiff Cohort

The Alzheimer's Disease Cardiff Cohort (ADCC) was collected between 2004 and 2020 using MRC, Moondance Foundation and Health and Care Research Wales (HCRW) funding. The cohort collection used a standardized clinical and comprehensive neuropsychological assessment (validated by Holmes et al.²⁹), see more details in Section 1 of the [Supplementary material](#). AD diagnosis was not supported by any biochemical or imaging measures (e.g. CSF or PET) due to the funds allocated to the study collecting the data.

We used plasma samples collected from 1439 early and late onset sporadic AD cases and 508 screened elderly controls. Information on age at assessment, sex, APOE genotype and genome-wide array genotyping was available for all 1947 samples. Within cases, information was also available for $n = 1319$ individuals on age at onset, and duration of disease was calculated for these samples. Details of the sample demographics are in [Table 1](#).

Biomarkers

Biomarkers were tested for 1986 individual plasma samples from the ADCC. P-tau181 concentration was measured using the Simoa P-tau181 Advantage Kit, whilst A β ₄₀, A β ₄₂, NfL and GFAP concentrations were measured using the Simoa Human Neurology 4-Plex E (N4PE) assay (Quanterix). The measurements were performed in one round of experiments using one batch of reagents with the analysts blinded to diagnosis and clinical data. All measurements for all five analytes were above the limit of detection of the assays.

Table 1 Summary of demographics and plasma biomarker summary characteristics in ADCC, post-outlier removal

	Controls (n = 508)	Cases (n = 1439)
Demographics		
Age, years	82.2 (6.72)	68.1 (8.03)
Sex, male/female	221/287	748/691
Age at onset	N/A	62.4 (7.9)
Duration, years	N/A	5.3 (3.6)
Biomarkers		
A β ₄₀	140 (40.0)	94.5 (34.4)
A β ₄₂	7.50 (2.05)	5.00 (1.84)
GFAP	196 (85.3)	215 (103)
NfL	32.9 (13.7)	31.0 (13.9)
P-tau181	3.18 (1.54)	4.10 (1.90)
A β ₄₂ /A β ₄₀	0.0556 (0.013)	0.0543 (0.014)

Values are mean (SD). Biomarker values are in $\mu\text{g/ml}$.

Intra-assay coefficients of variation were below 10%. These data were then matched to phenotype information. Thirty-nine samples were removed at this stage based on missing/mismatching data for age and gender or due to ID duplication, leaving 1947 individuals for further analysis. Samples were excluded for each biomarker analysis on a case-by-case basis, based on outlier thresholds calculated using Median Absolute Deviation (MAD).³⁰ This method is more robust to remote outliers than the mean and SD method and copes better with skewed data due to its reliance on non-parametric measures of central tendency and variation. Pearson's correlations between biomarkers were calculated for the 1735 samples which had no outlier measurements for any biomarker. Details of biomarker distributions are in [Table 1](#).

Genetics

Individuals for this analysis were included if both genetic and biomarker information were available, totalling 1947 individuals in the final dataset. All individuals had information available on APOE genotype ($\epsilon 2\epsilon 2 = 8$, $\epsilon 2\epsilon 3 = 145$, $\epsilon 2\epsilon 4 = 33$, $\epsilon 3\epsilon 3 = 844$, $\epsilon 3\epsilon 4 = 620$ and $\epsilon 4\epsilon 4 = 239$). Quality control (QC) of the genetic data was performed for cases and controls together, the QC steps used are reported elsewhere^{31,32} and in Section 2 of the [Supplementary material](#). Genotyped data were aligned to human genome assembly GRCh37/hg19 and imputed via Michigan Imputation server using Minimac3³³ with the Haplotype Reference Consortium (HRC)³⁴ reference panel. Post-imputation QC used thresholds of minor allele frequency (MAF) < 5%, poor accuracy of imputation (INFO) < 0.8, MISS > 5% and Hardy-Weinberg equilibrium $P \leq 10^{-6}$. This resulted in a final dataset containing 4 618 496 variants.

Statistical analysis

The association of biomarkers with age at onset and disease duration in cases, and with age at interview in cases and in controls (separately), was tested with linear regression where the biomarker was the outcome variable, controlling for sex. For all following analyses the biomarkers were adjusted for age and standardized to have a mean of zero and standard deviation of one. The correlations between the biomarkers were assessed with Pearson's correlation.

The association of AD case/control status by the biomarkers was tested using logistic regression, accounting for sex, APOE and PRS without the APOE region (chromosome 19:44.4–46.5 Mb) using the `glm()` function in R. The most parsimonious model was derived with the backwards stepwise approach [`step()` function in R]. The prediction accuracy was assessed by means of the AUC, using the `auc()` function in R.

The APOE region was represented by the number of $\epsilon 2$ and $\epsilon 4$ alleles which we used as two predictor variables. The PRS without APOE region (PRSnoAPOE) was used to account for the remaining genetic effect. For the PRS calculation we used the summary statistics from the largest clinically assessed late-onset case-control GWAS study on AD available at the time of analysis ($n = 63\,926$)³⁵. PRS were generated with the PLINK genetic data analysis toolset³⁶ for P -value threshold $P \leq 0.1$ on LD-clumped SNPs by retaining the SNP with the smallest P -value excluding variants with $r^2 > 0.1$ in a 1000 kb window, see details in.³⁷ Prior to analyses PRSnoAPOE was adjusted for five principal components and then standardised.

All statistical analyses were performed in R-statistical software (<https://www.R-project.org/>). The plots were generated using the `ggplot2` package with custom scripts generated in house.

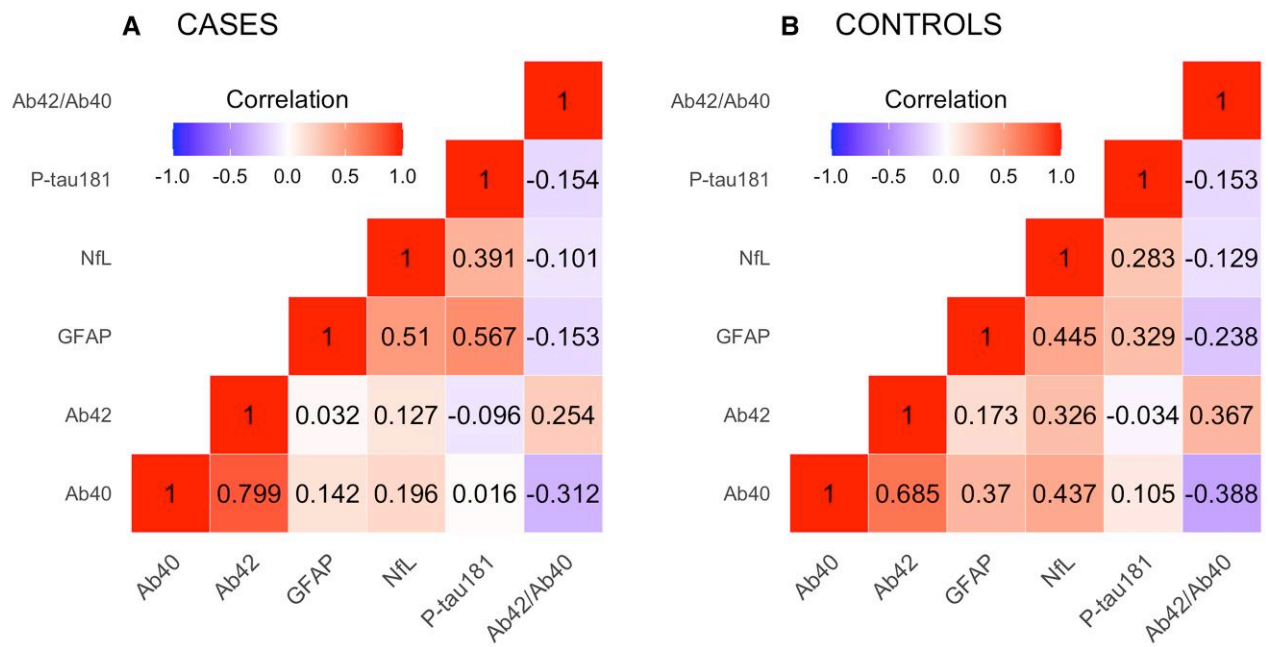


Figure 1 Pearson correlation between biomarkers in cases and controls. (A) Cases and (B) controls.

The results of the biomarkers' association with the clinical/demographic characteristics are presented without correction for multiple testing, since these analyses are hypothesis-driven.

Genetic analysis

SNP-based association analyses were performed for each biomarker using linear regression model with PLINK. Association analyses of SNPs with the biomarkers were adjusted for age and sex, five principal components (PCs) and case-control status ('caseness'). The adjustment for caseness was introduced to reduce the variation due to potential differences in association pattern of biomarkers between cases and controls, whilst using all available samples to maintain the statistical power. In addition, association analyses for cases and controls were also conducted separately. Since the APOE region is not well covered by the Illumina arrays used to genotype the ADCC dataset, we tested association of the biomarkers with the number of directly genotyped APOE-ε4 alleles. PCs were computed using PLINK and the number of PCs was determined via visual inspection of the pairwise PC scatter plots. The GWAS significance level was set to the commonly accepted $P < 5 \times 10^{-8}$. We did not further adjust this for the six biomarkers as the biomarker levels were measured in the same sample and are not independent.

To investigate further the variants of interest, we used Combined Annotation-Dependent Depletion (CADD) and RegulomeDB (RDB) scores for SNPs accessible within the Functional Mapping and Annotation of Genetic Associations (FUMA) on-line tool.³⁸ CADD is a tool for scoring the deleteriousness of single nucleotide variants as well as insertion/deletions variants in the human genome.^{39,40} RDB⁴¹ is a categorical score from 1a to 7, representing regulatory functionality of SNPs based on expression quantitative trait loci (eQTLs) and chromatin marks. 1a is the highest score, indicating that the SNP has the most biological evidence to be a regulatory element.

We compared our GWAS biomarker association results to AD genome-wide significant findings,³⁵ assessing all SNPs in the

ADCC GWAS within ± 20 kB of the GWAS-significant SNPs. The replication significance level was set to nominal significance level $P < 0.05$.

To summarize the association results from all variants in a gene, accounting for number of variants and linkage disequilibrium (LD) between them, we used Multi-marker Analysis of GenoMic Annotation (MAGMA, v1.09b).⁴² For the gene-based analysis, we mapped a SNP to a gene (as defined by NCBI 37.3) if it resided within the gene boundaries. The LD between SNPs was estimated with the European reference panel in 1000 Genomes phase 3. The significance level for the gene-based analysis results was set to the commonly accepted $P < 2.5 \times 10^{-6}$.

For the pathway analyses, 10271 gene sets were downloaded from Reactome, Biocarta, KEGG and Pathway Interaction Databases.³² The pathway analyses were performed using the 'competitive' option in MAGMA, assessing whether the genes in a gene set are more strongly associated with the phenotype than in other gene sets in the genome. We adopted the false discovery rate ($FDR \leq 0.05$) approach [`p.adjust()` function in R with method = 'fdr'] to correct for multiple testing the results of the pathway analyses.

Data availability

GWAS summary statistics for the top results ($P \leq 1 \times 10^{-5}$) are listed in the main text of the paper and [Supplementary material](#). Full GWAS summary statistics are available from the authors upon request.

Results

Biomarker results in relation to Alzheimer's disease, age at onset and disease duration

The correlation pattern between the biomarkers was similar for cases and controls and agreed with the results reported by Cullen

Table 2 Beta coefficients, SE and P-values for linear regressions predicting biomarkers from age at onset and disease duration in AD cases, controlling for age and sex

	n	Age at onset			Duration		
		B	SE	P	B	SE	P
A β ₄₀	1219	0.042	0.003	1.9×10^{-35}	0.016	0.007	0.027
A β ₄₂	1219	0.034	0.003	4.2×10^{-23}	0.013	0.007	0.077
GFAP	1301	0.034	0.003	7.1×10^{-24}	0.034	0.007	2.9×10^{-6}
NfL	1275	0.048	0.003	1.1×10^{-44}	0.050	0.007	1.2×10^{-12}
pTau-181	1309	0.011	0.003	0.0023	0.020	0.008	0.008
A β ₄₂ /A β ₄₀	1215	-0.012	0.003	0.0005	-0.004	0.008	0.592

Table 3 Beta coefficients, SE and P-values for linear regressions predicting biomarkers from age at interview in cases and controls, controlling for sex

	Cases (max n = 1439)				Controls (max n = 508)			
	n	B	SE	P	n	B	SE	P
A β ₄₀	1415	0.041	0.003	2.9×10^{-37}	492	0.064	0.006	1.2×10^{-22}
A β ₄₂	1417	0.034	0.003	4.6×10^{-25}	486	0.036	0.007	1.2×10^{-7}
GFAP	1394	0.034	0.003	8.4×10^{-28}	501	0.052	0.006	4.6×10^{-16}
NfL	1361	0.051	0.003	1.2×10^{-54}	478	0.074	0.006	1.9×10^{-30}
P-tau181	1389	0.014	0.003	4.3×10^{-5}	472	0.038	0.007	3.5×10^{-08}
A β ₄₂ /A β ₄₀	1413	-0.010	0.003	0.0018	481	-0.044	0.007	1.2×10^{-10}

et al.⁴³ The correlation between A β ₄₂ and A β ₄₀ values was high ($r = 0.8$ in cases and 0.7 in controls, $P < 10^{-16}$). The lowest correlation was observed between P-tau181 and A β -related biomarkers, see Fig. 1.

To assess whether the disease stage is captured by the biomarkers, we explored the relationship between biomarkers, age of onset and disease duration in cases. Table 2 summarizes the results. In this case-only analysis, age at onset was strongly positively associated with A β ₄₀, A β ₄₂, GFAP and NfL (P -values $\leq 4.2 \times 10^{-23}$), moderately with P-tau181 ($P = 0.0023$) and negatively with A β ₄₂/A β ₄₀ ($P = 4.8 \times 10^{-4}$). The biomarkers GFAP, NfL and P-tau181 show significant increase in females as compared to males ($P = 9.0 \times 10^{-23}$, 1.4×10^{-7} and 2.1×10^{-8} , respectively). This in part replicates the finding in Kumar-Singh et al.,⁴⁴ who showed that age-of-onset of PSEN1-linked familial AD correlated negatively with A β ₄₂/A β ₄₀ but positively with A β ₄₀ levels. Longer disease duration was strongly associated with elevated levels of GFAP and NfL ($P = 2.9 \times 10^{-6}$ and 1.2×10^{-12} , respectively) and moderately associated with increase of A β ₄₀ and P-tau181 levels ($P = 0.027$ and 0.008 , respectively).

In controls, all biomarkers were positively associated with age at interview (P -value ranked between 1.2×10^{-7} for A β ₄₂ and 1.9×10^{-30} for NfL) and negatively with the ratio A β ₄₂/A β ₄₀ ($P = 1.2 \times 10^{-10}$) (see Table 3), indicating that all biomarkers are sensitive to age and will show less discrimination between AD cases and controls if AD cases with earlier onset (~65–68 years) are compared with elderly screened controls (Supplementary Fig. 1).

Next, we assessed the prediction accuracy of disease status in our sample. The prediction accuracy of the case-control status by sex and APOE genotype resulted in AUC = 0.74 and $R^2 = 0.21$. All biomarkers were significantly associated with AD status when tested separately (Table 4 and Supplementary Fig. 2). The prediction accuracies, however, were moderate with the highest prediction accuracy AUC = 0.66 and 0.65 for A β ₄₂ and P-tau181, respectively.

The prediction accuracy of a model combining all biomarkers and genetics (APOE- ϵ 4, APOE- ϵ 2, PRS without APOE region) was

AUC = 0.81, $R^2 = 0.29$. The most parsimonious model that predicted the outcome with the same accuracy as above (derived using stepwise regression) included all predictors except A β ₄₂ and P-tau181 (APOE- ϵ 4 B = 1.3, $P = 2.02 \times 10^{-24}$; APOE- ϵ 2 B = -0.45, $P = 0.011$; PRSnoAPOE B = 0.14, $P = 0.033$; A β ₄₀ B = -0.62, $P = 6.6 \times 10^{-18}$; GFAP, B = 0.29, $P = 3.9 \times 10^{-4}$; NfL B = 0.45, $P = 4.6 \times 10^{-8}$; A β ₄₂/A β ₄₀ B = -0.20, $P = 0.003$).

This model highlights the importance of all genetic predictors and the A β ₄₀, GFAP and NfL biomarkers. The variance of A β ₄₂ was captured by A β ₄₀, as the correlation between these biomarkers was high. Indeed, when A β ₄₀ was dropped from the model, then A β ₄₂ became a significant predictor (B = -0.59, $P = 9.6 \times 10^{-12}$). In both models, the ratio of A β ₄₂/A β ₄₀ was significant, but it changed its direction of effect depending on which marker was included (B = 0.20, $P = 0.005$ and B = -0.20, $P = 0.003$, when A β ₄₂ or A β ₄₀ was included, respectively) P-tau181 was dropped from the model by the stepwise regression, however this should not be interpreted as P-tau181 being fully explained by the genetic predictors. In a model with only P-tau181 and genetics (APOE- ϵ 4, APOE- ϵ 2, PRSnoAPOE), P-tau181 remained highly significant over and above genetics (B = 0.38, $P = 4.5 \times 10^{-8}$).

The model with all biomarkers but without genetic predictors had an accuracy of AUC = 0.75 and explained variance of $R^2 = 0.18$. In this model, the same biomarkers as above showed significant association, with the addition of the P-tau181 biomarker (B = 0.18, $P = 0.022$), indicating that the P-tau181 signal may be explained by genetics, whereas the other significant biomarkers (A β -related, GFAP and NfL) add to the prediction over and above genetics.

Genome-wide association study

We performed three sets of GWAS (cases only, controls only, all samples) in ADCC with the five biomarkers (A β ₄₀, A β ₄₂, NfL, P-tau181, GFAP) and the A β ₄₂/A β ₄₀ ratio as outcome measures. The top SNPs with an association P -value $\leq 1 \times 10^{-5}$ are presented

Table 4 Results of logistic regressions predicting AD status from each biomarker, adjusted for age and sex

	B	SE	P	R ²	AUC
A β ₄₀	−0.44	0.058	3.5×10^{-14}	0.05	0.63
A β ₄₂	−0.56	0.059	2.8×10^{-21}	0.08	0.66
GFAP	0.55	0.067	2.4×10^{-16}	0.07	0.64
NfL	0.47	0.066	1.1×10^{-12}	0.05	0.63
P-tau181	0.55	0.067	1.4×10^{-16}	0.07	0.65
A β ₄₂ /A β ₄₀	−0.18	0.055	0.0009	0.01	0.56

Data include 1302 cases and 421 controls after excluding the missing values list-wise.

in [Supplementary Tables 1–6](#). In the case-control analysis, APOE- ϵ 4 was associated with all biomarkers ($P=0.011$ – 4.78×10^{-8} ; [Supplementary Tables 1, 2 and 4–6](#)), except NfL ([Supplementary Table 3](#)).

We compared the GWAS we performed for biomarkers to the genome-wide significant SNPs from a large clinically assessed AD GWAS study³⁵ (see [Supplementary Table 7](#)). The strongest associations for the GWAS index APOE SNP (rs429358) were for P-tau181 and GFAP ($P=0.001$ and 0.002 , respectively; [Supplementary Table 7](#)). Interestingly, SNPs in or near the WWOX gene were at least nominally associated with all biomarkers. The strongest association was found for GFAP ($P=1.2 \times 10^{-5}$) for a SNP situated 2.7 kb away from the GWAS index WWOX SNP.

The GWAS of the five biomarkers and the A β ₄₂/A β ₄₀ ratio in controls only and in all samples did not reveal any genome-wide significant loci. In the cases only GWAS, however, we observed two genome-wide significant loci for the A β ₄₂/A β ₄₀ ratio ([Supplementary Table 6](#) and [Fig. 2](#)). The lead SNPs for these loci lie within the intronic region of their respective genes (COPG2 and WWOX), with the WWOX variant predicted to function as an enhancer.

The first genome-wide significant locus was a high LD region on chromosome 7 spanning from 130.2–130.4 Mb and covering genes COPG2 (chr7:130 146 080–130 353 598) and TSGA13 (chr7:130 353 486–130 371 406) with the lead SNP rs17165066 [chr7:130 370 267, $B=0.15$, standard error (SE)=0.026, $P=8.9 \times 10^{-9}$]. This SNP tags 50 other SNPs with $r^2>0.8$; see Manhattan plot ([Fig. 2](#)) and LocusZoom plot ([Fig. 3A](#)). Moreover, this region contains two SNPs (rs10264429 and rs375839317, MAF=0.06, 0.07, respectively), which are in high LD with the lead SNP ($r^2=0.84$ and 0.71 , respectively) and have CADD scores = 13.6, 12.48, which are greater than the suggestive threshold for a SNP to be deleterious (CADD > 12.37). The rs77696591 (MAF=0.06) intergenic variant is also tagged by the lead SNP ($r^2=0.87$) and has an RDB score = 3a, i.e. has ‘putatively functional impact on gene regulation’. The lead SNP rs17165066 was not statistically significant in the clinically assessed AD GWAS.³⁵

The second genome-wide significant region was on chromosome 16 in the WWOX gene (chr16:78 133 327–79 246 564), which has also been linked to AD by GWAS.³⁵ The lead SNP rs34946778 (chr16:78989116, $B=0.15$, SE=0.026, $P=4.36 \times 10^{-9}$) was not statistically significant in the AD GWAS.³⁵ The linkage disequilibrium was $r^2=0.0014$ between the AD GWAS lead SNP (rs62039712) and the SNP identified in our study (rs34946778).

Finally, the number of APOE- ϵ 4 alleles was associated with A β ₄₀ ($B=-0.072$, $P=1.1 \times 10^{-2}$), A β ₄₂ ($B=-0.015$, $P=6.3 \times 10^{-7}$), A β ₄₂/A β ₄₀ ($B=-0.15$, $P=1.05 \times 10^{-5}$), GFAP ($B=0.1$, $P=1.3 \times 10^{-3}$) and P-tau181 ($B=0.18$, $P=4.7 \times 10^{-8}$) but not with NfL ($P=0.40$).

Discussion

We demonstrated that the prediction accuracy for AD status by the combination of blood biomarkers, sex, APOE and PRS reaches AUC = 0.81 ($R^2=0.29$) with the most significant contributors being APOE- ϵ 4, A β ₄₀ and GFAP. This AUC value is lower than that reported in Palmqvist et al.,²² likely due to our controls being systematically older than cases, with the diagnostic accuracies for AD being decreased with age.⁴⁵ Note that A β ₄₂ becomes a highly significant predictor when A β ₄₀ is dropped from the model and vice versa, although a stepwise regression recommended dropping A β ₄₂ over A β ₄₀. The prediction accuracy by all biomarkers without genetic predictors was AUC=0.75, which is slightly higher than the accuracy by genetic predictors alone (AUC=0.73 in our sample). Interestingly, P-tau181 was not significant if genetic predictors were included in the model and became significant only when no genetic predictors were used, indicating that genetic factors, APOE- ϵ 4 in particular, influence plasma P-tau181 levels. However, an advantage of P-tau181 as a biomarker over other predictors (e.g. genetics) is that it is a relatively inexpensive blood biomarker and does not reveal any sensitive genetic information.

In controls, age at interview was positively associated with all biomarkers (P -value ranged between 1.2×10^{-7} for A β ₄₂ and 1.9×10^{-30} for NfL) and negatively associated with the ratio A β ₄₂/A β ₄₀ ($P=1.2 \times 10^{-10}$), indicating that all biomarkers are sensitive to age or pre-clinical age-related neurodegenerative pathologies.

In case-only analyses, age at onset was significantly associated with all biomarkers, in particular, positively with A β ₄₀, A β ₄₂, GFAP, NfL and P-tau181 and negatively with the ratio A β ₄₂/A β ₄₀. In addition to age at onset, GFAP, NfL and P-tau181 were also associated with the disease duration, with similar effect sizes indicating that the associations can be attributed to age in general, rather than to a particular feature of the disease development and progression. These findings are in line with other recent studies. Chatterjee et al.⁴⁶ demonstrate that plasma GFAP levels are elevated in cognitively normal older adults at risk of AD. Aschenbrenner et al.⁴⁷ conclude that NfL can be used to monitor both cognitive decline due to normal ageing and dementia. Lantero Rodriguez et al.¹⁰ report that the main increase in plasma P-tau181 occurred between 8 and 4 years prior to death in patients with AD neuropathology, whereas patients without pathology and controls exhibited minor, although significant, increases in P-tau181 up until death.

The A β ₄₀ and A β ₄₂ results showing increasing concentration with age in both cases and controls support the earlier finding that A β ₄₀ and A β ₄₂ levels are increased before the onset of sporadic AD.^{48–50} It has also been shown that the biomarker distributions are more similar between subjects with and without AD in elderly subjects than in young subjects.⁴⁵ When comparing cases and controls in our sample, we found that cases have lower concentrations of A β ₄₀ and A β ₄₂ in plasma, accounting for age. This might indicate that cases, despite early onset, are in the advanced stage of the disease [mean disease duration 5.3 years (SE=3.6)] in our sample). An earlier study⁵⁰ showed that A β ₄₀ and A β ₄₂ levels are elevated in some patients before and during the early stages of AD but decline thereafter. Our results show similar association patterns (lower A β ₄₂/A β ₄₀ is associated with increased age) to the recent report⁷ for participants of all ages and diagnoses who were enrolled in a longitudinal study of memory and ageing. Another study,⁵ which included cognitively normal individuals, patients with mild cognitive impairment and patients with AD, found no significant correlations between the biomarker values and age. A population-based study⁵¹ reports results in a cohort where all individuals were born in the same week, but blood samples were

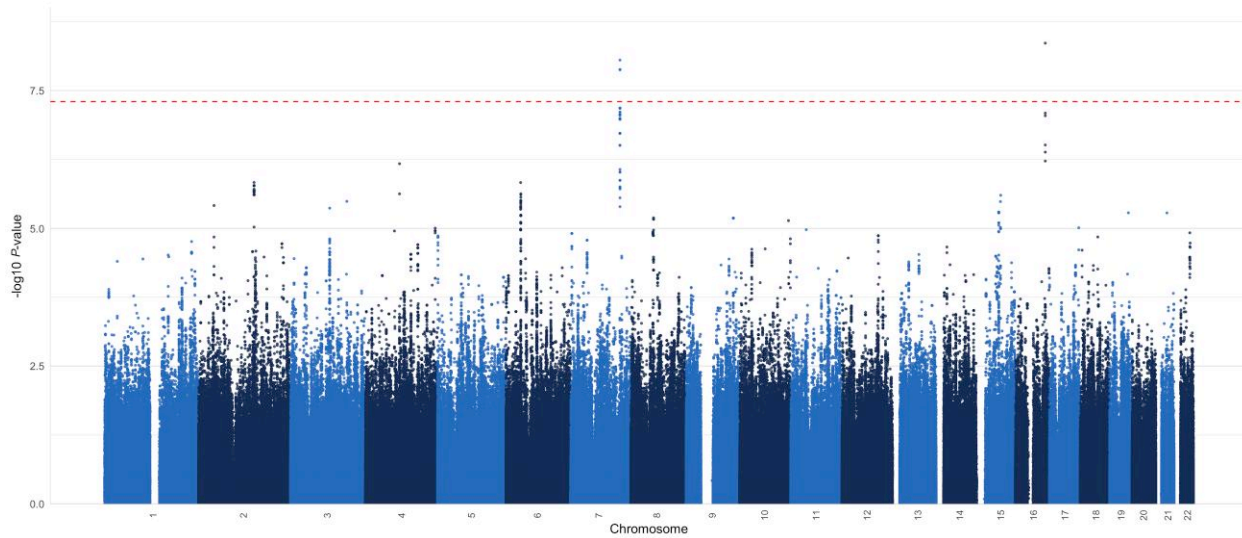


Figure 2 $A\beta_{42}/A\beta_{40}$ case-only GWAS ($n = 1420$ cases).

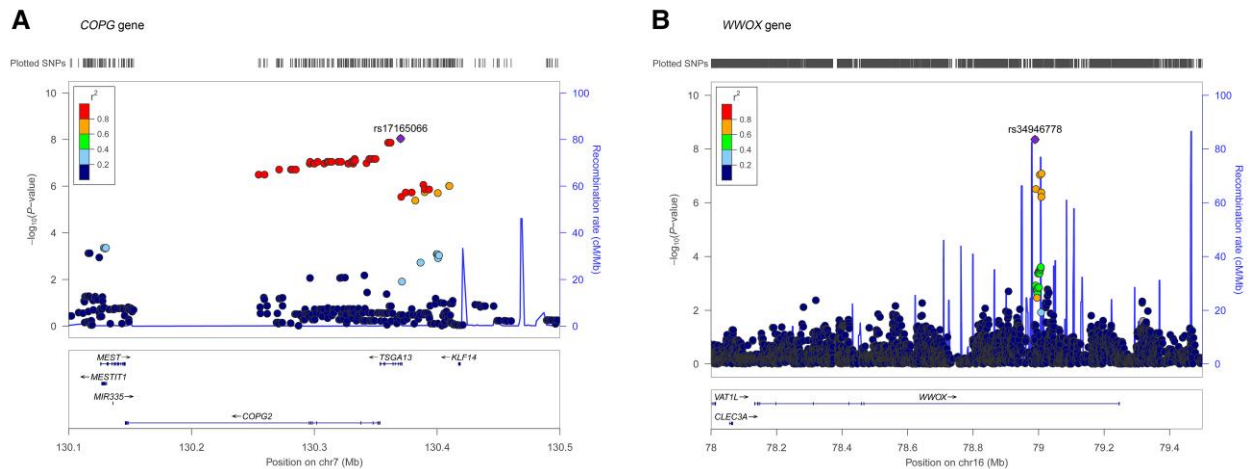


Figure 3 Genome-wide significant regions associated with $A\beta_{42}/A\beta_{40}$ in case-only analysis ($n = 1420$ cases). (A) COPG and (B) WWOX.

collected within the testing period of 2.6 years. Within this very limited age range, $A\beta_{42}$ (but not $A\beta_{40}$) was significantly positively associated with age. Therefore $A\beta_{42}/A\beta_{40}$ was also positively associated with age. In our study with a much wider age range, both $A\beta_{42}$ and $A\beta_{40}$ were significantly positively associated with age. The ratio $A\beta_{42}/A\beta_{40}$ was negatively associated with age because the increase in $A\beta_{40}$ was greater than that in $A\beta_{42}$ (Table 4 and Supplementary Fig 2). To summarize the $A\beta$ data, the biomarker is sensitive to age and potentially other clinical conditions and phenotypes unmeasured and unaccounted for in our and others' reports. Given this, interpretation of $A\beta$ measurements in the absence of other clinical information is uncertain at best.

In addition, the biomarkers measuring $A\beta_{40}$, $A\beta_{42}$ and P-tau181 levels also have complex trajectories as the disease develops, and this is all in the context of 80% AD diagnostic accuracy. Counterintuitively, it seems that P-tau181 is largely a plaque amyloid marker⁵²: it does not go up in progressive supranuclear palsy, it goes up in amyloid mice after onset of plaque pathology⁵³ (although it may also increase in tau-overexpressing mice⁵⁴). $A\beta$, however,

goes down when plaque deposition starts and APOE correlates with plaque number in a dose-dependent manner.⁵⁵ Thus, APOE and P-tau181 correlate positively, because they both largely mark amyloid deposition. When P-tau181 increases, $A\beta_{42}/A\beta_{40}$ decreases, because $A\beta_{42}$ sticks to the amyloid plaques, preventing it from leaking into plasma or CSF. An advantage of using the $A\beta_{42}/A\beta_{40}$ ratio over the individual biomarkers is that the ratio normalizes high versus low $A\beta$ producers to each other and is a more reliable qualitative test for $A\beta$ status in the brain than $A\beta_{42}$ alone.

We found two independent genome-wide significant associations with the ratio of $A\beta_{42}/A\beta_{40}$ in the COPG2 and WWOX genes in a case-only analysis (the lead SNPs in controls were not significant). In the analysis, which included both cases and controls, these SNPs were not genome-wide significant despite the increased sample size compared to cases-only. The GWAS SNPs found in cases were not statistically significant in controls and had effect sizes in the opposite direction. This may indicate that there are genetic-protein associations that can only be identified when looking at disease-relevant groups (AD in this case).

COPG2 is a part of the coat protein complex I (COPI) which is responsible for retrograde transport from Golgi-to-endoplasmic reticulum. Genetic modulation of the COPI complex leads to changes in amyloid precursor protein processing and a decrease in the amyloid plaque burden in an AD mouse model.⁵⁶

The WW domain-containing oxidoreductase gene (WWOX) maps to the ch16q23.1–23.2 region and encodes a 414-amino acid protein composed of two WW domains in its N-terminus and a central short-chain dehydrogenase/reductase domain.⁵⁷ In recent years, abundant evidence from multiple studies has causally linked WWOX loss of function with various central nervous system pathologies. WWOX dysfunction induced sequential aggregation of tau and A β , and caused apoptosis.⁵⁸ The role of WWOX/WOX1 in AD pathology and in cell death signalling has previously been reported,⁵⁹ as has its role in brain development and pathology.⁶⁰

In conclusion, our results demonstrate that the currently available plasma biomarkers reflect different aspects of AD, some of which can be attributed to ageing in addition to the disease-specific features, while others are specifically related to disease progression mechanisms. Our study shows that biomarker-based diagnosis is not perfect because the biomarker measurements in older controls are similar to those in younger clinically diagnosed AD cases (which likely represents increased prevalence of pre-clinical Alzheimer's changes in older controls). Biomarkers, however, have the advantage of specificity over clinical assessments, which may confuse dementia subtypes due to phenotypic similarities. Therefore, blood plasma biomarkers can only be a useful tool for the assessment and prediction of AD in the context of other genetic and/or clinical information. The idea that biomarkers alone might provide more accurate prediction for AD remains to be fully validated. Longitudinal studies which use a combination of genetics, plasma biomarkers, brain imaging, and pathology confirmation to differentiate cases and controls could provide accurate analyses moving away from prediction of dementia towards prediction of AD.

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Competing interests

B.D.S. has no direct conflict of interests with the results reported in this manuscript. He has however consulted for several major drug companies and is scientific founder of Augustin TX and Muna TX. He has a small amount of shares in Muna TX. H.Z. has served at scientific advisory boards and/or as a consultant for Abbvie, Alector, Annexon, Artery Therapeutics, AZTherapies, CogRx, Denali, Eisai, Nervgen, Novo Nordisk, Pinteon Therapeutics, Red Abbey Labs, Passage Bio, Roche, Samumed, Siemens Healthineers, Triplet Therapeutics and Wave, has given lectures in symposia sponsored by Collectricon, Fujirebio, Alzecure, Biogen and Roche, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (outside the submitted work). The other authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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