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FEATURED ARTICLE

Amyloid-beta misfolding and GFAP predict risk of clinical Alzheimer's disease diagnosis within 17 years

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Abstract

Introduction: Blood-based biomarkers for Alzheimer's disease (AD) are urgently needed. Here, four plasma biomarkers were measured at baseline in a community-based cohort followed over 17 years, and the association with clinical AD risk was determined.

Methods: Amyloid beta (Aβ) misfolding status as a structure-based biomarker as well as phosphorylated tau 181 (P-tau181), glial fibrillary acidic protein (GFAP), and neurofilament light (NfL) concentration levels were determined at baseline in heparin plasma from 68 participants who were diagnosed with AD and 240 controls without dementia diagnosis throughout follow-up.

Results: Aβ misfolding exhibited high disease prediction accuracy of AD diagnosis within 17 years. Among the concentration markers, GFAP showed the best performance, followed by NfL and P-tau181. The combination of Aβ misfolding and GFAP increased the accuracy.

Discussion: Aβ misfolding and GFAP showed a strong ability to predict clinical AD risk and may be important early AD risk markers. Aβ misfolding illustrated its potential as a prescreening tool for AD risk stratification in older adults.

KEYWORDS
Alzheimer's disease, blood biomarkers, immuno-infrared sensor, risk stratification, single molecule array

1 | BACKGROUND

The World Health Organization (WHO) estimates that more than 55 million people are living with dementia, and this number is predicted to rise to 139 million by 2050. In 2019, the estimated global cost of dementia was US$ 1.3 trillion.[1] Alzheimer's disease (AD) is a continuum, which can be categorized by biomarker status according to the ATN classification system.[2] This system rates individuals upon the presence of amyloid β (Aβ) alterations in cerebrospinal fluid [CSF] or positron emission tomography [PET] as "A", hyperphosphorylated tau (CSF or PET as "T"), and neurodegeneration (atrophy on structural magnetic resonance imaging [MRI], fluorodeoxyglucose [FDG]–PET, or CSF total tau as "N"). In addition, blood biomarkers have emerged and include candidates such as $A\beta_{1-42}/A\beta_{1-40}$ ratio, phosphorylated tau (P-tau), neurofilament light (NfL) chain, and glial fibrillary acidic protein (GFAP). Elevated levels of plasma P-tau181, P-tau217, and P-tau231 were indicative of prodromal and mild cognitive impairment (MCI) stages and predicted amyloid and tau pathology, whereas NfL has shown a high correlation with neurodegeneration in general, lacking AD specificity.[3–11] GFAP exhibited the ability to predict dementia and

AD.[12–15] Moreover, combinations of $A\beta_{1\text{-}42}/A\beta_{1\text{-}40}$ ratio, GFAP, and NfL revealed the potential to identify cerebral amyloidosis and/or disease severity.[16] These biomarkers have been quantified by immunoassays or mass spectrometry–based methods, which both detect low protein concentrations in blood plasma.[15–19] The single molecule array (Simoa) technology is an established immunoassay that we used in our analyses to determine P-tau181, GFAP, and NfL levels.

Complementary to widely used concentration-based protein biomarkers, the misfolding of Aβ in the initial phases of the disease in peripheral fluids has been established as a structure-based biomarker.[20–22] Misfolding and aggregation of native soluble forms into oligomeric and fibrillar, beta-sheet enriched structures are thought to be crucial in the development and progression of AD, where beta-sheet enriched species form amyloid plaques.[23,24] The immuno-infrared sensor assay provides the only technology to directly measure Aβ misfolding in blood plasma and has been validated previously.[22,25,26] Plasma Aβ misfolding was able to predict the risk of clinical AD diagnosis up to 14 years in advance in a population-based cohort[25,27] and up to 6 years in a cohort comprising participants with subjective cognitive decline.[26] In addition, Aβ misfolding in plasma in combination with plasma Aβ ratios led to higher disease prediction accuracy.[26] Because recent evidence has shown that plasma biomarkers identify pathological changes more than a decade before clinical manifestation, early risk prediction is crucial for successful therapy.[28–30]

In this study, we compared the Aβ structure–based biomarker performance with P-tau181, GFAP, and NfL levels in plasma to predict clinical AD diagnosis within 17 years in a population-based cohort. In addition, the genetic risk marker apolipoprotein E gene (*APOE*) was considered when combining biomarkers.

RESEARCH IN CONTEXT

1. **Systematic Review**: Characterization of Alzheimer's disease (AD) using biomarkers has increasingly entered the research field in recent years. Blood-based biomarkers are a non-invasive and cost-effective option. Studies have so far tended to focus on patients in whom AD has already manifested clinically. Accordingly, there is a need for studies that are more reflective of the population to better assess risks for AD.
2. **Interpretation**: We stratified the potential of amyloid beta (Aβ) misfolding and concentration biomarkers phosphorylated tau (P-tau)181, glial fibrillary acid protein (GFAP), and neurofibrillary light (NfL) in blood plasma to reliable predict incident AD diagnosis in a community-based cohort within 17 years. Aβ misfolding and GFAP in combination showed the best performance for disease prediction accuracy.
3. **Future Directions**: Due to the approval of a disease-modifying therapy, screening of older adults and the time point for interventions become more crucial. Aβ misfolding in plasma could be an early AD risk marker, and further studies in community-based settings could verify this hypothesis.

2 | METHODS

2.1 | The ESTHER cohort

Analyses presented here are based upon a nested case-control (NCC) study with available Aβ misfolding measurements within the Epidemiologische Studie zu Chancen der Verhütung, Früherkennung und optimierten Therapie chronischer Erkrankungen in der älteren Bevölkerung (ESTHER) cohort. Details of the cohort, which is a community-based prospective longitudinal study of older adults in Germany, have been described elsewhere.[31] Briefly, the cohort includes 9940 participants 50–75 years of age, who were recruited by their general practitioners (GPs) due to a general health examination in a statewide study in Saarland, Germany in 2000–2002. Participants completed standardized health questionnaires and provided blood samples, including heparin plasma samples. Medical information was provided by GPs, and comprehensive follow-up was conducted through questionnaires given to both, participants, and GPs at time points 2, 5, 8, 11, 14, and 17 years after recruitment. Major disease incidence and mortality were monitored throughout follow-up. Follow-up is still ongoing, and data are linked to the Saarland cancer registry. The provision of death certificates was obtained by local authorities. The ESTHER study was approved by the ethics committee of the Medical Faculty at Heidelberg University and the Physician's Board of Saarland.

Diagnoses of AD were collected from the GPs during the 14- and 17-year follow-ups as reported previously.[32,33] Briefly, GPs were contacted, and the participants' dementia status was queried, including the date of diagnosis for determination of the duration since baseline. German Guidelines for AD diagnosis follow the National Institute on Aging and the Alzheimer's Association[34] or the International Working group (IWG)-2 criteria.[35–37] Diagnoses stated in this work are based solely on the questionnaires, which were sent out to GPs, who had access eventually to additional medical reports from specialists.

In this study, participants that received an AD diagnosis within 17 years (n = 68), and dementia diagnosis free controls as confirmed by GPs (n = 240) were included to be characterized biochemically. The sample is based on a previously described NCC[38] and those participants with available Aβ misfolding measurements were included in this study (n = 308).

2.2 | Determination of Aβ misfolding status in plasma

Details about immuno-infrared measurements have been described previously.[20–22] Briefly, the immuno-infrared sensor provides a relative measurement of the structural properties of proteins and therefore

can be used to monitor aggregation or misfolding of proteins, specifically Aβ, tau, and TAR-DNA binding protein 43 (TDP-43).[20–22,25,26,39] The Aβ misfolding status in plasma, represents relative ratios of misfolded, β-sheet enriched, and pathological Aβ species compared to monomeric, non-toxic Aβ species. The secondary structure distribution is reflected by the amide I band and indicates the degree of misfolding of Aβ that is increased during disease progression. Throughout the analysis the whole Aβ fraction is extracted. Infrared readout values are given in wavenumbers (cm^{-1}). With lower readouts, the probability for a pathological transition to AD increases. We determined a priori a threshold at $\leq$1642 cm^{-1} that is indicative of the proposed biomarker-based transition to AD.[25] Each sample was analyzed with a freshly prepared sensor surface. In this study, 50 μL of lithium heparin samples was used. Measurements were carried out in a blinded manner.

2.3 | Determination of P-tau181, GFAP, and NfL levels using Simoa technology

The Simoa technology was used to measure P-tau181, GFAP, and NfL in plasma drawn at baseline. Plasma collected in lithium-heparin tubes was stored upon arrival at −80°C. Before analysis on the Simoa HD-X Analyzer by Quanterix, samples were thawed at room temperature and mixed thoroughly. Samples were applied to a conical 96-well plate (Quanterix, MA, USA) after centrifugation at 10,000 × *g* for 5 minutes and measurements were carried out in a single batch immediately. In this study commercially available Simoa Neurology 4-Plex E Advantage Kits and Simoa P-tau181 Advantage V2 Kits (Quanterix) were used according to manufacturer's instructions and with onboard automated 4x sample dilution. Measurements were carried out in a blinded manner. It is recommended to use ethylenediaminetetraacetic acid (EDTA) plasma for analyzing blood biomarkers in neurodegenerative diseases.[40] Unfortunately, levels of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ measured in our sample utilizing heparin samples were extremely low or not detected. Therefore, Aβ values were excluded in these analyses.

2.4 | *APOE* genotype

APOE genotype was determined based on allelic combinations of the single nucleotide polymorphisms (SNPs) rs7412 and rs429358 using predesigned TaqMan SNP genotyping assays (Applied Biosystems, CA, USA). Genotypes were analyzed in an end point allelic discrimination read using the Bio-RAD CFX Connect System (Bio-Rad Laboratories, CA, USA). For statistical analysis, *APOE* ε4 status (ε4 allele) was considered as positive (≥1 ε4 allele) or negative (no ε4 allele). When directly genotyped *APOE* data were missing, available quality controlled, imputed genetic data were utilized (imputation conducted using the Michigan Imputation Server, where SHAPEIT2 was used to phase the data and Minimac 4 was used to impute to the HRC Version r1.1 24 reference panel[33]). In analyses including *APOE*, participants were excluded if genotyped or imputed *APOE* information was not available ($n = 12$).

2.5 | Statistical methods

Descriptive statistics were used for summarizing participant characteristics, whereas chi-square, *t*-tests, and Mann-Whitney *U* tests were carried out to compare incident AD cases and controls. Additional chi-square tests were completed to compare incident AD cases and controls according to the distribution of concentration biomarker (P-tau181, GFAP, and NfL) quartiles, Aβ misfolding status (Aβ misfolding −, amide I maximum frequency >1642 cm^{-1}; Aβ misfolding +, amide I maximum frequency ≤1642 cm^{-1}), and combination categories of biomarker quartiles and Aβ misfolding status.

Multiple imputations ($n = 5$) for data missing at random ($n = 2$ for GFAP, $n = 2$ for NfL) was carried out following the Markov chain Monte Carlo method.[41] In the analyses including *APOE*, those individuals who did not have genotyped or imputed *APOE* information available were excluded. Logistic regression analyses adjusted for age and sex were utilized to calculate odds ratios (ORs) and 95% confidence intervals (CIs) for incident AD diagnosis within 17 years based on the predictors: age, sex, *APOE* status, and each biomarker. All biomarker predictors were considered as continuous variables (per standard deviation [SD] increase in log-transformed values of P-tau181, GFAP, and NfL and per SD decrease in Aβ Amide I maximum values) for the OR estimates.

For discriminating incident AD cases from controls based upon Aβ misfolding, receiver-operating characteristic (ROC) analyses including calculation of area under the curve (AUC) were performed and sensitivity and specificity were calculated for each wavenumber ranging between 1630.5 and 1659.5 cm^{-1}. Values below the threshold of 1642 cm^{-1} were assumed to be indicative for AD. The combined ROC curves for Aβ misfolding + *APOE*, Aβ misfolding + GFAP, and Aβ misfolding + GFAP + *APOE* were also calculated. The ROC curves were calculated based on continuous log-transformed P-tau181, GFAP, and NfL levels, whereas continuous Aβ misfolding values, and *APOE* was considered categorically (*APOE* ε2ε2, ε2ε3, ε3ε4, ε4ε4 vs ε3ε3). ROC contrast analysis using the DeLong test was conducted to test for significant differences between curves.[42] All ROC curve analyses utilized a logistic regression model adjusted for age and sex. Codes can be shared upon request.

All analyses were conducted two-sided at a significance level of 0.05 using SAS software, version 9.4 (SAS Institute, Cary, NC, USA) and OriginPro 2019, version 9.6 (OriginLab Corporation, Northampton, MA, USA).

3 | RESULTS

In this study, plasma AD biomarkers were assessed at baseline in a subset of participants from the ESTHER cohort ($n = 308$; 68 incident AD cases and 240 controls) (Figure 1). Table 1 summarizes the study population baseline characteristics. Table S1 shows in addition the participant characteristics compared to the overall ESTHER study. AD participants were on average 69 years of age, whereas controls were on average 66 years of age at baseline. Furthermore, 63% of

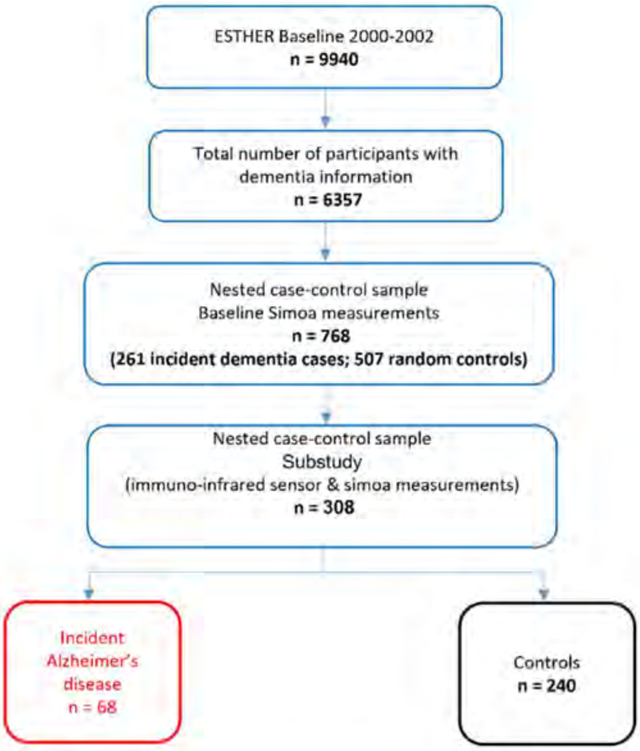


FIGURE 1 Overview of participants in ESTHER and samples included in the analyses presented here. (ESTHER = Epidemiologische Studie zu Chancen der Verhütung, Früherkennung und optimierten Therapie chronischer Erkrankungen in der älteren Bevölkerung)

AD subjects and 53% of controls were female. *APOE* ε4 genotype was positive in 49% of the AD group and 28% of the control group.

Aβ misfolding was determined by immuno-infrared measurements. AD cases had a mean amide I maximum frequency of 1641 cm^{-1} (SD ± 4 cm^{-1}), whereas controls had a mean amide I maximum frequency of 1646 cm^{-1} (SD ± 4 cm^{-1}). AD cases had significantly lower immuno-infrared sensor readout values as compared to controls, which means participants with an AD diagnosis within 17 years had a significantly higher degree of Aβ misfolding in plasma at baseline ($P < .001$) (Figure 2). Of interest, 65% of the AD cases diagnosed 9 to 17 years after study entry showed increased pathological misfolding of Aβ, compared to 71% of those diagnosed 0 to 9 years after study entry.

AD cases showed significantly increased mean levels of P-tau181, with 2.3 pg/mL (SD ± 1.4 pg/mL) compared to controls with 1.9 pg/mL (SD ± 1.0 pg/mL) ($P < .01$) (Figure 3A). Significant differences in mean concentrations of GFAP between AD cases with 159.0 pg/mL (SD ± 111.1) and controls with 99. 6 pg/mL (SD ± 46.7 pg/mL) were also evident ($P < .001$) (Figure 3B). In addition, NfL concentrations were increased in AD subjects (23.9 pg/mL SD ± 11.8 pg/mL) as compared to controls (18.9 pg/mL SD ± 9.4 pg/mL) at baseline ($P < .001$) (Figure 3C). Logistic regression results revealed that Aβ misfolding per SD decrease had the highest OR for incident AD, with 4.24 (95% CI 2.68–6.67), followed by *APOE* (≥1 ε4 allele vs none) with 2.36 (95% CI 1.32–4.24) and GFAP per SD increase with 2.08 (95% CI 1.44–3.01) (Table 1). In Contrast, NfL (1.37 95% CI 0.98–1.91) and P-tau181 per SD (1.25 95% CI 0.93–1.68) showed lower ORs.

TABLE 1 Participant characteristics at baseline and association with incident AD diagnosis within 17 years

	N total	AD cases (0–17 years)	Controls	*P*-value[a]	OR (95% CI)[b]
N total	308	68	240		
Age at baseline	308	68.8 ± 4.3	66.1 ± 4.6	<.0001	**1.16 (1.08–1.25)**
Female	169	43 (63.2)	126 (52.5)	.12	Ref.
Male	139	25 (36.8)	114 (47.5)		0.62 (0.35–1.10)
APOE ε4 –	200	34 (51.5)	166 (72.2)	<.01	Ref.
APOE ε4 +	96	32 (48.5)	64 (27.8)		**2.36 (1.32–4.24)**
Aβ Amide I maximum frequency (cm^{-1})	308	1641.2 ± 4.3 cm^{-1} (1631–1657 cm^{-1})	1645.7 ± 4.5 cm^{-1} (1633–1667 cm^{-1})	<.0001	**4.24 (2.68–6.67)**
P-tau181	308	2.3 ± 1.4 pg/mL (0.2–7.5 pg/mL)	1.9 ± 1.0 pg/mL (0.1–7.6 pg/mL)	.01	1.25 (0.93–1.68)
GFAP	306	159.0 ± 111.1 pg/mL (6.0–875.0 pg/mL)	*n* = 238 99.6 ± 46.7 pg/mL (13.3–408.0 pg/mL)	<.0001	**2.08 (1.44–3.01)**
NfL	306	23.9 ± 11.8 pg/mL (0.7-80.0 pg/mL)	*n* = 238 18.9 ± 9.4 pg/mL (6.4-79.3 pg/mL)	<.0001	1.37 (0.98–1.91)

Note: Non-imputed data are presented as frequency (%) for categorical values and mean ± SD (range) for continuous variables.
Abbreviations: Aβ, amyloid beta; *APOE4* +, ≥1 ε4 allele; AD, Alzheimer's disease; CI, confidence interval; GFAP, glial fibrillary acidic protein; *N*, number of participants; NfL, neurofilament light; OR, odds ratio; P-tau181, phosphorylated tau181; SD, standard deviation.
[a]*P*-value for comparison between AD cases (0–17 years) and controls (chi-square, *t*-test, Mann–Whitney *U* test results as appropriate).
[b]Results of multivariate logistic regression utilizing the imputed data set to account for GFAP and NfL missing values ($n = 4$) for AD diagnosis within 17 years adjusted for age and sex. All biomarker predictors were considered as continuous variables (per SD increase in log-transformed values of P-tau181, GFAP, and NfL and per SD decrease in Aβ Amide I maximum values).

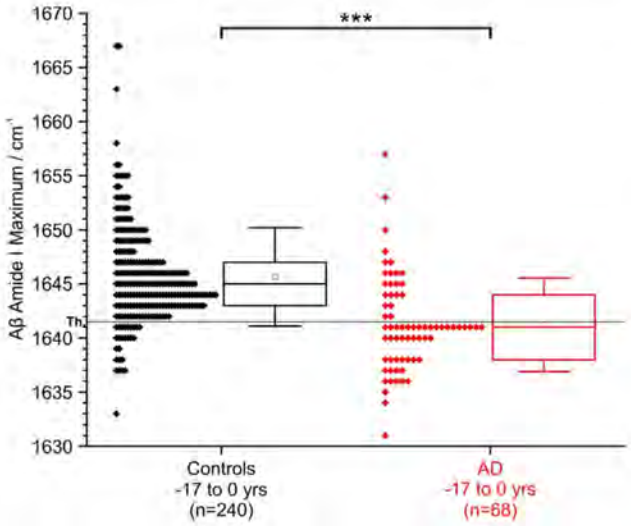


FIGURE 2 Immuno-infrared sensor analysis of amyloid beta (Aβ) misfolding in heparin plasma at baseline from ESTHER study participants categorized with diagnoses after 17 years. Amide I band frequencies of patients with Alzheimer's disease (AD) and cognitively unconcerned age- and sex-matched controls (every diamond represents a single patient). A threshold of 1642 cm^{-1} (dotted line) discriminates AD versus controls. Box and whisker plots show median (vertical line), mean (square), interquartile range (boxes), and standard deviation (whiskers). Mann–Whitney *U* tests was used to test for statistically significant differences between AD cases and controls: $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$

The predefined discriminative threshold of ≤1642 cm^{-1} for Aβ misfolding indicating a pathological biomarker transition toward AD. ROC analysis revealed the highest AUC of of all biomarkers measured: 0.78 (95% CI 0.71–0.85) (Figure 4A). Among the concentration markers, GFAP showed the highest AUC value (AUC 0.74, 95% CI 0.67–0.82), followed by NfL (AUC 0.68, 95% CI 0.61–0.75) and P-tau181 (AUC 0.61, 95% CI 0.53–0.70) (Figure 4A). Differences in concentration markers when categorized in misfolding positive and negative for AD cases and controls are provided in Figure S1. The distribution of concentration biomarker quartiles and Aβ misfolding status at baseline in incident AD cases and controls can be found in supplementary Table S2.

The combination of *APOE* and Aβ misfolding increased the AUC slightly to 0.80 (95% CI 0.73–0.86) (Figure 4B). AUC was further increased to 0.83 (95% CI 0.76–0.89) when GFAP and Aβ misfolding were combined. However, combining all three markers did not further improve the AUC (AUC 0.83, 95% CI 0.77–0.90) (Figure 4B). In addition, DeLong analyses showed that neither concentration markers nor *APOE* combined with Aβ misfolding led to a statistically significant difference between ROC curves of biomarker combinations and Aβ misfolding alone. GFAP + Aβ misfolding had the highest prediction accuracy of incident clinical AD conversion within 17 years. Adding *APOE* did not further improve the AUC.

4 | DISCUSSION

Studies on predictive biomarkers for AD utilizing population-based cohorts, including symptom-free individuals, are still lacking. Here we presented results from a sub-study of the ESTHER cohort, a large population-based cohort study of older adults from Germany. The presence of Aβ misfolding, which had recently been verified and validated as an AD-specific biomarker,[20–22,25,26] showed higher accuracy than concentration markers—P-tau181, GFAP, or NfL—to predict AD diagnosis within 17 years of follow-up. The combination of Aβ misfolding and GFAP exhibited the highest AD-prediction accuracy.

71% of the study participants who were diagnosed after 0 to 9 years showed Aβ misfolding, whereas 65% of participants who were diagnosed with AD between 9 and 17 years after baseline collection had increased misfolding. This suggests that Aβ misfolding could be a prescreening biomarker for risk of clinical AD conversion up to 17 years before diagnosis. Due to the approval of a disease-modifying therapy, many more people will need to be screened periodically starting at about 60 years old. This helps to identify time points of biomarker changes and start of interventions based on blood tests.

Most recently, elevated levels of P-tau181 showed great potential for predicting amyloidosis and tau pathology.[3,4,7] However, the performance of this biomarker in our study was at 17 years inferior to GFAP and NfL, with an AUC of 0.61, although levels were elevated in manifested AD cases compared to controls, as shown in other studies.[3,4,7,9,10] We have shown previously that P-tau181 levels were associated with risk of clinical AD incidence only within 9 years of diagnosis.[43] Here, we identified 22 AD cases without Aβ misfolding, but with already elevated P-tau181 levels on average compared to controls (Figure S1A). Because Aβ misfolding was negative, these individuals might have non-AD pathological changes instead of being in the Alzheimer's continuum, as suggested by the ATN classification system. Considering, that Aβ misfolding is currently not included in the ATN system in contrast to PET or CSF ratios, this biomarker could be added in the future to amyloid "A" section, not only but also because of the AD specificity. Further analyses revealed that the largest group of AD cases with positive misfolding status had rather low P-tau181 values (Table S2). This might be an indication that Aβ misfolding occurs before P-tau181 rises in plasma. However, a negative correlation of P-tau levels with Aβ misfolding was significant for the individuals with incident AD within 9 years ($P < .01$; $r = -.53$). Therefore, the question of which biomarker alteration occurs first in the disease progression needs further investigation.

NfL is a general neurodegenerative marker and not specific for AD. It did not improve the disease-prediction accuracy of AD diagnosis. Nineteen of 23 individuals (83%) with positive misfolding status and NfL levels in the highest quartile developed AD (Table S2). However, 40% of AD cases were Aβ misfolding positive but had no elevated NfL values. This also supports the idea that Aβ misfolding may be an early and NfL a later risk prediction marker. Our study with biomarker measurements at baseline provides insight into the risk of developing AD with 17 years of follow-up, whereas other studies had shorter

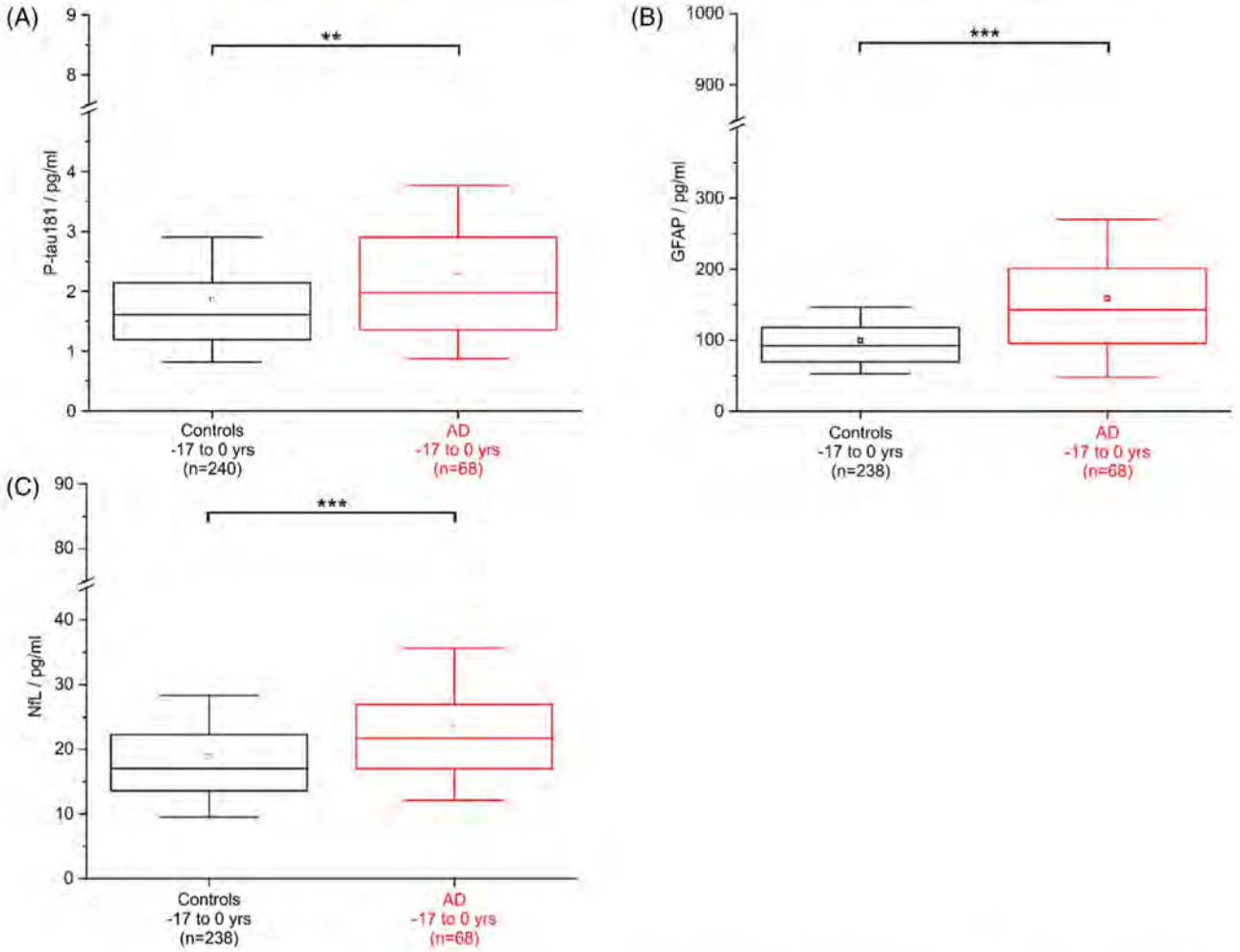


FIGURE 3 Simoa measurements of P-tau181 (A), NfL (B), and GFAP (C) in heparin plasma at baseline from ESTHER study participants categorized with diagnoses after 17 years. Concentration values in pg/mL of patients with Alzheimer's disease (AD, red) and cognitively unconcerned age and sex matched controls (black) (every diamond represents a single patient). Box and whisker plots show median (vertical line), mean (square), interquartile range (boxes), and standard deviation (whiskers). Mann–Whitney *U* tests were used to test for statistically significant differences between AD cases and controls: $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$

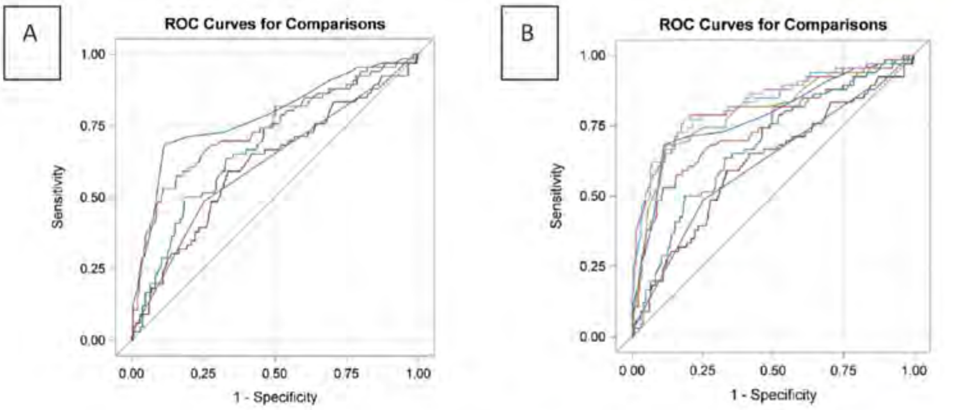
follow-up time, only up to 11 years,[3,5,7,11,12] or other types of AD such as familial cases,[44,45] which may not be representative of the general population.

As a marker of astrogliosis, GFAP is currently of research interest for analyzing AD-specific associations.[46] In our study, GFAP levels were significantly higher in participants who were diagnosed with AD within 17 years. However, whether an increased GFAP level is AD specific needs further investigation. Recent study reporting has indicated that this is not necessarily the case.[46,47] But the performance of GFAP alone is in concordance with recent results.[43,46,48] Of interest, there were 24 participants who had GFAP levels within the highest quartile and who were also Aβ misfolding positive at baseline, of whom 22 (92%) went on to receive an AD diagnosis during follow-up (Table S2).

In our study, a combination of Aβ misfolding and GFAP levels showed especially good predictive potential. Aβ misfolding alone showed the best discriminative performance and is on the same level as recently published plasma Aβ assays of prodromal and MCI stages.[17] Furthermore, the combination of Aβ misfolding and GFAP brings an added value to the disease-prediction accuracy of incident AD within 17 years. This might be because elevated GFAP levels indicate abnormal activation of astrocytes that often surround amyloid plaques and may therefore be associated with Aβ misfolding.

The addition of *APOE* as a genetic risk factor did not improve disease-prediction accuracy. This could be because genetic predisposition, calculated from *APOE* genotype, indicates only increased AD risk. Blood-based biomarkers, however, directly indicate pathological changes that lead to neuronal loss and the development of the disease. In our study, Aβ misfolding had the strongest prognostic ability, as it directly detects the postulated first pathological process, Aβ misfolding and oligomerization, the base for plaque formation.[28] In addition, the measurement provides an advantage compared to quantitative assays with enabling analyses of heparin plasma samples. Furthermore, the misfolding measurement is a relative measure and the readout of the amide I maximum position is not dependent on concentration.

There are some limitations to this study. First, the dementia diagnoses may be inaccurate and not all participants visited a neurologist



Predictor	AUC (95% CI)
Aβ misfolding	0.784 (0.713-0.854)
GFAP	0.741 (0.666-0.816)
NfL	0.680 (0.606-0.755)
P-tau181	0.615 (0.534-0.696)
APOE	0.615 (0.541-0.690)

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P-tau181	0.615 (0.534-0.696)
APOE	0.615 (0.541-0.690)
Aβ misfolding + *APOE*	0.798 (0.730-0.865)
Aβ misfolding + GFAP	0.828 (0.764-0.891)
Aβ misfolding + GFAP + *APOE*	0.835 (0.772-0.897)

FIGURE 4 Receiver-operating curve (ROC) analyses to determine the discriminative power of all biomarkers to distinguish between patients with Alzheimer's disease (AD) and controls within 17 years. Simoa biomarkers are log-transformed values. Participants with missing apolipoprotein E (*APOE*) status have been excluded ($n = 12$). (A) ROC analyses revealed an area under the curve (AUC) for AD versus controls of 0.78 with respect to the degree of amyloid beta (Aβ) misfolding (blue), 0.74 for GFAP (red), 0.68 for NfL (mint), and 0.61 for P-tau181 (brown), underscoring the status of Aβ misfolding as the best performing solo biomarker. (B) Combined ROC curve analyses showed the highest AUC for the combination of biomarkers Aβ misfolding, *APOE* status, and GFAP (AUC 0.83, pink), followed with the same value by Aβ misfolding and GFAP (AUC 0.83, light blue) and Aβ misfolding and APOE (AUC 0.80, yellow). The data showed that *APOE* status did not increase the discriminative power of Aβ misfolding and GFAP, suggesting that only these two blood-based biomarkers are favored. Note: ROC Contrast Analyses (DeLong test). Aβ mis. − (Aβ mis. + GFAP): $P = .09$. Aβ mis. − (Aβ mis. + P-tau181): $P = .59$. Aβ mis. − (Aβ mis. + NfL): $P = .34$Aβ mis. − (Aβ mis. + *APOE*): $P = .28$

or other specialist. However, this characteristic of our community-based cohort study may better reflect real-life practice of AD diagnosis and care in community settings than cohorts conducted in specialized academic settings with highly selective study populations. Furthermore, another weakness is that A$\beta_{1\text{-}40}$ and A$\beta_{1\text{-}42}$ values were not measurable in Simoa analyses, thereby limiting comparability between structural and concentration changes of Aβ. One possible cause could be that heparin inside the collection tubes became unstable during long storage and precipitated during thawing. Because Aβ has a consensus sequence for binding heparin and the samples were centrifuged before Simoa measurements, most of the Aβ species may have been pelleted during processing and were therefore no longer in the solution.[49] Here, the immuno-infrared sensor revealed an advantage, since preprocessing was unnecessary and structural changes of the whole Aβ fraction could be analyzed regardless of the type of blood- collection tube. Other studies have recommended using EDTA tubes instead of heparin because of the analyte recovery.[40] Unfortunately, the ESTHER study has insufficient EDTA.

5 | CONCLUSION

In summary, we presented results from a community-based cohort with AD diagnoses after 17 years of follow-up. A combination of Aβ misfolding and GFAP showed the best discrimination between AD and controls and the greatest potential for risk stratification. By the correct identification, these participants then avoid invasive and costly diagnostic tests like lumbar puncture or PET imaging. Our approach could lead to a non-invasive and cost-effective multifactorial diagnostic tool for prescreening older adults regarding the risk of developing AD. In further studies in which EDTA plasma samples were collected, it is also conceivable to expand the biomarker panel to include A$\beta_{1\text{-}40}$ and A$\beta_{1\text{-}42}$, and the A$\beta_{1\text{-}40}$/A$\beta_{1\text{-}42}$ ratio to improve the assay and biomarker panel. By identifying those individuals at high risk of AD development, disease-modifying therapies could be administered early in the disease's progression, thereby preventing symptomatic clinical AD. These findings must be validated in additional longitudinal studies like ESTHER, preferably with PET and/or CSF data available, to confirm and enclose the time point when biomarker alterations appear.

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CONFLICTS OF INTEREST

The authors L.B., H.S., B.H., J.S., A.N., H.B., and K.G. have no competing interests to declare. D.R. has received consulting fees from and served on a Data Safety Monitoring Board/Advisory Board for Janssen, Germany. Author disclosures are available in the supporting information.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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