Highly Specific and Ultrasensitive Plasma Test Detects Abeta(1-42) and Abeta(1-40) in Alzheimer's Disease

Elisabeth H Thijssen (✉ e.thijssen@amsterdamumc.nl)

Vrije Universiteit Amsterdam, Amsterdam UMC, Amsterdam <https://orcid.org/0000-0002-9723-5441>

Inge M.W. Verberk

Amsterdam UMC - Locatie VUMC: Amsterdam UMC Locatie VUmc

Jeroen Vanbrabant

ADx Neurosciences

Anne Koelewijn

Amsterdam UMC - Locatie VUMC: Amsterdam UMC Locatie VUmc

Hans Heijst

Amsterdam UMC - Locatie VUMC: Amsterdam UMC Locatie VUmc

Philip Scheltens

Amsterdam UMC - Locatie VUMC: Amsterdam UMC Locatie VUmc

Wiesje van der Flier

Amsterdam UMC - Locatie VUMC: Amsterdam UMC Locatie VUmc

Hugo Vanderstichele

Biomarkable

Erik Stoops

ADx Neurosciences

Charlotte E. Teunissen

Amsterdam UMC - Locatie VUMC: Amsterdam UMC Locatie VUmc

Research article

Keywords: Plasma biomarkers, amyloid beta, Alzheimer's disease, commercial assays

Posted Date: September 18th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-72749/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

BACKGROUND

Plasma biomarkers that reflect specific amyloid beta (Abeta) proteoforms are essential to monitor treatment effects of Alzheimer's disease (AD) therapies. Our aim was to develop and validate ready-to-use Simoa 'Amyblood' assays that measure full length Abeta₁₋₄₂ and Abeta₁₋₄₀ and compare their performance with two commercial assays.

METHODS

Linearity, intra- and inter-assay %CV were compared between Amyblood, Quanterix Simoa triplex, and Euroimmun ELISA. Sensitivity and selectivity were assessed for Amyblood and the Quanterix triplex. Clinical performance was assessed in CSF biomarker confirmed AD (n=43, 68±6 years) and controls (n=42, 62±5 years).

RESULTS

Prototype and Amyblood showed similar calibrator curves and differentiation (20 AD vs 20 controls, $p<0.001$). Amyblood, Quanterix triplex, and ELISA showed similar linearity (96%-122%) and intra-assay %CVs ($\leq 3.1\%$). A minor non-specific signal was measured with Amyblood of +2.4 pg/mL Abeta₁₋₄₂ when incubated with 60 pg/mL Abeta₁₋₄₀. A substantial non-specific signal of +24.7 pg/mL Abeta_{x-42} was obtained when 40 pg/mL Abeta₃₋₄₂ was measured with the Quanterix triplex. Selectivity for Abeta₁₋₄₂ at physiological Abeta₁₋₄₂ and Abeta₁₋₄₀ concentrations was 125% for Amyblood and 163% for Quanterix. Amyblood and Quanterix ratios ($p<0.001$) and ELISA Abeta₁₋₄₂ concentration ($p=0.025$) could differentiate AD from controls.

CONCLUSIONS

We successfully developed and upscaled a prototype to the Amyblood assays with similar technical and clinical performance as the Quanterix triplex and ELISA, but better specificity and selectivity than the Quanterix triplex assay. These results suggest leverage of this specific assay for monitoring treatment response in trials.

Background

Alzheimer's disease (AD) is the most common form of dementia, affecting 50 million people worldwide.¹ *In vivo* AD diagnosis and monitoring of treatment response in clinical trials is based on changes in amyloid beta proteins (Abeta), measured by positron emission tomography (PET) or in cerebrospinal fluid (CSF).^{2,3,4} However, both methods are associated with major disadvantages in terms of high costs (PET) and invasiveness (lumbar puncture to collect CSF), which prohibit repetitive analysis. Therefore, it is

critical to have access to blood-based biomarkers as a less invasive method for monitoring of treatment effects.

Since the introduction of highly sensitive technologies such as bead-based immunoassays or immunoprecipitation combined with mass spectrometry (IP-MS), consistent reductions in plasma Abeta_{42/40} ratio are reported in AD.⁵⁻¹⁰ With IP-MS, a 10-15% reduction in the plasma Abeta_{42/40} ratio has been measured in amyloid PET or CSF positive cases, compared to amyloid negative cases,⁷ and the ratio could differentiate the groups with an area under the curve (AUC) of 84-97%.⁸ IP-MS is often more sensitive than immunoassays, but requires a high sample volume and a high level of expertise which may limit broad clinical implementation. The Abeta_{42/40} ratio measured with the Single Molecule Array (Simoa, Quanterix),¹¹ showed promising results in identifying individuals with amyloid pathology (AUC of 68-87%^{5,9}) across controls, mild cognitive impairment (MCI), and AD. This bead-based technology offers high throughput and is ultrasensitive. However, the commercial Quanterix assay lacks antibody specificity, since it measures Abeta_{x-42} and Abeta_{x-40}, whereas the specificity for full-length Abeta₁₋₄₂ and Abeta₁₋₄₀ is important for monitoring the effect of treatments that target specific Abeta isoforms.

At Amsterdam University Medical Centers (Amsterdam UMC) and ADx Neurosciences (ADx) novel ready to use (RTU) immunoassays, called “Amyblood”, were developed for detection of full length Abeta₁₋₄₂ and Abeta₁₋₄₀ with Simoa technology.¹² This way both the high specificity of the antibodies and the high sensitivity of Simoa technology could be leveraged. The aim of the present study is to analytically validate the Amyblood assays, including specificity and selectivity evaluation, and explore their clinical value in a CSF-biomarker confirmed cohort of AD patients and controls. We compared the analytical and clinical performance of the Amyblood assays with the commercially available Quanterix triplex kit (Abeta_{x-42}/Abeta_{x-40}) and the Euroimmun ELISA assays (Abeta₁₋₄₂/Abeta₁₋₄₀).

Materials And Methods

Prototype and Amyblood assay development and transfer

For proof of concept, a prototype assay (**supplementary methods**) was developed in-house by Amsterdam UMC using a Simoa homebrew assay development kit (Quanterix, MA), utilizing capture antibodies ADx102 (21F12)¹³ for Abeta₁₋₄₂ or ADx103 (2G3)¹³ for Abeta₁₋₄₀ and detector antibody ADx101 (3D6)¹³ for Abeta₁ (ADx, Ghent, BE). These prototype assays formed the basis for the Amyblood assays that were further developed and upscaled by ADx. The sample diluent formulation and capture antibody conjugation were optimized to improve sensitivity. Reproducibility in %CV was tested on 3 monoclonal antibody lots and on the small batch compared to the upscaled RTU assays (volumes equivalent to 50 assay kits of 96 data points) based on duplicate measurements of the average enzyme per bead (AEB) signal, excluding the blank. Inter- and intra-assay variation were tested in six independent runs by ADx and five runs at Amsterdam UMC based on duplo measurements of two plasma quality

control samples. Initial clinical performance of the Abeta_{1-42/1-40} ratio was confirmed in 20 CSF Abeta₁₋₄₂ positive AD cases and 20 CSF Abeta₁₋₄₂ negative controls. Subsequently, the Amyblood RTU kits were shipped to Amsterdam UMC for further assay characterization and clinical validation. The Amyblood reagent preparation and assay set-up are detailed in the **Supplementary methods**.

Analytical characterization of the six assays

Analytical validation in all six assays (Abeta₄₂ and Abeta₄₀ measured with Amyblood, Quanterix, and ELISA) included measurement of the Lower Limit of Quantification (LLOQ) (mean of 16 blanc samples + 10 standard deviations (SD)), linearity upon dilution (15 EDTA plasma samples, three dilutions: dilution factor (df) 4, 6 and 8 for Abeta₁₋₄₂ and df 8, 10, and 12 for Abeta₁₋₄₀), intra-assay %CV (SD of duplicate measurement divided by the mean *100%) of 85 clinical samples and inter-assay %CV of 14 samples over two runs. Additionally, selectivity and specificity were investigated, including physiological, low, medium and high concentrations, detailed in the **Supplementary methods**.

Comparison of analytical assay characteristics

To compare the analytical performance of the novel Amyblood assays, we selected two commercially available plasma amyloid immunoassays. The Quanterix Neurology 3-plex A assay kit employs the Simoa HD-1 analyzer, with a different capture antibody for Abeta_{x-42} (H31L21) and the same capture antibody for Abeta_{x-40} (2G3), combined with a different detector antibody (6E10), comprising the RHD motif (aa5-7) that is not N-terminus specific and therefore results in binding of x-42 and x-40.¹⁴ We also selected the Euroimmun ELISA assays that employ the same antibodies as the Amyblood assays to measure Abeta₁₋₄₂ and Abeta₁₋₄₀. The Quanterix and ELISA analytical validation methods are specified in the **Supplementary methods**.

Clinical samples

We included 85 participants from the Amsterdam Dementia Cohort¹⁵ with available EDTA plasma samples in the Biobank, 43 were AD dementia patients and 42 controls (n = 35 with subjective cognitive decline (SCD), n = 7 with psychiatric disease). All subjects visited the Alzheimer Center of the Amsterdam UMC between August 2002 and January 2017 for extensive dementia screening that consisted of neurological, physical, and neuropsychological evaluation, electroencephalography, brain magnetic resonance imaging, and CSF AD biomarker analysis.¹⁵ The diagnosis was made upon multidisciplinary consensus based on applicable clinical criteria.^{3,16} CSF Abeta₁₋₄₂, P-tau181, and T-tau were measured using Innostest ELISA (Fujirebio, Ghent, BE) by research staff blinded for clinical diagnoses.¹⁷ CSF Abeta₁₋₄₂ concentrations were adjusted for the drift in CSF biomarker analyses that occurred over the years and subsequently dichotomized as CSF amyloid positive (≤ 813 pg/ml Abeta₁₋₄₂) and amyloid negative (> 813 pg/ml Abeta₁₋₄₂).¹⁸ All AD-dementia cases were selected to be CSF amyloid positive, and all controls were required to be CSF amyloid negative (Table 1).

Table 1
Cohort characteristics and biomarker values in the clinical validation

	Cohort	Controls (42)	AD (43)	Total (85)	<i>p</i>
	Sex, M/F	25/17	21/22	39/46	<i>0.33</i>
M (SD)	Age, y	61.7 (5)	68.1 (6)	64.9 (6)	< 0.001
M (SD)	MMSE	27.2 (3)	22.0 (5)	24.6 (5)	< 0.001
n		42	42	84	
M (IQR)	CSF Tau (pg/mL)	223 (117)	615 (399)	427 (395)	< 0.001
n		42	43	85	
M (IQR)	CSF P-tau (pg/mL)	39 (15)	89 (37)	63 (36)	< 0.001
n		41	43	84	
M (IQR)	CSF Abeta 42 (pg/mL)	1132 (308)	658 (153)	804 (475)	< 0.001
n		42	43	85	
Abeta, Amyloid beta; CSF, Cerebrospinal fluid; ELISA, Enzyme-Linked Immuno Sorbent Assay; MMSE, Mini-Mental State Examination; pTau, phosphorylated tau. M (SD) shows the mean value and standard deviation. M (IQR) shows the median value and interquartile range. P values show the difference between the CSF Aβ negative and the CSF Aβ positive group. Significant p-values are shown in bold (<i>p</i> < 0.05).					

EDTA-plasma samples were obtained through venipuncture. After 10-minute centrifugation at 1800xg within 2 hours, plasma was aliquoted in 0.5 mL polypropylene tubes and stored at – 80 °C. Samples were thawed at room temperature and centrifuged at 14,000xg prior to analyses.

Written consent to use medical data and biomaterials for research purposes was in place, in accordance with the ethical committee of the Amsterdam UMC, location VUmc, and with the Helsinki Declaration act of 1975.

Plasma amyloid measurement

Abeta_{1–42} and Abeta_{1–40} were measured with the Amyblood kit on the Simoa HD-1 analyzer and by Euroimmun ELISA. Concentrations of Abeta_{x–42} and Abeta_{x–40} were measured with the Neurology 3-Plex assay A (Quanterix) on the Simoa HD-1 analyzer. All samples were manually diluted (**eTable 1**) and analyzed in duplicate, following manufacturer's instructions.

Statistical analysis

Differences in Abeta₄₂ and Abeta₄₀ concentrations between patients with AD and controls were tested with linear regression analysis for group differences, both unadjusted and adjusted for sample storage time, sample run, age, and sex. The Abeta_{42/40} ratios measured with the Amyblood and Quanterix assays

were normally distributed. The Abeta_{42/40} ratios measured with the ELISA assay were not normally distributed, neither after natural log transformation, nor after excluding the extreme outlier. Therefore, Spearman correlations were used to investigate all correlations. $P < 0.05$ was considered statistically significant. All analyses were performed using IBM SPSS Statistics, version 26 and graphs were constructed using R version 3.5.3.

Results

Robustness of the upscaled Amyblood RTU assays

Differences in antibody lot were determined by comparing three lots of ADx102 and ADx103 conjugated bead batches. The variation in AEB values of the calibrator across the lots was 19%CV for Abeta₁₋₄₂ and 14% for Abeta₁₋₄₀ (**eFigure 1**). The calibration curves %CV of the small and upscaled bead batches based on calibrator AEB values was 10.0% (range: 3.8%-17.6%) for Abeta₁₋₄₂ (**eFigure 2A**) and 7.0% (range: 0.03%-13.9%) for Abeta₁₋₄₀ beads (**eFigure 2B**). Amyblood Abeta_{1-42/1-40} inter-assay %CV was 2.9% (sample 1: 3.9% at AUMC, 3.5% ADx, sample 2: 2.2% at AUMC, 2.0% at ADx) and inter-center %CV was 17.2% (sample 1: 14% at AUMC, 23% ADx, sample 2: 14% at AUMC, 13% at ADx) (**eFigure 3**).

The Abeta₁₋₄₂ and Abeta₁₋₄₀ concentrations were measured in a feasibility sample set of 20 CSF Abeta₁₋₄₂ positive AD cases and 20 CSF Abeta₁₋₄₂ negative SCD cases. The fold change in Abeta_{1-42/1-40} ratio between AD and controls was 1.39 for the prototype and 1.29 for the Amyblood (both: $p < 0.001$, Fig. 1). There was a good correlation between the prototype and Amyblood results of Abeta₁₋₄₂ ($R = 0.77$, $p < 0.001$), Abeta₁₋₄₀ ($R = 0.89$, $p < 0.001$), and the Abeta_{1-42/1-40} ratio ($R = 0.69$, $p = 0.001$) (**eFigure 4**).

Analytical validation of all six assays in this study

Upon serial dilution, all six Abeta assays showed acceptable linearity (i.e. in the range 80–120%), except for a small deviation of the Quanterix Abeta_{x-42} assay (122%) (**eTable 1**). All assays showed intra-assay %CV values $< 10\%$ for clinical samples. The inter-assay %CV of the Amyblood Abeta₁₋₄₂ assay was 13.3%, and 10.4% for Abeta₁₋₄₀. The inter-assay %CV of Quanterix Abeta_{x-42} assay was 7.9%, and 5.4% for Abeta_{x-40}. The LLOQ's were 1.6 pg/mL for Amyblood Abeta₁₋₄₂ and 1.7 pg/mL for Abeta₁₋₄₀; 0.34 pg/mL for Quanterix Abeta_{x-42} and 0.16 pg/mL for Abeta_{x-40}; and 5.4 pg/mL for ELISA Abeta₁₋₄₂ and 11.9 pg/mL for Abeta₁₋₄₀. One sample measured with the Amyblood Abeta₁₋₄₂ assay and eight samples measured with the ELISA Abeta₁₋₄₂ assay were lower than their blank. All Abeta₄₀ concentrations were above the blank (**eFigure 5**).

Assay Specificity and Selectivity

Selectivity and specificity of the Abeta₄₂ and Abeta₄₀ measurements were tested for the Amyblood and Quanterix assays. Detailed specificity concentrations and %recovery are described in **eTable 2** and **eTable 3**. Different spike concentrations were used for the Amyblood and Quanterix assays, to align with their difference in absolute Abeta₄₂ and Abeta₄₀ concentrations (**eTable 4**).

Specificity of the Amyblood and Quanterix assays

Low, medium and high concentrations of Abeta fragments 1–42, 1–40, 1–43, 2–42, and 3–42 spiked in sample buffer were measured with the Abeta_{1–42} and Abeta_{x-42} assays. For Amyblood, a minor signal above blank was measured when other Abeta fragments were incubated, with a maximum reported result of 2.4 pg/mL for 60 pg/mL of spiked Abeta_{1–40} (Fig. 2A). A substantial increase compared to the blank was observed for Quanterix Abeta_{x-42} values, especially for Abeta_{2–42}, and Abeta_{3–42} fragments, with a maximum of + 24.7 pg/mL Abeta_{x-42} for 40 pg/mL of Abeta_{3–42} (Fig. 2B). When measuring Abeta fragments 1–42, 1–38, 1–39, and 11–40 with the Amyblood Abeta_{1–40} and Quanterix Abeta_{x-40} assays, none of the other Abeta isoforms yielded a signal above blank. The Abeta 1–40 fragment was accurately measured with the Amyblood assay, but yielded a lower formal concentration when measured with the Quanterix assay (Fig. 2C and 2D).

Selectivity of the Amyblood and Quanterix assays

We tested selectivity by measuring the signal of one analyte in sample diluent (either Abeta_{1–42} or Abeta_{1–40}), in the presence of a known concentration of the other analyte. Described physiological concentrations were based on concentrations measurements in plasma after sample dilution (**eTable 4**).

For the Amyblood assay, the %recovery of 6 pg/mL Abeta_{1–42} measured with the Amyblood assay increased from 115% in the presence of the low concentration (3 pg/mL) of Abeta_{1–40} to 154% for the high concentration (60 pg/mL) of Abeta_{1–40}. The %recovery of 25 pg/mL Abeta_{1–42} was 89% in presence of the low and 105% for in the presence of the high concentration Abeta_{1–40}. The %recovery of 60 pg/mL Abeta_{1–42} was 101% in the presence of the low and 105% in the presence of the high Abeta_{1–40} concentration. At 6 pg/mL Abeta_{1–42} and 15 pg/mL Abeta_{1–40}, which approximate the physiological concentrations measured in plasma after correction for dilution factor, the %recovery of Abeta_{1–42} was 125% (Fig. 3A).

For the Quanterix assay, the %recovery of 1 pg/mL plasma Abeta_{x-42} measured with the Quanterix assay increased from 105% in the presence of the low concentration (4 pg/mL) Abeta_{1–40} to 1021% in the presence of the high concentration (130 pg/mL) Abeta_{1–40}. The %recovery of 15 pg/mL Abeta_{1–42} was 96% in the presence of a low and 188% in the presence of a high concentration of Abeta_{1–40}. The %recovery of 40 pg/mL Abeta_{1–42} was 88% in the presence of the low concentration of Abeta_{1–40} and 140% in the presence of the high Abeta_{1–40} concentration. At 1 pg/mL Abeta_{1–42} and 25 pg/mL Abeta_{1–40}

$_{40}$, which approximate the physiological concentrations, the %recovery of Abeta $_{1-42}$ was 163% (Fig. 3B). For both Abeta $_{42}$ assays, the increase in %recovery returned to normal upon increasing Abeta $_{42}$ concentrations relative to the Abeta $_{40}$ concentration.

The %recovery of 3 pg/mL plasma Abeta $_{1-40}$ measured with the Amyblood assay was 113% for the low concentration (6 pg/mL) Abeta $_{1-42}$ and 110% for the high concentration (60 pg/mL) Abeta $_{1-42}$. The %recovery of 15 pg/mL Abeta $_{1-40}$ was 110% for the low and 95% for the high concentration Abeta $_{1-42}$. The %recovery of 60 pg/mL Abeta $_{1-40}$ was 102% for the low and 96% for the high Abeta $_{1-42}$. At physiological concentrations of 15 pg/mL Abeta $_{1-40}$ and 6 pg/mL Abeta $_{1-42}$ the %recovery of Abeta $_{1-40}$ was 110% (Fig. 3C).

The %recovery of 4 pg/mL plasma Abeta $_{x-40}$ measured with the Quanterix assay was 89% for the low concentration (1 pg/mL) Abeta $_{1-42}$ and 92% in the presence of the high concentration (40 pg/mL) Abeta $_{1-42}$. The %recovery of 25 pg/mL Abeta $_{1-40}$ was 94% in the presence of the low and 97% in presence of the high concentration Abeta $_{1-42}$. The %recovery of 130 pg/mL Abeta $_{1-40}$ was 112% for the low and 106% for the high concentration Abeta $_{1-42}$. The highest %recovery was 115%, found at 130 pg/mL Abeta $_{1-40}$ and 15 pg/mL Abeta $_{1-42}$. At physiological concentrations of 25 pg/mL Abeta $_{1-40}$ and 1 pg/mL Abeta $_{1-42}$ the %recovery of Abeta $_{x-40}$ was 94% (Fig. 3D). The %recovery of both Abeta $_{40}$ assays seems relatively unaffected by an increase in Abeta $_{42}$.

Assay validation in clinical samples

Plasma Abeta $_{42}$ was reduced in AD patients compared to controls for all three assays: -8.5% for Amyblood (uncorrected: $p = 0.075$; corrected for age, sex, storage time, and sample run: $p = 0.012$), -3.6% for Quanterix (uncorrected $p = 0.096$, corrected $p = 0.005$), and -9.5% for ELISA (uncorrected $p = 0.008$, corrected $p = 0.025$). There were no differences in plasma Abeta $_{40}$ levels between AD patients and controls after correction for the indicated covariates: Amyblood; uncorrected: $p = 0.023$; corrected: $p = 0.98$, Quanterix: uncorrected: $p = 0.16$, corrected: $p = 0.96$, ELISA: uncorrected: 0.06, corrected: 0.62 (**eTable 4, eFigure 6**). The Amyblood Abeta $_{1-42/1-40}$ ratio was decreased by -21% in AD patients compared to controls ($p < 0.001$ uncorrected, corrected $p < 0.001$). The Quanterix Abeta $_{x-42/x-40}$ ratio was decreased by -13% (uncorrected $p = 0.001$, corrected $p < 0.001$). The ELISA Abeta $_{1-42/1-40}$ ratio was decreased by -22% (uncorrected $p = 0.004$, corrected $p = 0.24$). Excluding a Abeta $_{1-42/1-40}$ ELISA outlier that was 3.9 times the median ratio for controls from the statistical analysis changed the p-values for the ELISA analyses (uncorrected $p < 0.001$, corrected $p = 0.06$) (Fig. 4).

Correlations of results of the three immuno-assays

The plasma Amyblood Abeta $_{42}$ concentrations correlated with the Quanterix and ELISA Abeta $_{42}$ results ($\rho > 0.48$, $p < 0.001$) (Fig. 5A and B). Amyblood Abeta $_{40}$ concentrations correlated with the Quanterix and

ELISA Abeta₄₀ results ($\rho > 0.74$, $p < 0.001$) (Fig. 5C **and D**). The Amyblood Abeta_{42/40} ratio correlated with the Quanterix ratio ($\rho = 0.68$, $p < 0.001$), not with the ELISA ratio ($\rho = -0.01$, $p = 0.95$) (Fig. 5E **and F**).

The CSF Abeta₁₋₄₂ concentrations did not correlate with plasma Abeta₄₂ measured with Amyblood ($\rho = 0.10$, $p = 0.36$), Quanterix ($\rho = 0.11$, $p = 0.32$) or ELISA ($\rho = 0.25$, $p = 0.09$), whereas the CSF Abeta₁₋₄₂ concentrations did correlate with the Abeta_{42/40} ratio, measured with Amyblood ($\rho = 0.25$, $p = 0.02$), Quanterix ($\rho = 0.28$, $p = 0.01$) and ELISA ($\rho = 0.36$, $p = 0.001$).

Discussion

With this study we introduce the novel Amyblood assays developed on ultrasensitive Simoa technology for detection of the specific N-terminal Abeta peptides Abeta₁₋₄₂ and Abeta₁₋₄₀ in plasma. The validation was successful and the results were highly comparable to technical and clinical validation results obtained for two commercially available assays: the Quanterix triplex and Euroimmun ELISA. The specificity of the Amyblood assays for the specific isoforms was higher compared to the Quanterix assay. Moreover, the Amyblood Abeta_{1-42/1-40} ratio could successfully differentiate AD cases from controls, similar to the Quanterix assay. The ELISA ratio could not differentiate the groups, though the ELISA Abeta₁₋₄₂ concentration by itself could. Our validation data show that the Amyblood assays are suitable for robust measurement of 1-42 and 1-40 amyloid isoforms in plasma.

The development of the Amyblood assays was motivated by the need for an assay that simultaneously offers high specificity, high sensitivity and high throughput. Therefore, we employed the known specificity of the ADx102, ADx103, and ADx101 antibodies as demonstrated earlier in CSF,^{19,20} together with the sensitivity of the Simoa technology.¹¹ The promising prototype assay results (**eFigure 3**) urged us to upscale for widespread validation. Upscaling is challenging since production of larger stock volumes can influence the reagent performance and may affect the sensitivity. Therefore, performance of the prototype and upscaled batch were thoroughly tested. Upscaling did not affect the calibrator curve, reproducibility was demonstrated at Amsterdam UMC and ADx (variation < 20%), and lastly, the assay remained successful in differentiating 20 AD patients and 20 controls ($p < 0.001$). These results indicate that the transformation of our initial prototype to the RTU Amyblood assays was successful.

Full length Abeta₁₋₄₂ and Abeta₁₋₄₀ are the most abundant Abeta proteoforms in AD brain and CSF.^{19,21} These proteoforms are more soluble and more toxic than N-terminal truncated proteoforms, and are therefore important therapeutic targets.²¹ This stresses the need for highly specific amyloid assays that can proof target engagement in clinical trials. Our specificity analyses showed that the Amyblood Abeta₁₋₄₂ assay showed minor cross-reactivity for other proteoforms in sample buffer. At 60 pg/ml Abeta₁₋₄₀ in sample diluent, a non-specific concentration of 2.4 pg/mL was measured with the Amyblood Abeta₁₋₄₂ assay. However, this nonspecific signal could be clinically meaningful, being similar to the group difference of also 2.4 pg/mL Abeta₁₋₄₂ as observed between AD and controls. With the Quanterix Abeta_{x-42} assay, a non-specific signal of 0.9 pg/mL was read at 40 pg/mL Abeta₁₋₄₀, which is three

times the group difference of 0.3 pg/mL Abeta_{x-42} as measured with this assay. In addition, a minor increase of 0.8 fold the group difference was measured with the Amyblood Abeta₁₋₄₂ assay when Abeta₃₋₄₂ in sample buffer was incubated, where a large increase of 80 fold the group difference was measured with the Quanterix Abeta_{x-42} assay for this proteoform. The difference in assay specificity could be explained by the Amyblood N-terminal ADx102 (21F2) antibody that is specific for the first amino acid of the Abeta peptide, whereas the Quanterix assay detects N-terminal amino acid 4–10. However, our specificity experiments were performed in sample buffer, whereas a wide variety of endogenous proteins are present in plasma which would make low affinity non-specific binding less likely.²² Indeed, our selectivity analyses showed a recovery closer to 100% for Amyblood Abeta₁₋₄₂ for higher Abeta₁₋₄₂ concentrations next to the presence of varying concentrations of Abeta₁₋₄₀ peptide. At physiological concentrations of Abeta₁₋₄₂ and Abeta₄₀ in buffer, the %recovery was 125% for Abeta₁₋₄₂. For the Quanterix assay, the Abeta_{x-42} recovery was higher (163%) at physiological concentrations. Our data suggest that the Amyblood Abeta₁₋₄₂ assay had better specificity and selectivity than the Quanterix Abeta_{x-42} assay. Finally, there was no cross reactivity for both Abeta₄₀ assays, indicating the high specificity of the 2G3 antibody for Abeta₄₀ employed in both formats.

The Amyblood and Quanterix assays could successfully differentiate CSF Abeta₁₋₄₂ positive AD patients and Abeta₁₋₄₂ negative controls. The ELISA ratio could not differentiate AD from controls, but the ELISA Abeta₁₋₄₂ concentration alone could. Interestingly, a recent study did show promising results with the Abeta_{42/40} ratio measured with yet another ELISA assay and similarly showed a reduction in Abeta PET positive cases in a large cohort of controls, MCI and AD. This was against the expectations for ELISA assays being less sensitive towards low protein concentrations.²³ Similar to other studies, we found no correlation between CSF Abeta₁₋₄₂ and plasma Abeta₄₂ concentrations, and a weak correlation with the plasma Abeta_{1-42/1-40} ratio.^{5,6} An explanation could be that amyloid produced peripherally, for example by platelets,²⁴ distorts the association of Abeta measured in plasma with Abeta produced only by the central nervous system, as measured in CSF.

It has been suggested that SCD cases have a higher risk of converting to AD compared to healthy elderly controls.²⁵ We carefully selected controls with normal CSF biomarker values, who are not likely to convert.²⁶ We wish to stress that the focus of our study was to compare analytical performance and clinical samples were included to indicate potential clinical value, since other studies are on their way on early diagnostic use of the Amyblood assays. Our positive results show that the assay is suitable for diagnostic studies.

A next step in the development of these assays is to leverage the multiplexing possibilities of Simoa technology and simultaneously detect multiple biomarkers, to reflect different aspects of AD within one assay run, saving time and resources. In addition, development of Certified Reference Material is critical to calibrate and compare different amyloid assays and to enable clinical implementation. In collaboration with biotechnology companies, the assay will be made available on a global level.

Conclusions

We have developed an exceptionally specific blood test that measures full length Abeta₁₋₄₂ and Abeta₁₋₄₀ using high throughput semi-automated ultrasensitive technology. This study shows that the Amyblood assay has the potential to specifically and sensitively measure the concentrations of full length Abeta₁₋₄₂ and Abeta₁₋₄₀, and as such could be a specific test for target engagement in future clinical trials.

Abbreviations

Abeta = Amyloid beta

AD = Alzheimer's disease

AEB = average enzyme per bead

AUC = area under the curve

CSF = Cerebrospinal fluid

LLOQ = Lower Limit of Quantification

MCI = Mild Cognitive Impairment

PET = Positron Emission Tomography

RTU = ready to use

SCD = Subjective Cognitive Decline

Declarations

Ethical Approval and Consent to participate

Written consent to use medical data and biomaterials for research purposes was in place, in accordance with the ethical committee of the Amsterdam UMC, location VUmc, and with the Helsinki Declaration act of 1975.

Consent for publication

All authors approved this manuscript for publication

Availability of supporting data

The datasets used and analyzed during the current study can be made available by the corresponding author upon reasonable request

Competing interests

None of the authors have competing interests with regard to this manuscript.

Funding

Elisabeth Thijssen is supported by a TKI research grant from Health Holland (LSHI17001). Inge Verberk is supported by research grants Gieskes-Strijbis Fonds and Alzheimer Nederland (NL-17004). Charlotte Teunissen is supported by the European Commission (Marie Curie International Training Network, JPND), the Dutch Research Council (ZonMW), The Weston Brain Institute, Alzheimer Netherlands. Wiesje van der Flier holds the Pasman chair, and is recipient of NCDC, which is funded in the context of Deltaplan Dementie from ZonMW Memorabel (projectnr 73305095005) and Alzheimer Nederland. Research of the Alzheimer Center Amsterdam is part of the neurodegeneration research program of Amsterdam Neuroscience. This research is performed in close collaboration with ADx NeuroSciences, Ghent, Belgium. Funding agencies had no role in the design and conduct of the study, collection, management, analysis or interpretation of the data, preparation, review or approval of the manuscript or decision to submit the manuscript for publication.

Authors' contributions

ET, IV, ES, HV, and CT contributed to study concept and design. All authors contributed to data acquisition and/or analysis. ET, IV, JV, HV, and ES contributed to Amyblood kit manufacturing. ET, IV, and CT contributed to drafting the text and figures. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

References

1. Patterson, C. World Alzheimer report 2018. *Alzheimer's Disease International (ADI), London* (2018).
2. Rabinovici, G. D. *et al.* Association of Amyloid Positron Emission Tomography With Subsequent Change in Clinical Management Among Medicare Beneficiaries With Mild Cognitive Impairment or Dementia. *Jama* **321**, 1286-1294, doi:10.1001/jama.2019.2000 (2019).
3. Jack, C. R., Jr. *et al.* NIA-AA Research Framework: Toward a biological definition of Alzheimer's disease. *Alzheimers Dement* **14**, 535-562, doi:10.1016/j.jalz.2018.02.018 (2018).

4. Lewczuk, P. *et al.* Cerebrospinal Fluid A β 42/40 Corresponds Better than A β 42 to Amyloid PET in Alzheimer's Disease. *Journal of Alzheimer's Disease* **55**, 813-822, doi:10.3233/jad-160722 (2016).
5. Janelidze, S. *et al.* Plasma beta-amyloid in Alzheimer's disease and vascular disease. *Sci Rep* **6**, 26801, doi:10.1038/srep26801 (2016).
6. Verberk, I. M. W. *et al.* Plasma Amyloid as Prescreener for the Earliest Alzheimer Pathological Changes. *Ann Neurol* **84**, 648-658, doi:10.1002/ana.25334 (2018).
7. Ovod, V. *et al.* Amyloid beta concentrations and stable isotope labeling kinetics of human plasma specific to central nervous system amyloidosis. *Alzheimers Dement* **13**, 841-849, doi:10.1016/j.jalz.2017.06.2266 (2017).
8. Nakamura, A. *et al.* High performance plasma amyloid-beta biomarkers for Alzheimer's disease. *Nature* **554**, 249-254, doi:10.1038/nature25456 (2018).
9. Li, W. W. *et al.* Brain Amyloid-beta Deposition and Blood Biomarkers in Patients with Clinically Diagnosed Alzheimer's Disease. *J Alzheimers Dis* **69**, 169-178, doi:10.3233/JAD-190056 (2019).
10. Palmqvist, S. *et al.* Performance of Fully Automated Plasma Assays as Screening Tests for Alzheimer Disease-Related beta-Amyloid Status. *JAMA Neurol*, doi:10.1001/jamaneurol.2019.1632 (2019).
11. Rissin, D. M. *et al.* Single-molecule enzyme-linked immunosorbent assay detects serum proteins at subfemtomolar concentrations. *Nat Biotechnol* **28**, 595-599, doi:10.1038/nbt.1641 (2010).
12. QuanterixCorp. Whitepaper 5.0 - Enabling Ultrasensitive Protein Quantification with the Quanterix Homebrew Kit. (2014).
13. K. Johnson-Wood, M. L., R.Motter, K.Hu, G.Gordon, R. Barbour, K.Khan, M.Gordon, H. Tan, D. Games, I. Lieberburg, D. Schenk, P. Seubert*, AND L. McConlogue. Amyloid precursor protein processing and Ab42 deposition in a transgenic mouse model of Alzheimer disease. *The National Academy of Sciences of the USA* **94**, 1550– 1555 (1997).
14. Baghallab, I., Reyes-Ruiz, J. M., Abulnaja, K., Huwait, E. & Glabe, C. Epitomic Characterization of the Specificity of the Anti-Amyloid Abeta Monoclonal Antibodies 6E10 and 4G8. *J Alzheimers Dis* **66**, 1235-1244, doi:10.3233/JAD-180582 (2018).
15. van der Flier, W. M. & Scheltens, P. Amsterdam Dementia Cohort: Performing Research to Optimize Care. *J Alzheimers Dis* **62**, 1091-1111, doi:10.3233/JAD-170850 (2018).
16. Jessen, F. *et al.* A conceptual framework for research on subjective cognitive decline in preclinical Alzheimer's disease. *Alzheimers Dement* **10**, 844-852, doi:10.1016/j.jalz.2014.01.001 (2014).
17. Mulder, C. *et al.* Amyloid-beta(1-42), total tau, and phosphorylated tau as cerebrospinal fluid biomarkers for the diagnosis of Alzheimer disease. *Clin Chem* **56**, 248-253, doi:10.1373/clinchem.2009.130518 (2010).
18. Tijms, B. M. *et al.* Unbiased Approach to Counteract Upward Drift in Cerebrospinal Fluid Amyloid-beta 1-42 Analysis Results. *Clin Chem* **64**, 576-585, doi:10.1373/clinchem.2017.281055 (2018).

19. Struyfs, H. *et al.* Diagnostic Accuracy of Cerebrospinal Fluid Amyloid-beta Isoforms for Early and Differential Dementia Diagnosis. *J Alzheimers Dis* **45**, 813-822, doi:10.3233/JAD-141986 (2015).
20. Vanderstichele, H. M. *et al.* Optimized Standard Operating Procedures for the Analysis of Cerebrospinal Fluid Abeta42 and the Ratios of Abeta Isoforms Using Low Protein Binding Tubes. *J Alzheimers Dis* **53**, 1121-1132, doi:10.3233/JAD-160286 (2016).
21. Wildburger, N. C. *et al.* Diversity of Amyloid-beta Proteoforms in the Alzheimer's Disease Brain. *Sci Rep* **7**, 9520, doi:10.1038/s41598-017-10422-x (2017).
22. Kratz, A., Ferraro, M., Sluss, P. M. & Lewandrowski, K. B. Case records of the Massachusetts General Hospital. Weekly clinicopathological exercises. Laboratory reference values. *The New England journal of medicine* **351**, 1548-1563, doi:10.1056/NEJMcpc049016 (2004).
23. Doecke, J. D. *et al.* Total Abeta42/Abeta40 ratio in plasma predicts amyloid-PET status, independent of clinical AD diagnosis. *Neurology* **94**, e1580-e1591, doi:10.1212/WNL.0000000000009240 (2020).
24. Humpel, C. Platelets: Their Potential Contribution to the Generation of Beta-amyloid Plaques in Alzheimer's Disease. *Curr Neurovasc Res* **14**, 290-298, doi:10.2174/1567202614666170705150535 (2017).
25. Slot, R. E. R. *et al.* Subjective cognitive decline and rates of incident Alzheimer's disease and non-Alzheimer's disease dementia. *Alzheimers Dement* **15**, 465-476, doi:10.1016/j.jalz.2018.10.003 (2019).
26. Ebenau, J. L. *et al.* ATN classification and clinical progression in subjective cognitive decline: The SCIENCE project. *Neurology* **95**, e46-e58, doi:10.1212/WNL.0000000000009724 (2020).

Figures

Initial clinical performance comparison of Amyblood and Prototype Abeta_(1-42/1-40)

Figure 1

Abeta1-42/1-40 ratio measured in AD and control samples with the Amyblood and Prototype assays. Comparison of the prototype and Amyblood assays was based on measuring a feasibility sample set of 40 samples, 20 CSF Abeta1-42 positive AD cases and 20 CSF Abeta1-42 negative controls, measured in duplicate in both assays. The average Abeta1-42/1-40 ratio measured with the Amyblood assays were 0.151 ± 0.022 for controls and 0.116 ± 0.026 for AD, the average Abeta1-42/40 ratio measured with the prototype was 0.139 ± 0.015 for controls and 0.100 ± 0.017 for AD.

Figure 2

Specificity of the Amyblood and Quanterix assays A. Amyblood Abeta1-42 concentrations were measured above blank reading for all abeta fragments, with a maximum of 2.4 pg/mL for 60 pg/mL of Abeta1-40 for the fragments other than Abeta1-42. B. Quanterix Abetax-42 signal were measured above blank for Abeta2-42, and Abeta3-42, with a maximum of 24.7 pg/mL for 40 pg/mL of Abeta3-42 C. No increase in Amyblood Abeta1-40 signal was measured above blank reading for any of the Abeta fragments other than Abeta1-40. D. No increase in Quanterix Abetax-40 concentration was measured above blank reading for any of the Abeta fragments other than Abeta1-40. Dashed line represents the LLOQ per assay.

Figure 3

Selectivity of the Amyblood and Quanterix amyloid assays A. The highest %recovery measured with the Amyblood Abeta₁₋₄₂ assay was 125% in the presence of 6 pg/mL Abeta₁₋₄₂ and 15 pg/mL Abeta₁₋₄₀. B. The highest %recovery measured with the Quanterix Abeta_{x-42} assay was 1021% in the presence of 1 pg/mL Abeta₁₋₄₂ and 130 pg/mL Abeta₁₋₄₀. C. The highest %recovery measured with the Amyblood Abeta₁₋₄₀ assay was 110% in the presence of 3 pg/mL Abeta₁₋₄₀ and 6 pg/mL Abeta₁₋₄₂. D. The highest %recovery measured with the Quanterix Abeta_{x-40} assay was 115% in the presence of 130 pg/mL Abeta₁₋₄₀ and 15 pg/mL Abeta₁₋₄₀. The concentrations in buffer nearest to physiological Abeta₁₋₄₂ and Abeta₁₋₄₀ combinations in plasma as measured with this assay are indicated with an asterisk.

Figure 4

The plasma Abeta42/40 ratio measured with three different immunoassays Plasma Abeta42/40 ratio's measured in CSF Abeta1-42 negative controls and CSF Abeta1-42 positive AD groups. A. Abeta1-42/1-40 measured with the Amyblood assays. B. Abeta_x-42/_x-40 measured with the Quanterix triplex. C. Abeta1-42/1-40 measured with the Euro-immun ELISA assays. P-values are corrected for sample batch, sample storage time, age, and sex.

Figure 5

Correlations between Abeta42, Abeta40, and the ratio measured with Amyblood, Quanterix and ELISA A, B. Abeta42 concentrations measured with Amyblood were correlated with Quanterix and ELISA. C, D. Abeta40 concentrations measured with Amyblood were correlated with Quanterix and ELISA. E, F. The plasma Abeta42/40 ratio measured with the Amyblood and Quanterix assays correlated. The plasma Abeta42/40 ratio measured with ELISA did not correlate with Amyblood. The Abeta42/40 outlier in ELISA has been excluded from the figure for visualization, but was included in the calculated correlation coefficient. All correlations coefficients are Spearman's rho.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [20200904LTSupplement.docx](#)