Let's not forget: Peptidases in Alzheimer’s disease
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QUENCH Aβ PEPTIDE DEGRADATION AS A DIAGNOSTIC AND PROGNOSTIC MARKER FOR EARLY SPORADIC ALZHEIMER’S DISEASE

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QUENCHED Aβ PEPTIDE DEGRADATION AS A DIAGNOSTIC AND PROGNOSTIC MARKER

ABSTRACT

Alzheimer’s disease (AD) affects as a progressive neurodegenerative disease 35 million people worldwide. This number is expected to grow, raising the need for a cure and a way to diagnose AD. The definite diagnosis of AD relies on post mortem analysis of plaques and neurofibrillary tangles, however, to modify or stop disease progression preclinical diagnosis of AD is necessary. Therefore, biomarkers are needed that detect people at risk to develop AD. The amyloid cascade hypothesis states that Aβ accumulation is the initiator of AD pathology, probably caused by decreased clearance of the Aβ peptide. Based on this hypothesis, we investigated a quenched Aβ40 peptide that only becomes fluorescent upon degradation as a potential novel biomarker for early AD. We show that its degradation is significantly decreased in early Braak stages in post mortem cerebrospinal fluid (CSF) of AD patients, but not in blood plasma. In CSF this decrease was coinciding with a decline in insulin-degrading enzyme levels. However, in ante mortem CSF obtained by lumbar puncture, qAβ40 degradation did not correlate with clinical diagnosis and could not differentiate stable mild cognitive impaired (MCI) subjects from MCI subjects that converted to AD within 2 years. In conclusion, qAβ40 degradation was not proven to be a diagnostic or prognostic marker by itself, however combining the qAβ40 degradation assay with other biomarkers such as Aβ42 levels, increased diagnostic accuracy.

INTRODUCTION

Alzheimer’s disease (AD) is the most common neurodegenerative disorder currently affecting over 35 million people worldwide. Due to the growing world population and the fact that people reach a higher age, this number is expected to raise to 115 million in 2050 (Mapstone et al., 2014). No cures or disease-modifying therapies are available and clinical diagnosis is based on impairment of memory and other cognitive functions as measured with the MMSE (Mini-Mental State Examination) introducing a high misdiagnosis rate (Blennow et al., 2014). However, early detection and accurate diagnosis are crucial to modify or stop disease progression in an early stage before AD patients are too advanced to derive clinical benefit from treatment. Therefore, biomarkers are needed that can detect cognitively healthy people at risk to develop AD. Amyloid-β42 (Aβ42), total tau (t-tau) and phosphorylated tau (p-tau) proteins in cerebrospinal fluid (CSF) are well-established biomarkers for AD and are increasingly used for diagnosis in clinical practice (Vos et al., 2014). However, variability of CSF analyses between laboratories influence their diagnostic accuracy. In addition, increased levels of t-tau and p-tau in CSF are only detected from the MCI (mild cognitive impairment) stage onwards, when clinical symptoms are already apparent. The decline in Aβ42 already occurs in cognitively normal
individuals who later develop dementia but is not correlated with the severity of the disease in later stages (Hampel et al., 2014). Therefore, there is a need for more sensitive biomarkers to detect preclinical AD.

According to the amyloid cascade hypothesis, Aβ accumulation is the initiator of AD (Hardy and Allsop, 1991) and drives further AD pathogenesis such as tau aggregation, synaptic dysfunction and eventually neuronal death (Hardy and Selkoe, 2002; Tam and Pasternak, 2012; Stargardt et al., 2013). Several lines of evidence indicate that Aβ accumulation is caused by decreased clearance of the Aβ peptide (Qiu and Folstein, 2006; Zhao et al., 2007). To study alterations in Aβ clearance during AD progression, we developed a sensitive Aβ degradation assay by generating a quenched Aβ40 (qAβ40) peptide containing a small fluorescein group and a quenching dabcyl group (Stargardt et al., 2013). The qAβ40 only becomes fluorescent upon separation of the quencher and fluorophore, which occurs after degradation, enabling a direct measurement of Aβ clearance in tissues or body fluids. We have shown that degradation of the qAβ40 was already decreased in presymptomatic stages of sporadic AD in hippocampal brain tissue and that this decrease was correlated with decreased protein levels of the insulin-degrading enzyme (IDE), the main peptidase degrading the qAβ40 peptide (Stargardt et al., 2013).

In the present study we investigated whether decreased qAβ40 degradation can be detected in CSF and blood in preclinical stages of AD, thereby providing a potential diagnostic and prognostic tool for early AD.

**RESULTS**

**Decreased qAβ40 degradation and IDE protein levels in post mortem CSF of AD patients**

To investigate whether the ability to degrade qAβ40 is affected in CSF of AD patients, we examined post mortem CSF of control subjects and sporadic AD patients in different stages of the disease. Included were Braak stage 0 (control, no AD), Braak I-II (cognitively healthy but starting AD pathology in brain) and IV – VI (mild to severe AD). Information regarding the post mortem CSF samples is listed in Table 1. Fig. 1A (left panel, solid bars) shows that qAβ40 degradation was decreased already in Braak stage I (two-tailed t-test, \(P = 0.04\)), II (two-tailed t-test, \(P = 0.03\)), IV (two-tailed t-test, \(P = 0.03\)) and VI (two-tailed t-test, \(P = 0.02\)) when compared to Braak stage 0. Analysis of qAβ40 degradation in post mortem CSF from subjects with different amyloid scores ranging from O (no plaques), to C (severe plaque pathology) showed less efficient clearance of qAβ40 in amyloid score B and C (Fig. 1A, right panel). By grouping CSF samples based on the post mortem diagnosis of the
Figure 1 - qAβ40 degradation in CSF during the progression of sporadic AD.

(A) Left panel: qAβ40 degradation in post mortem CSF at different Braak stages, in the absence of inhibitor (solid bars) and in presence of the IDE inhibitor bacitracin (dotted bars). qAβ40 degradation is significantly decreased in Braak stages I, II, IV and VI compared to Braak stage 0. Error bars represent standard error of the mean (SEM) (*P < 0.05). Right panel: qAβ40 degradation in post mortem CSF at different amyloid scores. (B) Left panel: qAβ40 degradation is significantly decreased in post mortem CSF of AD patients compared to cognitively healthy control, grouped by their post mortem definite diagnosis. Right panel: qAβ40 degradation in subjects grouped by their clinical diagnosis: cognitively healthy control, mild cognitive impairment (MCI) and AD. Error bars represent SEM. (C) Upper panel: Western blot analysis of IDE in post mortem CSF at different Braak stages. Proteins were separated on a 7.5% SDS-PAGE gel. Lower panel: Western blot quantification of IDE levels in post mortem CSF at different Braak stages.
subjects (Braak stage 0: control versus Braak stage I-VI: AD) the rate of qAβ40 degradation was significantly different in cognitively healthy subjects compared to subjects with AD (two-tailed t-test, \( P = 0.0004 \)) (Fig. 2B, left panel). However, when CSF samples were grouped on the basis of the clinical diagnosis of the subjects, in which Braak stage I-II subjects are cognitively healthy controls, the qAβ40 degradation rate could not differentiate control subjects from Braak stage III – VI subjects that correspond to MCI and AD patients (Fig. 1B, right panel). To test for effects of age, sex, post mortem delay, pH and brain weight on qAβ40 degradation, Spearman correlation analysis and a Chi-square test were performed that did not show a correlation between these variables (Table 2). Together, our data show that in post mortem CSF the rate of qAβ40 degradation is decreased in Braak stages I-IV when compared to Braak stage 0, likely caused by a decrease in IDE levels and activity.

<table>
<thead>
<tr>
<th>Table 1 - Detailed donor information</th>
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<tbody>
<tr>
<td>Age</td>
</tr>
<tr>
<td>Br 0 (n = 6)</td>
</tr>
<tr>
<td>Br I (n = 6)</td>
</tr>
<tr>
<td>Br II (n = 7)</td>
</tr>
<tr>
<td>Br IV (n = 7)</td>
</tr>
<tr>
<td>Br V (n = 7)</td>
</tr>
<tr>
<td>Br VI (n = 6)</td>
</tr>
</tbody>
</table>

PMD, post mortem delay (hours); BW, brain weight (grams).

<table>
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<tr>
<th>Table 2 - Correlation subject variables with qAβ40 degradation</th>
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<tbody>
<tr>
<td>Spearman correlation</td>
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<tr>
<td>Post mortem delay (h)</td>
</tr>
<tr>
<td>Age at death</td>
</tr>
<tr>
<td>pH</td>
</tr>
<tr>
<td>Brain weight (grams)</td>
</tr>
</tbody>
</table>

Spearman correlation values and significance for correlation between qAβ40 degradation in post mortem CSF and post mortem delay (hours), age at death, pH and brain weight (grams).
No decrease in $\text{qA}\beta_{40}$ degradation in blood plasma of AD patients

To test whether the $\text{qA}\beta_{40}$ degradation assay can be used in blood plasma to detect early AD, we examined the degradation rates of $\text{qA}\beta_{40}$ in post mortem blood samples of control and subjects with sporadic AD covering different Braak stages of the disease. We analyzed most representative subjects in each Braak stage from our previous study (Stargardt et al., 2013). Detailed donor information is listed in Table 3. There was a high inter-individual variation and no correlation was found between $\text{qA}\beta_{40}$ degradation and the different Braak stages or amyloid scores (Fig. 2).

$\text{QA}\beta_{40}$ degradation in ante mortem CSF to predict AD development

A biomarker should distinguish cognitively healthy subjects at risk to develop AD from those not at risk to develop AD and predict further cognitive decline in MCI patients. To examine whether measurements of $\text{qA}\beta_{40}$ degradation meet these requirements, we analyzed $\text{qA}\beta_{40}$ degradation rates in 75 ante mortem CSF samples of subjects with subjective memory complaints (SMC, $n = 19$), MCI subjects that remained stable over a period of 2 years (MCI stable, $n = 18$), MCI subjects that converted to clinical AD-type dementia within a 2 year follow-up (MCI converter, $n = 18$), and subjects diagnosed with probable AD (Prob AD, $n = 20$). Detailed subject information is listed in Table 4. $\text{QA}\beta_{40}$ degradation showed a trend towards reduced degradation rates in CSF from subjects diagnosed with prob AD compared to SMC and MCI stable subjects (respectively $P = 0.11$ and $P = 0.05$; SMC mean: $4.25 \pm 1.34$; MCI stable mean: $4.36 \pm 1.24$; MCI converter mean: $4.07 \pm 1.42$; Prob AD mean: $3.69 \pm 0.79$; Fig. 3A). Furthermore, we analyzed $\text{qA}\beta_{40}$ degradation rates in subjects grouped by their AD profiles based on their CSF levels of $\text{A}\beta_{42}$ ($\leq 550 \text{ pg/mL}$), total tau ($t$-tau; $\geq 375 \text{ pg/mL}$) and phosphorylated tau ($p$-tau $\geq 52 \text{ pg/mL}$) (Duits et al., 2014). Fig. 3B shows less efficient $\text{qA}\beta_{40}$ degradation in subjects with the AD profile, which was however not significant (non-AD mean: $4.25 \pm 1.34$; AD mean: $3.8 \pm 0.99$). A Spearman correlation analysis showed that there was no significant correlation between CSF levels of $\text{A}\beta_{42}$, $t$-tau, $p$-tau, age and MMSE scores and a Chi-Square test showed no effect of sex (Table 5).

In summary, $\text{qA}\beta_{40}$ degradation seems slightly lower in ante mortem CSF of AD subjects, however, there was a high inter-individual variation within groups and could therefore not significantly differentiate stable MCI subjects from MCI subjects that converted to AD within 2 years. To assess whether a combination of the $\text{qA}\beta_{40}$ degradation assay with other potential biomarkers increases diagnostic accuracy, we performed a forward stepwise logistic regression analysis including all biomarkers ($\text{A}\beta_{42}$, $p$-tau, $t$-tau and $\text{qA}\beta_{40}$ degradation). The model including both $\text{A}\beta_{42}$ and $\text{qA}\beta_{40}$
degradation classified subjects with SMC and probable AD patients with a higher accuracy from subjects with SMC (94.9%, \( P = 0.01 \)) than without qAβ40 degradation (87.2%, \( P = 0.001 \)).

![Figure 2](image)

**Figure 2** - QAβ40 degradation in post mortem blood plasma during the progression of sporadic AD. Left panel: qAβ40 degradation in post mortem blood plasma of patients with different Braak stages. Right panel: qAβ40 degradation in post mortem blood plasma of patients with different amyloid scores. Error bars represent standard error of the mean (SEM).

### Table 3 - Detailed donor information

<table>
<thead>
<tr>
<th>Br 0 (n = 3)</th>
<th>Age ± SD</th>
<th>Sex</th>
<th>PMD ± SD</th>
<th>pH ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>64.0 ± 5.0</td>
<td>1F-2M</td>
<td>5.3 ± 0.1</td>
<td>6.7 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Br I (n = 2)</td>
<td>79.0 ± 3.6</td>
<td>0F-3M</td>
<td>7.7 ± 1.2</td>
<td>6.7 ± 0.6</td>
</tr>
<tr>
<td>Br II (n = 3)</td>
<td>78.0 ± 7.1</td>
<td>1F-2M</td>
<td>6.9 ± 0.1</td>
<td>6.3 ± 0.1</td>
</tr>
<tr>
<td>Br III (n = 3)</td>
<td>84.3 ± 1.5</td>
<td>1F-2M</td>
<td>7.0 ± 2.6</td>
<td>6.4 ± 0.2</td>
</tr>
<tr>
<td>Br IV (n = 3)</td>
<td>89.0 ± 3.6</td>
<td>3F-0M</td>
<td>6.2 ± 2.4</td>
<td>6.6 ± 0.4</td>
</tr>
<tr>
<td>Br V (n = 3)</td>
<td>81.7 ± 10.4</td>
<td>2F-1M</td>
<td>6.1 ± 2.4</td>
<td>6.4 ± 0.2</td>
</tr>
<tr>
<td>Br VI (n = 3)</td>
<td>82.0 ± 10.8</td>
<td>3F-0M</td>
<td>5.0 ± 0.7</td>
<td>6.4 ± 0.2</td>
</tr>
</tbody>
</table>

PMD, post mortem delay (hours).

### Table 4 - Detailed subject information

<table>
<thead>
<tr>
<th>Age ± SD</th>
<th>Sex</th>
<th>First MMSE score ± SD</th>
<th>Last MMSE score ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMC (n = 19)</td>
<td>61.4 ± 9.1</td>
<td>12F-8M</td>
<td>27.8 ± 1.2</td>
</tr>
<tr>
<td>MCI stable (n = 18)</td>
<td>66.0 ± 9.1</td>
<td>8F-12M</td>
<td>27.4 ± 1.5</td>
</tr>
<tr>
<td>MCI converter (n = 18)</td>
<td>65.8 ± 6.6</td>
<td>11F-9M</td>
<td>25.5 ± 3.0</td>
</tr>
<tr>
<td>Prob AD (n = 20)</td>
<td>64.5 ± 8.4</td>
<td>7F-13M</td>
<td>23.3 ± 5.5</td>
</tr>
</tbody>
</table>

SMC, subjective memory complaints; MCI, mild cognitive impairment; Prob AD, probable Alzheimer’s disease.
DISCUSSION

In the present study we investigated the potential of the qAβ\textsubscript{40} degradation assay as a diagnostic and prognostic tool to detect early sporadic AD. In our previous study we showed decreased degradation of qAβ\textsubscript{40} in post mortem hippocampal brain tissue of AD patients already in the first two Braak stages (Stargardt et al., 2013). In the present study, we investigated whether this decrease could also be detected in CSF and blood plasma. Since the definite diagnosis for AD and the determination of the Braak stages can only be performed post mortem, we investigated in post mortem blood plasma and
whether qAβ_{40} degradation rates and Braak stages were correlated. In post mortem blood plasma, qAβ_{40} degradation was not different between control subjects and AD patients. However, in post mortem CSF qAβ_{40} degradation was significantly decreased already in the early Braak stages and this decrease was associated with decreased IDE protein levels. Although IDE protein levels correlated with the progression of the disease, qAβ_{40} degradation rates were similar in Braak stages I, II, IV and VI. This suggests that in Braak stages I - VI peptidases other than IDE contribute to qAβ_{40} degradation in CSF, compensating for the increasing loss of IDE. This is different from what we observed in hippocampal tissue (Stargardt et al., 2013) where the decrease in IDE protein levels corresponded to decreased qAβ_{40} degradation rates, suggesting no compensation for the loss of IDE by other peptidases. However, this was investigated in hippocampal tissue, which is highly susceptible for AD pathology, and may explain the susceptibility of the hippocampus for Aβ accumulation as there may be no compensation by other Aβ degrading enzymes. It would be interesting to examine whether other brain areas can compensate for decreased levels of IDE by upregulating other peptidases during AD development, thereby limiting their susceptibility for Aβ accumulation.

When grouping subjects by their post mortem diagnosis, qAβ_{40} degradation was significantly decreased in subjects diagnosed with AD (Braak stage I - VI) compared to control subjects (Braak stage 0). However, clinical diagnosis is only possible when symptoms are apparent, starting at Braak stage III. Consequently, qAβ_{40} degradation was not significantly different between controls and AD subjects when grouped by clinical diagnosis. Also, changes in qAβ_{40} degradation in ante mortem CSF did not predict further cognitive decline in MCI patients. Although qAβ_{40} degradation may in theory allow for diagnosis of early, presymptomatic AD, we could not confirm this in ante mortem CSF samples. The reason may be that the definite post mortem diagnosis does not correspond with the clinical diagnosis in our cohort and ‘masks’ the correlation between qAβ_{40} degradation and disease progression. In addition, post mortem CSF was obtained from ventricles, whereas ante mortem CSF was obtained by lumbar puncture. Ventricular CSF is enriched in brain-derived proteins as compared to lumbar CSF (Lewczuk, 2014) what potentially explains why qAβ_{40} degradation differences between control subjects and subjects with AD are more pronounced in post mortem CSF.

In conclusion, our study shows a significant decrease in qAβ_{40} degradation rates in post mortem CSF of AD patients, but the qAβ_{40} degradation assay cannot be applies as a diagnostic or prognostic marker in ante mortem blood or CSF.
MATERIALS AND METHODS

Human post mortem CSF and blood plasma

Human post mortem CSF (ventricular) and blood plasma were obtained from the Netherlands Brain Bank (NBB; Amsterdam, The Netherlands). The donors have given informed consent for using the tissue and for accessing the extensive neuropathological and clinical information for scientific research, in compliance with ethical and legal guidelines (Huitinga et al., 2008). Included in the post mortem CSF study were Braak stage 0 \( (n = 6) \), I \( (n = 6) \), II \( (n = 7) \), IV \( (n = 7) \), V \( (n = 7) \) and VI \( (n = 6) \). Detailed subject information is presented in Table 1. Included in the post mortem blood plasma study were Braak stage 0 \( (n = 3) \), I \( (n = 3) \), II \( (n = 3) \), III \( (n = 3) \), IV \( (n = 3) \), V \( (n = 3) \) and VI \( (n = 3) \). Detailed subject information is listed in Table 3. All samples were matched as closely as possible for age, sex, post mortem delay and pH-CSF.

Human ante mortem CSF

CSF from different patient groups were included from the Alzheimer Centre Memory Cohort (van der Flier et al., 2014). Diagnosis was defined in a multidisciplinary meeting according to state of the art criteria, but excluding CSF biomarker results (van der Flier et al., 2014). Human ante mortem CSF was collected by lumbar puncture, centrifuged within 2 hours \( (1800 \times g, 10 \text{ min at room temperature}) \) and stored at -80°C in polypropylene tubes in agreement with consensus guidelines (Teunissen et al., 2009). CSF levels of Aβ42, t-tau and p-tau were measured with an InnoTest sandwich ELISA (Innogenetics, Ghent, Belgium) as described previously (Mulder et al., 2010). Detailed subject information is listed in Table 4.

Quenched Aβ40 degradation assay

HFIP-treated aliquots of the qAβ40 peptide were resuspended in DMSO followed by vortexing and sonication for 10 min, immediately before use. Lysates were diluted in KMH buffer \( (110 \text{ mM KAc, } 2 \text{ mM MgAc and } 20 \text{ mM Heps-KOH, pH 7.2}) \) and bacitracin (Sigma, St. Louis, MO, USA) was added to a final concentration of 200 µM followed by 30 min incubation at 4°C. Then, peptides were added to a final concentration of 340 nM in 50 µL total volume. Degradation of the qAβ40 peptides was analyzed at 37°C using the FLUOstar OPTIMA (BMG Labtec, Jena, Germany).
IDE protein levels on Western blot

Post mortem CSF samples (100 µL per sample) were concentrated by centrifugation in a SpeedVac (Savant, Hicksville, NY, USA) for 1.5 hours. 6 x SDS-sample buffer (350 mM Tris-HCl pH 6.8, 10% SDS, 6% β mercaptoethanol, 30% glycerol and 0.02% Bromphenol blue) was added to the concentrated CSF samples and heated for 5 min at 95°C. Samples were separated on 7.5% SDS-PAGE gels. After electrophoresis, proteins were transferred onto a nitrocellulose membrane. Blots were blocked in 5% milk in TBS and incubated with the primary antibody against IDE (1:500; Abcam, Cambridge, UK) overnight at 4°C. Subsequently, membranes were washed and incubated with the secondary antibody IRDye 680 (1:15.000; LI-COR Biosciences, Lincoln, NE, USA) for 1.5 hours at room temperature. Signal was detected using an Odyssey imaging system (LI-COR, Lincoln, NE, USA). Protein bands were quantified using ImageJ software (Developed at National Institutes of Health, http://rsb.info.nih.gov/ij).

Statistical analysis

Statistical analyses were performed with the SPSS version 19.0 software (Chicago, IL, USA). Nonparametric Student's t-tests were used for 2 groups analysis. Correlation analysis was performed using Spearman test. In addition, logistic regression was performed using the forward stepwise method to assess whether a combination of markers resulted in a higher diagnostic accuracy. P-values < 0.05 were considered significant.

ACKNOWLEDGEMENTS

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