Serum beta-secretase 1 (BACE1) activity as candidate biomarker for late-onset Alzheimer's disease

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Abstract Beta-secretase (BACE1) is a key enzyme in the formation of amyloid- β ; its activity/concentration is increased in brain and cerebrospinal fluid of patients with late-onset Alzheimer's disease (LOAD). Since BACE1 was found also in blood, we evaluated its potential as peripheral biomarker. To this aim, serum BACE1 activity was assessed in 115 subjects with LOAD and 151 controls. We found that BACE1 changed across groups ($p < 0.001$) with a 25% increase in LOAD versus controls. High levels of BACE1 (IV quartile) were independently associated with the

diagnosis of LOAD (OR 2.8; 1.4–5.7). Diagnostic accuracy was 76% for LOAD. Our data suggest that increased BACE1 activity in serum may represent a potential biomarker for LOAD. Additional studies are needed to confirm the usefulness of BACE1, alone or in combination with other markers, in discriminating patients and predicting LOAD onset and progression.

Keywords Late-onset Alzheimer's disease · Mild cognitive impairment · Mixed dementia · Blood-based biomarkers · BACE1

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Introduction

Currently, the diagnosis of late-onset Alzheimer's disease (LOAD) mainly relies on clinical criteria and, despite intense research, only a few cerebrospinal fluid (CSF) biomarkers have shown an acceptable performance in terms of diagnosis/prognosis (Zetterberg 2019). However, lumbar puncture remains an invasive and costly collection technique, especially considering the age of the patients to be examined.

By definition, an ideal disease-related biomarker should be easily accessible and measurable, inexpensive, time-effective, quantitative, and informative of the pathophysiological processes. Of these criteria, blood (serum/plasma) and urine represent more suitable biological sources of potential biomarkers compared to CSF. On the other hand, CSF biomarkers should better reflect the pathophysiology of diseases affecting the central nervous system (CNS) compared with those detected in the periphery (Thambisetty and Lovestone 2010; Zetterberg 2019). A peripheral biomarker could be a surrogate biomarker for LOAD diagnosis/prognosis if (1) it is involved in the pathogenesis of the disease, (2) its level in the brain is altered in association with disease, and (3) it can cross the blood–brain barrier and/or is a protein that is expressed and secreted in non-CNS tissues/cells.

Converging evidence from postmortem brain and CSF clearly suggests that beta-secretase (BACE1) (Bruford et al. 2008) could fulfill these criteria. This aspartyl protease actively participates in the formation of the amyloid- β (A β) peptide, which in turn plays a central role in LOAD neuropathogenesis. A β is generated from the sequential enzymatic cleavage of the amyloid precursor protein (APP) by enzymes bearing β - and γ -secretase activities (Vassar et al. 1999). Notably, BACE1 catalyzes the rate-limiting step of the amyloidogenic process; moreover, unlike other β -secretases, BACE1 is mainly expressed in the brain. Consistent with the role of BACE1 in AD (Luo et al. 2001), it was shown that the production of A β was abolished in BACE1 knockout mice, whereas its over-expression in APP transgenic mice led to increased A β levels (Bodendorf et al. 2002). The protein and activity of BACE1 was found to be higher in the brain (frontal cortex) of AD patients compared to that in controls (Fukumoto et al. 2002). Subsequently, altered levels of BACE1 were found also in the CSF of AD patients (Zetterberg et al. 2008), as well as subjects with mild

cognitive impairment (MCI) (Zhong et al. 2007). Finally, BACE1 might fulfill the third aforementioned requirement for a possible peripheral biomarker for AD; although it is expressed mainly in the brain, the protease is also expressed and potentially secreted in systemic circulation in several non-CNS tissues including the pancreas, liver (Vassar et al. 1999; Decourt and Sabbagh 2011), and blood cells such as platelets (Johnston et al. 2008; Liu et al. 2009). Notably, in the largest study on platelet membrane BACE1, the enzyme's activity was shown to significantly increase in individuals with AD compared to that in age-matched controls (Johnston et al. 2008). However, the authors reported large variation in inter-individual activity, most likely due to the laborious procedure for platelet preparation. This high variability was not reported by the only, to the best of our knowledge, published work dealing with the measurement of BACE in blood (plasma) (Shen et al. 2018). This small-scale study showed a significant increase in its activity in patients with LOAD ($n = 75$) and with MCI ($n = 96$) converting to LOAD (Shen et al. 2018). Most likely, the uniqueness of this work is due to the technical shortcomings of BACE1 detection in a complex environment such as blood (Decourt and Sabbagh 2011).

The present study aimed to evaluate the validity of BACE1 as a biomarker for the diagnosis of LOAD. With this purpose, we optimized the assay methodology and investigated the serum activity of BACE1 in a sample of elderly individuals, including patients affected by LOAD and cognitively healthy controls.

Materials and methods

Study design

From a sample of 607 older subjects (>60 years) evaluated at the Memory Clinics of Casa Sollievo della Sofferenza, San Giovanni Rotondo, or the Department of Internal Medicine, S. Anna University Hospital, Ferrara (Italy), 279 were enrolled into the present study:

- 115 patients (73% females) with mild to moderate probable LOAD by the National Institute on Aging–Alzheimer's Association (NIA–AA) workgroups criteria (McKhann et al. 2011); Mini Mental State Examination (MMSE) range: 18–23; Clinical Dementia Rating (CDR) range: 1–2;

- 151 older subjects (52% females) without any signs or symptoms of cognitive impairment were included as controls; MMSE range: 26–30.

Five percent of these subjects ($n = 13$) refused to participate. Subjects with MCI, other forms of dementia, severe liver or kidney disease, congestive heart failure or chronic obstructive pulmonary disease, cancer, acute illnesses, or using non-steroidal anti-inflammatory drugs, antibiotics, or steroids at the time of recruitment were excluded ($n = 328$).

The research was conducted according to the Declaration of Helsinki (<http://www.wma.net>) and the European Guidelines for Good Clinical Practice (<http://www.ema.europa.eu>). All participants (and their caregiver, if demented) were informed about the research project and provided written consent. The study was approved by the local ethics committee of Casa Sollievo della Sofferenza, San Giovanni Rotondo (protocol no. 3877/DS) and the local ethics committee of Azienda Arcispedale S. Anna, Ferrara (protocol no. 170579). Personal data and anamnesis were collected by a structured interview from patients/caregivers. All patients underwent a general physical/neurological examination and neuropsychological assessment by a battery of tests, as previously described (Cervellati et al. 2015). All patients underwent a brain MRI (Philips Achieva 1.5 T) or a brain computed tomography scan (SIEMENS SOMATON HQ) when MRI was contraindicated or the patient's age was over 85 years. Clinical chemistry analyses were routinely performed to exclude causes of secondary cognitive impairment.

Criteria used for the diagnosis of diabetes, hypertension, and cardiovascular disease (CVD), and smoking classification (greater than or equal to 10 pack-year history of ever smoking) were described in details elsewhere (Cervellati et al. 2013).

Albumin, creatinine, C-reactive protein, hemoglobin, total cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) concentrations were measured by routine laboratory methods.

BACE1 assessment

Peripheral blood samples were collected by venipuncture into Vacutainer® tubes without anticoagulant after overnight fasting. After 30 min of incubation at room temperature, the blood samples were centrifuged at

4650×*g* for 20 min, and sera were collected and stored in single-use aliquots at -80°C until analysis.

Substrate synthesis

The substrate was designed following Grüninger-Leitch et al. (2002) with chemical modification of the C terminus. The BACE substrate SEVNLDAEFR was C-terminally labeled with the fluorescent group Luciferase Yellow (LY) and N-terminally labeled with the quenching group Dabsyl, forming a FRET donor–acceptor pair. Following the synthetic scheme described in Grüninger-Leitch et al. (2002), the Dabsyl moiety was inserted as a lysine side chain. On the contrary, the light-sensitive LY was added to the C terminus during the last step of the substrate synthesis. To do this, we adopted a convergent synthetic strategy in which the peptide sequence H-K(Dabsyl)SEVNLDAEFR-C-NH₂ and the maleimido-functionalized LY were synthesized and purified separately. The further Thiol–Michael reaction between the sulfhydryl group of the C-terminal Cys and the maleimide group of the LY provided the desired product with a quantitative yield and with high purity. Detailed information regarding the substrate synthesis is provided in the [Supplementary material](#) (Methods 1.1–1.3, Figs. 1–5).

BACE1 activity assay

The assay was carried out as previously described (Shen et al. 2018), with few modifications. Briefly, the substrate was dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 392 μM and stored in aliquots at -20°C up to 3 months. No substantial change in the basal signal was observed

Fig. 1 Serum BACE1 activity in controls and LOAD. LOAD patients had significantly higher activity of BACE1 compared to controls ($p < 0.001$), demonstrating almost 25% higher mean values

Fig. 2 Correlation between serum BACE1 activity and age in the whole sample and within controls or LOAD subsample. In the whole sample (a), BACE1 activity showed a significant

correlation with age ($r = 0.299$, $p < 0.001$). Considering subject subsamples, we observed a significant correlation in controls (b) ($r = 0.280$, $p < 0.001$) but not in patients with LOAD (c)

within the storage time. For the assay, the stock solution was diluted to a final concentration of 30 μ M in 50 mM acetate buffer, pH 4.5, and 0.1 M NaCl and 100 μ L were dispensed in triplicate to the wells of a black, flat-bottom microplate. After pre-incubation for 10 min at 37 $^{\circ}$ C, the reaction was started by the addition of 5 μ L of sample (wild-type enzyme or undiluted serum) in triplicate. The fluorescence was read every 30 s for 20 min using excitation and emission wavelengths of 430 nm and 520 nm, respectively, in a Tecan Infinite M200 (Tecan Group, Switzerland) microplate reader. The reaction rates were converted from relative fluorescence units (RFU) per

minute to enzyme units (U) by interpolation with a standard curve constructed using known concentrations of the wild-type enzyme (β -secretase human; Sigma-Aldrich, Cat. No. S4195).

Statistical analysis

Continuous variables were tested for normality using Kolmogorov–Smirnov and Shapiro–Wilk tests. Comparisons between groups were performed using a t test and Mann–Whitney U test for variables with normal and non-normal distributions, respectively. Chi-squared tests were used to compare differences in categorical variables. Associations between variables of interest were analyzed by Pearson’s and Spearman’s correlation according to their normal/non-normal distribution. Multivariable logistic regression analysis (using the highest quartile value as cut-off) was performed to determine the impact of multiple potential confounders on the relationship between the variables of interest. A receiver operating characteristic (ROC) curve was performed to evaluate the diagnostic accuracy of the examined biomarker in discriminating controls from diseased patients. GraphPad Prism (version 5) was used for the determination of the Michaelis–Menten constant (K_m) for the substrate using non-linear regression. Two-tailed probability values <0.05 were considered statistically

Fig. 3 Serum BACE1 activity in controls and LOAD after exclusion of older subjects. After exclusion of older subjects, BACE1 activity was still significantly higher in LOAD

Fig. 4 Levels of serum BACE1 activity in controls and LOAD, among women (left) and men (right). BACE1 activity was significantly (and similarly) higher in LOAD compared to controls ($p < 0.001$) in both men and women subsamples

significant. SPSS 17.00 for Windows (Chicago, Illinois, USA) was used for statistical analysis.

Results

Assay optimization

The Michaelis–Menten constant of the synthesized substrate was evaluated by incubating different concentrations of substrate in the range 0–40 μM with a fixed amount of recombinant enzyme. As reported by the analysis software, the calculated value of K_m was 17 μM (standard error, 12 μM); therefore, for further experiments, we chose to use a substrate concentration of 30 μM as optimal, which showed a good linearity within the timeframe of the assay.

Fig. 5 Levels of serum BACE1 activity in controls and LOAD, after exclusion of subjects with comorbidities. After exclusion of subjects with comorbidities (hypertension, diabetes, or CVD), BACE1 activity was still significantly higher in LOAD ($p < 0.001$) compared to controls. As reported in the text, the mean age of the groups considered in this analysis were comparable to each other

Demographics

The principal characteristics of the 266 subjects enrolled in the study are reported in Table 1. Controls were younger ($p < 0.001$) and presented a lower prevalence of female gender compared with LOAD. Comorbidities also differed between the groups, with controls having a lower prevalence of hypertension, diabetes, and CVD compared to LOAD patients.

BACE1 activity levels across the study groups

To test the hypothesis that BACE1 serum activity was increased in patients with LOAD compared to controls, we compared the serum levels of the enzyme in the two groups.

As shown in Table 1 and Fig. 1, BACE1 activity was found to be significantly (around 25%) increased in LOAD compared to that in controls ($p < 0.001$).

Effect of age and other potential confounding factors on BACE1 activity

As the second step of our investigation, we evaluated the influence of potential confounding factors on the association between BACE1 serum activity and cognitive diagnosis. Age was the most important among putative confounders, because (1) it is the foremost risk factor for dementia, (2) it significantly differed between groups, and (3) it was positively associated with BACE1 in the whole sample ($r = 0.299$, $p < 0.001$; Fig. 2a), although the relationship was substantially driven by controls ($r = 0.283$, $p < 0.001$; Fig. 2b). In order to eliminate the

Table 1 Principal characteristics of the sample according to diagnosis

	Controls (<i>n</i> = 151)	LOAD (<i>n</i> = 115)
Age (years)	73 ± 5	79 ± 5*
Female gender (%)	52	73*
Formal education (years)	11 (8–13)	5 (3–5)*
MMSE score (/30)	27 (26–30)	20 (18–23)*
Current smoker (%)	11	9
Hypertension (%)	44	69*
Diabetes (%)	6	13*
CVD (%)	5	9*
Total cholesterol (mmol/L)	5.6 (4.9–6.4)	5.5 (4.7–6.1)
Triglycerides (mmol/L)	2.7 ± 1.0	2.9 ± 1.4
LDL-C (mmol/L)	3.5 (2.8–4.1)	3.3 (2.7–3.8)
HDL-C (mmol/L)	1.7 ± 0.5	1.6 ± 0.5
Glucose (mmol/L)	5.5 ± 1.2	5.3 ± 1
Hemoglobin (g/dL)	135 ± 17	133 ± 16
Albumin (g/L)	40 ± 4	39 ± 4
BACE (kU/L)	18.5 (15.2–22.3)	24.6 (20.5–32.1)*

Continuous variables are expressed as mean ± SEM or median (interquartile range). Categorical variables are expressed as percentage within group

* $p < 0.05$ vs. controls

CVD cardiovascular disease, MMSE Mini Mental State Examination

possible effect of age on BACE1 levels, we performed a further comparison between the groups after the exclusion of older subjects (age > 83 years); in this way, we obtained a subsample ($n = 200$ subjects) composed by groups with equivalent age (mean age—controls, 73 years; LOAD, 74 years; $p = 0.10$). As shown in Table 2 and Fig. 3, the between-group difference in BACE1 activity was highly similar to that observed in the whole sample.

The possible interfering effect of gender on BACE1 was also explored because of the significant difference in men/women prevalence in the two sample groups (Table 1). The enzyme activity was significantly higher in women in the whole population ($p < 0.01$) (Supplementary Fig. 6); despite this gender gap, the

difference in BACE1 activity between controls and LOAD was similar in women and men (Fig. 4a, b). In a similar fashion, BACE1 did not differ in subjects with hypertension, diabetes, or CVD, compared to those without the given comorbidity (data not shown). Moreover, in a subsample including only subjects free from comorbidity, BACE1 was still increased in LOAD compared to controls (Fig. 5).

Finally, multivariable logistic regression analysis was performed to evaluate the ability of BACE1 to discriminate LOAD from controls, after adjusting for all potential confounders (Table 3). The covariates included in the models were age, gender, smoking, hypertension, diabetes, and CVD. Compared with controls, high serum BACE1 levels (IV quartile value = 62.7 kU/L) were independently associated with an increase in the likelihood of being diagnosed LOAD.

Diagnostic accuracy analysis

To appreciate the diagnostic accuracy (assessed as area under the curve, AUC) of BACE1 activity, we performed ROC analysis for controls versus LOAD (Supplementary Fig. 7). We identified the best

Table 2 Levels of serum BACE1 activity in non-demented individuals (controls) and LOAD, after exclusion of older patients

	Controls (<i>n</i> = 151)	LOAD (<i>n</i> = 49)	<i>p</i> value
Age (years)	73 ± 5	74 ± 2	0.10
BACE (kU/L)	18.5 (15.2–22.3)	24.3 (20.8–29.3)	<0.001

Data are expressed as median (interquartile range)

Table 3 Odds ratios (95% CI) for diagnosis of LOAD in subjects with high levels (IV quartile) of serum BACE1 activity

		Adjusted for age and gender	Adjusted for age, gender, smoking, and comorbidities
LOAD	5.16 (3.03–8.79)*	3.36 (1.78–6.34)*	2.79 (1.37–5.67)†

Covariates included into the models: age, gender, arterial hypertension, cardiovascular disease, diabetes, and smoking habit

* $p < 0.001$, † $p < 0.01$

compromises between sensitivity and specificity for the diagnosis of LOAD with a cut-off of BACE1 activity of 20.7 kU/L; sensitivity was 70% while specificity was 75%, with an accuracy of 76%. The positive and negative predictive values were 65% and 78%, respectively. A higher sensitivity (90%, with a specificity of 41%) was obtained with a cut-off of 27.5 kU/L, while a higher specificity (90%, with a sensitivity of 42%) was obtained with a cut-off of 16.0 kU/L.

Discussion

The demand for new biological biomarkers of LOAD, less intrusive and more easily obtainable than those from CSF, is dramatically increasing. Blood-based biomarkers could be the best answer to this need; however, unlike CSF, blood is not in direct contact with the CNS and cannot provide a comprehensive representation of the various biochemical/metabolic changes occurring in this organ (Zetterberg 2019). This gap is filled when, as in the case of BACE1, the biomarker is a central player in the pathogenesis of the disease and is expressed and secreted also from various non-CNS tissues.

BACE1 is also present in plasma, as demonstrated by a recent study by Shen et al. (2018), which showed that BACE1 activity was higher in patients with AD and MCI compared to that in controls. As acknowledged by the authors of that study, further steps forward were warranted, in particular the validation in independent clinical cohorts to definitely establish the clinical utility of these findings. Our study extends upon the previous findings by examining a larger population (more than doubled, considering controls and AD). We found that LOAD had significantly higher levels of serum BACE1 activity compared to controls. Notably, this difference was independent of age, gender, and other risk factors for dementia, such as hypertension, diabetes, and CVD (including stroke).

The demonstration of the clinical relevance of BACE1 in the field of Alzheimer's disease (AD) clearly

emerged from the increasing number of pharmacological studies dealing with the development of effective inhibitors of brain BACE1 (Schipke et al. 2018). However, the large consensus on the therapeutic potential of BACE1 inhibition has been not corroborated by clinical trials. As recently highlighted by Schipke et al. (2018), drugs that efficiently lower brain A β levels do not improve cognitive function when administered to mild-to-moderate LOAD patients. Similar failures have been reported for other drugs and this has led to the belief that the treatment was initiated too late to be effective (Baazaoui and Iqbal 2018). Indeed, it is well known that LOAD-related brain changes, including formation and aggregation of A β , occur a long time before the appearance of clinical symptoms. Being the key enzyme in A β production, BACE1 could be more suitable as a therapeutic target in the pre-clinical stage. This idea also found support in the study of Shen et al. (2018) and our study on peripheral BACE1, since the enzyme activity was found to be already increased in MCI patients. In particular, this alteration was shown to be significant in those MCI subjects that converted to LOAD, but not in those who did not, suggesting a potential utility in the prediction of clinical progression (Shen et al. 2018).

Independently of the relative proportions of BACE1 activity in LOAD, the majority of published studies suggest that the enzyme is more active in LOAD. The mechanisms underlying this up-regulation in brain/CSF are not completely understood, but they are likely to include post-translational modifications or increased expression from the remaining functional neurons and/or astrocytes (Fukumoto et al. 2002). To explain the apparent parallel increase in BACE1 activity in the CNS and systemic circulation, we need to identify factors/abnormalities that are known to influence the enzyme catalytic activity and coexist in the brain and periphery of MCI and LOAD patients. Convergent evidence shows that oxidative stress might be a reliable candidate in this framework (Guglielmotto et al. 2009; Tamagno et al. 2012; Tan et al. 2013). First, there is a general

consensus that an imbalanced cellular redox state, leading to biomolecular oxidative damage, is an early event in LOAD pathogenesis and progression (Sultana and Butterfield 2009; Cervellati et al. 2015, 2016); fingerprints of oxidative challenge have been repeatedly found in the brain (Smith et al. 1997; Butterfield et al. 2006), but also in the periphery of patients in the pre-clinical or clinical stage of the disease (Schrag et al. 2013; Cervellati et al. 2014a, 2014b). Second, as shown by in vivo (Chen et al. 2008; Guglielmotto et al. 2009; Mouton-Liger et al. 2012) and in vitro (Guglielmotto et al. 2009) evidence, reactive oxygen species (ROS) generated by dysfunctional mitochondria mediate the up-regulation of BACE1 transcription and activity via a lipoperoxidation process. Notably, mitochondrial deficits seem to play a central role in LOAD, and it was shown that dysfunction in these primary sources of ROS is not isolated to neurons, but occurs systemically (Sultana and Butterfield 2009; Morris et al. 2014; Valacchi et al. 2018).

Finally, some important limitations of the study must be acknowledged. First, the study was cross-sectional, thereby precluding our ability to establish any temporal relationship between BACE1 and cognitive impairment/dementia. Second, we cannot exclude that biases or unmeasured confounders might limit the reliability of our findings. Third, the lack of age and sex matching and the different distribution in comorbidities between groups may have introduced biases. However, the between-group differences in BACE1 activity were still significant after adjustment for age and gender, as well as in a subsample of 200 subjects with comparable age (Table 2). Moreover, we showed that neither gender nor comorbidities appeared to significantly interfere with the results (Figs. 4 and 5, Supplementary Fig. 6). Fourth, the diagnosis of LOAD was based on the NIA-AA workgroups criteria; it would be appropriate to replicate our results in LOAD patients diagnosed with the support of abnormal biomarkers (structural neuroimaging or cerebrospinal fluid analysis).

Conclusion

In conclusion, data from the present study suggest that increased BACE1 activity in serum may represent a suitable candidate as diagnostic biomarker for LOAD. However, since multiple pathophysiological processes are activated in the clinical course of LOAD, both in

brain and periphery, it is questionable that a single blood-based biomarker might have satisfactory diagnostic and prognostic accuracy. Although we have provided interesting findings on this potential serum biomarker, additional studies are needed in order to confirm the usefulness of BACE1, most likely in combination with other markers, in discriminating patients and in predicting disease onset and progression.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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