Research Article

Serum concentration of beta amyloid peptide and the activity of angiotensin converting enzyme in alzheimer's disease patients: search for a potential biomarker

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ABSTRACT

Background: Alzheimer's disease (AD) is a disorder that affects mainly aged population. There is still no diagnostic test for the disease. Over the past few years investigators have studied several plasma biomarkers, most frequently, plasma beta amyloid peptide (Aβ). Level of Aβ depends on balance between production and clearance, accomplished by proteases. Our purpose was to study differences in the concentrations of Aβ's (and their ratios) and also in the activity of ACE in blood serum of AD patients and control groups.
Methods: We measured the blood levels of beta amyloid 40 and 42 (Aβ40 & Aβ42) and their proportions in AD patients' blood samples. We also measured the angiotensin converting enzyme (ACE) activities.
Results: This study showed that amounts of Aβ40 and Aβ42 in the blood serum of AD patients were significantly lower than that in control samples. The ratio of Aβ42/Aβ40 was not significantly different from controls. With respect to age and severity of disease we observed that Aβ40 concentration was lower in AD male patients and decreased with severity of AD. We also observed that serum Aβ42 concentration was decreased by increasing the age of female patients.
Conclusions: Our results indicated that the ACE activity was significantly higher in patients in comparison with normal individuals. Also, it was revealed that increase in age resulted in reduced ACE activity in females and increased activity in males. This study also showed there was a positive relationship between ACE activity and severity of disease.

Keywords: Alzheimer's disease, Blood serum, Beta amyloid peptide, Angiotensin converting enzyme

INTRODUCTION

Most people with Alzheimer's disease are diagnosed at age 65 or older. Almost two-thirds of the Americans suffering from AD are women. The estimated risk is 17.2 percent for women and nearly 9.1 percent for men.[1] AD is characterized by the deposition of beta amyloid peptide (Aβ) in amyloid plaques.[2] Recent evidences have shown that the toxicity of amyloid aggregates is highest when they are in the form of oligomers, formed mainly by Aβ42, rather than fibrils.[3,4]

There is still no definitive diagnostic test or biological risk marker for the disease. The criteria followed in most research studies are those proposed by the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ARDA) for defining AD.[5,6]

Over the past few years a number of investigators have studied several plasma biomarkers, most frequently, plasma amyloid beta (Aβ40 and Aβ42).[7-10] Aβ can also be found in cerebrospinal fluid (CSF) and neuronal

cultures.[11-13] $A\beta_{42}$ is far more amyloidogenic than $A\beta_{40}$ and contributes to early parenchymal $A\beta$ deposition as diffuse plaques, whereas $A\beta_{40}$ tends to deposit in amyloid plaque cores as well as in blood vessel walls.[14-15] Soluble $A\beta$ is removed, via interstitial fluid (ISF) bulk flow, into the bloodstream. On the other hand, $A\beta$ circulating in the blood can influx into and/or efflux from the brain, through a receptor-mediated transport, mechanism by which $A\beta$ crosses the blood brain barrier (BBB).[16-17]

There are a wide variety of methodologies for the measurement of products of cellular processes in CSF, blood plasma and serum as biomarkers for the diagnosis of different diseases and disorders.[14] Evidently it is still unclear if the blood $A\beta$ levels (serum $A\beta_{40}$ and/or serum $A\beta_{42}$, or their ratio) would in any way reflect a diagnostic criteria for AD or not.[18-19] There are suggestive evidences that changes in plasma or serum $A\beta_{40}$, $A\beta_{42}$, or the ratio of $A\beta_{42}/A\beta_{40}$ may be associated with individuals at risk for developing AD.[20-21]

Some studies, comparing clinically diagnosed AD patients with normal elderly individuals, showed no differences in either plasma $A\beta_{40}$ or $A\beta_{42}$.[22,23] However, other studies revealed the occurrence of high plasma $A\beta_{42}$, but not $A\beta_{40}$, in the patients with mild cognitive impairment (MCI).[24,25] Still other studies showed an increase in $A\beta_{42}$ and/or $A\beta_{40}$ in plasma of AD patients.[26,27] Decrease in $A\beta_{42}$ and/or the ratio of $A\beta_{42}/A\beta_{40}$ in plasma of AD patients compared with non-AD demented individuals and controls has also been reported.[28-31] According to some studies it seems that a low level of Aβ42/Aβ40 ratio is associated with AD and generally with dementias.[26]

On the other hand, most prospective studies indicate that elevation in plasma $A\beta_{42}$ level is present before or just at the onset of a clinically diagnosed disease.[32] Interestingly, it was also shown that conversion to late onset AD (LOAD) was concomitant to a decline in $A\beta_{42}$ levels and also in $A\beta_{42}/A\beta_{40}$ ratio, although many studies showed no difference in $A\beta_{42}$ levels and in $A\beta_{42}/A\beta_{40}$ ratio in patients with MCI who progressed to LOAD.[33,34]

Steady-state level of $A\beta$ depends on a balance between its production and clearance which could be accomplished by several peptidases and proteases. Neprilysin, known as the principle $A\beta$ degrading peptidase and insulin-degrading enzyme (IDE), known to cleave multiple short polypeptides (including $A\beta$) are proteases that are recently proposed to be involved in stablising a balance between amyloid production and its clearance. There are also other enzymes associated with $A\beta$ degradation, such as endothelin-converting enzyme (ECE) and angiotensin-converting enzyme (ACE).[16,35,36]

ACE is also an endopeptidase that its principle action is to convert angiotensin I (AGT I) to AGT II, an activity that is strongly associated with hypertension and vascular dysfunction.[37] It is now clear that mouse and human brain homogenates contain an activity that converts $A\beta_{42}$ to $A\beta_{40}$ and that the major portion of this converting activity is mediated by ACE.[38] Using animal models, several investigators have found that ACE activity decreases with age but the situation in humans remains unclear.[39] Therefore, it seems that it would be very insightful to clarify the relationship between serum ACE activity and AD onset and progression with age. In addition, there might also be a relationship between ACE activity and $A\beta_{42}$ and $A\beta_{40}$ levels in serum.[40]

Interestingly, elevation of ACE activity has been reported in the brains of patients with AD.[41] In a study conducted by Akatsu et al, they have shown that AD patients with a higher ACE activity in the peripheral blood were at a more advanced age at onset.[37] Other studies have revealed that lower ACE activity in plasma could increase the risk of AD as it is known that ACE degrades $A\beta$.[37,42] They also showed that the level of $A\beta_{40}$ in the peripheral blood of AD patient was significantly higher than that of the completely normal group.[37] Other studies have shown that ACE level and activity in CSF and serum were lower in patients with AD compared to controls.[42-43]

Our prime hypothesis was that if there exist significant differences in the concentrations of amyloid peptides (and their ratios) and also in the activity of ACE in blood serum of AD patients (considering sex, age and severity of the disease) and control young (YC) and aged (AC) groups? Our results showed that even at relatively small sample sizes of patients and controls in this study, significant differences still could be observed in blood serum concentrations of $A\beta$ peptides and ACE activity, and this could be considered to be of considerable importance in search for anticipative measures in the onset and progression of AD.

METHODS

Patients

Blood samples were collected from AD patients diagnosed in Zanjan (North Western region of Iran), Zanjan University of Medical Sciences (ZUMS) general hospital as case group and randomly recruited normal healthy population as control group. Diagnosis was made by standard diagnostic criteria, i.e., NINCDS- ADRDA and MMSE. None of the subjects had hypertension, cardiovascular and renal disease and not received any ACE inhibitor, antihypertensive, antidepressant and addictive medications (Table 1a). Clinical information (Table 1b), was obtained from either clinical records or by interviewing patients, their families and legal guardians. Blood samples were taken only from diagnosed sporadic AD patients. Subjects were either AD group (12 individuals, aged 76 ± 9) and non-AD (Control), which themselves were divided into two subgroups: 1) Aged control group (AC) (10 individuals,

aged 67 ± 8) and young control group (YC) (18 individuals, aged 29 ± 2).

Table 1 a: Characteristics (sex, age and number) of AD and non-AD individuals, whose blood samples were prepared and examined for $A\beta_{40}$, $A\beta_{42}$ and ACE activity.

Category	Sex	Numbers	Age (average ± SD)
AD	M	6	75.5 ± 11.3
	F	6	78.2 ± 8.90
	T	12	76.8 ± 9.80
Non-AD (Age Control)	M	4	70.2 ± 10.1
	F	6	65.2 ± 6.50
	T	10	67.4 ± 8.20
Non-AD (Young Control)	M	11	29.3 ± 2.40
	F	7	28.7 ± 1.20
	T	18	29.0 ± 2.00
Total	M	21	-
	F	19	-
	T	40	-

N, sample numbers; SD, standard deviation; M, male; F, female; T, Total

Blood collection

Blood samples (4 mL) were collected from the intermediate cubital vein, transferred into tubes containing separator gel and clot activator (without EDTA), and maintained for 30 min at room temperature. Samples were then centrifuged at 1000 ×g for 10 min at 4°C, supernatants (serums) were kept in several vials (0.5 mL each) and stored at -80°C until use.

Measurement of $A\beta$ concentration

ELISA kit (Invitrogen) was used to quantify the concentration of $A\beta_{40}$ and $A\beta_{42}$ in the serum. Measurements were made at 450 nm in a Bio Tek Elx (Model 808) microplate reader. All measurements were performed at least four times.

All research protocols regarding human samples were approved by the Clinical Review Board and Ethics Committee at Zanjan University of Medical Sciences. All participating patients were thoroughly informed about the studies and signed their consent information and the suitability of the information received.

Table 1 b: The number and severity of disease in AD patients at the time of blood sampling according to sex and age.

Sex	51-60			61-70			71-80			81-90			Total
	Mi	Mo	S	Mi	Mo	S	Mi	Mo	S	Mi	Mo	S	
Male	1	-	-	-	-	-	2	1	-	1	1	-	6
Female	-	-	-	-	1	-	1	-	1	-	2	1	6
Total	1	-	-	-	1	-	3	1	1	1	3	1	12

N, sample numbers; SD, standard deviation; M, male; F, female; T, total; Mi, mild; Mo, moderate; S, severe

Measurement of ACE activity

ACE activity was determined at 382 nm by the use of a CamSpec M501 spectrophotometer, in which N-hippuryl-l-histidyl-l-leucine was used as the standard substrate of ACE. Measurements were performed at least four times.

One unit of ACE activity was defined as the amount of enzyme that could produce 1 µmol of hippurate under defined conditions per minute. The following equation was used to determine the ACE activities.[44]

Serum ACE activity (U/L) = $(\Delta A \times V_t \times 1000) / (33.5 \times 15 \times V_s)$, where ΔA was the absorbance of the test sample minus that of the blank sample, V_t was the total volume (5 mL), and V_s was the sample volume (0.1 mL). The number 1000 was the conversion factor of U/mL to U/L, and 33.5 was the molar absorptivity of the chromogen (cyanuric chloride) that was dissolved in 1, 4-dioxan.[44]

Statistical analysis

The Student T-test was used for the analysis of the differences between sample and control groups. The level of significance was either $p < 0.05$ or $p < 0.01$.

RESULTS

Serum $A\beta$ level

The results of our measurements on patients and controls (Table 1) are presented in Table 2 which shows the average concentrations of $A\beta_{40}$ and $A\beta_{42}$, together with their ratios in different samples. The results in Table 2 are shown separately in the forthcoming Figures. Figure 1a shows the blood serum concentrations of $A\beta_{40}$. $A\beta_{40}$ concentrations in different sexes are shown in Figure 1b. Effects of age and severity of disease is shown in Figures 1c and 1d. Figure 2a shows that $A\beta_{42}$ concentrations in the serum of AD patients were like $A\beta_{40}$ (Figure 1a)

lower than that of other groups. Effect of age on the concentration of $A\beta_{42}$ was only available for female patients (Figure 2b). Figure 2c shows that older male patients (81-90) have higher amounts of $A\beta_{42}$ than younger (71-80) male patients. The ratios of blood serum concentrations of $A\beta_{42}$ to $A\beta_{40}$ ($A\beta_{42}/A\beta_{40}$) in AD patients were compared with controls (Figure 3a). Comparisons were also made with respect to the age of AD patients in males (Figure 3b) and females (Figure 3c).

Table 2: The concentrations of $A\beta_{40}$ and $A\beta_{42}$, the ratios of concentrations of $A\beta_{42}/A\beta_{40}$ and ACE activity in the peripheral blood serum samples.

Category	Sex	$A\beta_{40}$ (pg/mL)	$A\beta_{42}$ (pg/mL)	$A\beta_{42}/A\beta_{40}$ (%)	ACE (U/L)
	M	155.4 ± 113.4	1.34 ± 1.30	1.00 ± 1.53	48.9 ± 15.6
AD	F	173.5 ± 125.6	1.30 ± 1.03	0.490 ± 0.520	55.1 ± 11.6
	Total	164.5 ± 114.5	1.32 ± 1.29	0.750 ± 1.12	52.0 ± 13.5
	M	587.9 ± 278.0 *	3.26 ± 2.17	0.630 ± 0.480	37.6 ± 19.0
AC	F	355.6 ± 207.9	3.51 ± 2.40	1.02 ± 0.500	54.6 ± 11.0
	Total	448.5 ± 253.3 **	3.41 ± 2.19 **	0.860 ± 0.510	42.2 ± 21.5
	M	544.8 ± 206.7 **	2.96 ± 1.42	0.610 ± 0.520	61.2 ± 16.7
YC	F	548.4 ± 180.5 **	3.88 ± 2.50	0.800 ± 0.670	51.2 ± 16.7
	Total	549.5 ± 190.9	3.34 ± 1.95 **	0.690 ± 0.570	57.0 ± 14.9

*p < 0.05, ** p < 0.01 compared with AD group
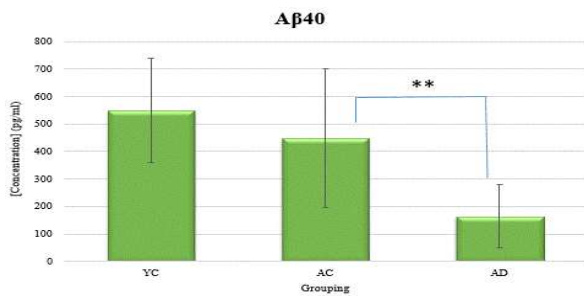M, male; F, female; AD, Alzheimer's disease; AC, aged control; YC, young control

Figure 1: a) Concentration of $A\beta_{40}$ in serum samples. b) Concentration of $A\beta_{40}$ in males and Females. c) Concentration of $A\beta_{40}$ in male patients (70-80 and 80-90). d) Concentration of $A\beta_{40}$ based on severity. AD, Alzheimer's disease; AC, aged control; YC, young control. (*p < 0.05, p < 0.01).**
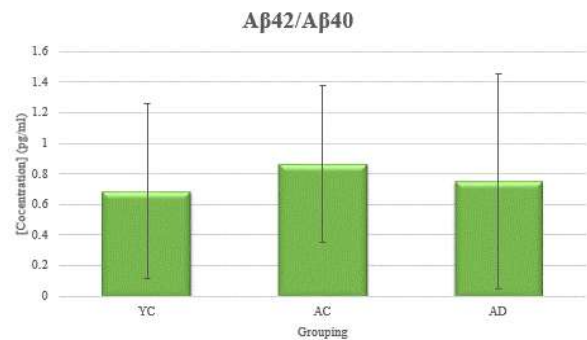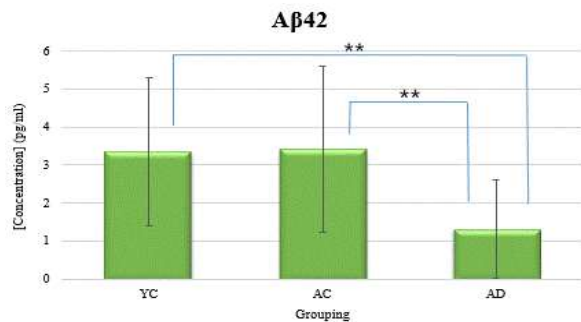

Figure 2: a) Concentration of $A\beta_{42}$. b) and c) Concentrations of $A\beta_{42}$ in the male (b) and female (c) patients with respect to age. AD, Alzheimer's disease; AC, aged control; YC, young control. (*p<0.05, ** p<0.01).

Figure 3: a) Concentration ratio of $A\beta_{42}$ to $A\beta40$. b and c) Concentration ratio of $A\beta_{42}$ to $A\beta_{40}$ in male and female patients in different age groups.
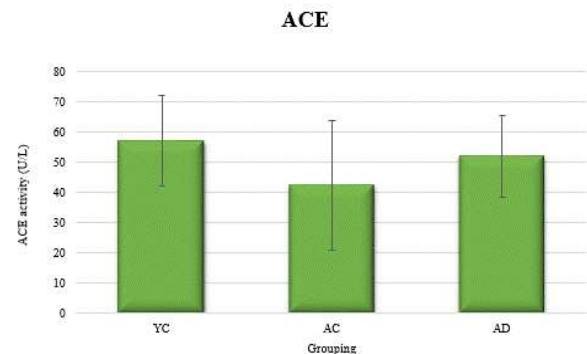

Figure 4: a) ACE activities in patients and controls. b) like a, but in different sexes. ACE activities in male (c) and female (d) patients. e) ACE activities in patients with respect to severity. AD, Alzheimer's disease; AC, aged control; YC, young control.

Serum ACE activity

Figure 4 shows our results of ACE activity measurements in the peripheral serum samples of AD patients in comparison with controls, generally, and in different sexes. There was no significant difference in ACE activities either in AD patients generally (Figure 4a) or in different sexes (Figure 4b). Effect of age on measured ACE activity in blood serum samples of AD patients are shown in males (Figure 4c) and females (Figure 4d). Figure 4e shows the effect of disease severity on ACE activity in blood serum samples.

DISCUSSION

As it was pointed out in the results section, it is clear that $A\beta_{40}$ concentration is decreased in AD patients (Figure 1a) and in both sexes (Figure 1b). Furthermore its concentration is decreased in male patients upon aging (Figure 1c) and because of severity, again in male patients (Figure 1d). In female AD patients we did not obtain a conclusive result (data not shown). We also observed that increase in severity of disease (from mild to moderate in males and from moderate to severe in females) was concomitant with decreases in $A\beta_{40}$ concentrations in blood serum samples (Figure 1d). So it seems that $A\beta_{40}$ concentration in peripheral blood samples of AD is lower than healthy individuals and decreases even more with the severity of AD. In case of $A\beta_{42}$ (Figure 2) almost the same conclusions could be derived as those of $A\beta_{40}$ except that our results showed that $A\beta_{42}$ concentration was increased in male patients upon aging (Figure 2c), which is in opposite to that of $A\beta_{40}$ that showed decreases (Figure 1c). At present we do not know if this opposite relationship also exists in $A\beta_{42}$ concentrations for female patients also. We may speculate that in female AD patients, upon increasing the age, greater amounts of $A\beta_{42}$ remain in the brain and consequently less appears in their serums. On the contrary, in male AD patients the amounts of $A\beta_{42}$ that remains in the brain is less than that of $A\beta_{40}$, and since the toxicity of $A\beta_{42}$ has been shown to be far more than $A\beta_{40}$ we may conclude that upon aging in AD patients, females would show more adverse cognitive decline than male AD patients. Our results does not affirm those studies that show an increase in $A\beta_{42}$ and/or $A\beta_{40}$ in plasma of AD patients[26-27] and is in line with those that report decreases in $A\beta_{42}$ and $A\beta_{40}$ in plasma of AD patients compared with non-AD demented individuals and controls.[28-31] Data in Figure 3, about the concentration ratios of $A\beta_{42}$ to $A\beta_{40}$, shows that the ratio does not change significantly when compared to control samples (Figure 3a), and also the differences are not significant when the effect of aging was studied in either males (Figure 3b) or females (Figure 3c). Generally, it seems that $A\beta_{42}/A\beta_{40}$ could not be considered as either a diagnostic or prognostic indicator.

In case of ACE activity, our results show decrease in ACE activity by aging (Figure 4a) and this trend is more

obvious in males than females (Figure 4b). Increase in ACE activity in AD group in comparison with AC group is again more evident in males than females (Figure 4b). Effect of aging on ACE activities is shown in Figures 4c and 4d. Here we also notice a difference between males and females in terms of ACE activity. Its activity in aged male patients is increased (Figure 4c) but in female patients there is decreases upon aging (Figure 4d). If ACE activity could be considered to play a protective role in the pathogenesis of AD, and this protective role could be related to the conversion of $A\beta_{42}$ to $A\beta_{40}$, considering the fact that $A\beta_{40}$ is far less toxic than $A\beta_{42}$, the above discussion about differences in ACE activities in females and males, together with lower amounts of $A\beta_{42}$ in the blood serum of female patients (Figure 2b), would mean that there could be far more $A\beta_{42}$ in the brain of female patients and hence more severity of AD in female patients than male patients. On the other hand, it can be postulated that increases in the levels of $A\beta$ in the brain of AD patients might trigger increased expression of ACE in the brain. Regarding evidences that ACE is up-regulated in AD patients,[45-47] it could be suggested that brain ACE may enter into the blood circulation and consequently would increase the ACE level in blood of AD patients.[37] Last but not least, correlations between ACE levels and AD may relate more to the effects on the renin-angiotensin system and brain vascular changes than to a major contribution to $A\beta$ clearance.[41]

CONCLUSIONS

There is still insufficient evidence to permit a definite conclusion regarding the use of plasma $A\beta_{40}$, $A\beta_{42}$ and ACE in the diagnosis or assessment of risk for AD.[16, 35, 48] In this study, we found that serum concentrations of $A\beta40$ and $A\beta_{42}$ could be considered as a potential biomarker for the diagnosis of AD and/or to determine the severity of the disease, particularly in females. Our data also showed that above statement could not be stated for the blood serum concentration ratio of $A\beta_{42}/A\beta_{40}$. In case of blood serum activity of ACE in AD patients our results showed that it could be anticipated that ACE activity increase, particularly in male patients and also this activity become increased upon aging in AD patients and with increase in the severity of the disease.

Ethical approval: The study was approved by the Institutional Ethics Committee

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