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A Method for Diagnosing Alzheimer's Disease Based on Salivary Amyloid-β Protein 42 Levels

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Abstract. We have developed a non-invasive method of diagnosing Alzheimer's disease (AD), which can also predict the risk of its future onset. It is based on measuring salivary levels of amyloid-β protein terminating at position 42 ($A\beta_{42}$). Brain deposits of this peptide are characteristic of AD. Biomarker studies indicate that such brain deposits commence a decade or more prior to clinical onset of the disease. We report here that $A\beta_{42}$ is produced in all peripheral organs tested, thus establishing the generality of its production. We used this information to develop simple and sensitive tests to determine salivary $A\beta_{42}$ levels. The levels were first stabilized by adding thioflavin S as an anti-aggregation agent and sodium azide as an anti-bacterial agent. We then quantitated the $A\beta_{42}$ in a series of samples with ELISA type tests. Control cases showed almost identical levels of salivary $A\beta_{42}$ regardless of sex or age. All AD cases secreted levels of $A\beta_{42}$ more than double those of controls. Individuals at elevated risk of developing AD secreted levels comparable to the AD cases. The results establish that salivary $A\beta_{42}$ levels can be used to diagnose AD as well as to predict the risk of its future onset.

Keywords: Alzheimer's disease, amyloid-β protein, amyloid-β protein precursor, ELISA type assays, saliva, thioflavin S

INTRODUCTION

It would be difficult to overstate the urgency of finding solutions to the problem of Alzheimer's disease (AD). Alzheimer Disease International, in their 2010 World Alzheimer Report, estimated that there were 35 million people suffering from this disorder, at an annual cost of $604 billion (http://www.alz.co.uk). The 2016 United States Alzheimer's Association Report estimated that there were 5.4 million cases in that country alone, with a new case being identified every 66 s. The annual cost in the US at that time was estimated to be $263 billion, not including the unpaid care costs of patients, which were estimated to be a further $221 billion per year [1].

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Rogers et al. [2] were the first to show that amyloid accumulation in AD is initiated by amyloid-β (Aβ) binding to the complement protein C1q. This binding initiates the complement cascade, which terminates by the formation of the membrane attack complex (MAC). The MAC, intended to attack foreign invaders, instead attacks brain neurons in a process known as bystander lysis. AD is characterized by brain deposits of Aβ protein terminating at position 42 ($A\beta_{42}$). $A\beta_{42}$ is a peptide fraction of amyloid-β protein precursor (AβPP), which is relatively insoluble. If it is allowed to accumulate in brain, it produces extracellular deposits in the form of senile plaques. These plaques stimulate an inflammatory response. The inflammatory response, in turn, fully activates the complement system. This results in formation of MAC that directly damages residual brain neurons. A progressive loss of these brain neurons occurs, which eventually results in the cognitive deficits that define clinical AD.

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This sequence of events can be avoided by early intervention with anti-inflammatory agents. Traditional non-steroidal anti-inflammatory drugs (NSAIDs) are one such class of agents. Complement inhibitors are another emerging treatment class. Multiple epidemiological studies have been carried out which indicate that those consuming NSAIDs prior to the expected time of AD onset have a substantially reduced risk of developing the disease [3]. Typical examples include the incidence studies of Vlad et al. [4] and In t' Veld et al. [5], the prevalence study of Landi et al. [6], and the case control study of Breitner et al. [7].

The diverse data are in remarkable agreement in that the longer the time interval between consuming NSAIDs and the anticipated onset of AD, the greater the reduction of risk. It can be as much as 5-6 fold [8]. Furthermore, traditional NSAIDs were shown to have an ameliorating effect on the pathology and behavior in 13 of 14 studies using transgenic mouse models of AD. Ibuprofen was the NSAID of choice in ten of these studies [9, 10].

Recent advances, using biomarkers for AD, have provided a plausible explanation for these results. It is now known that biomarkers become positive more than a decade before the clinical onset of AD. Bateman et al. [11], in a landmark study of 128 cases in which genetic mutations made AD development inevitable, found that concentrations of $A\beta_{42}$ in cerebrospinal fluid declined 25 years before the expected clinical onset. Aβ deposits in the brain, as revealed by Pittsburgh compound B PET studies, were detected 15 years before the expected clinical onset. Villemagne et al. [12] estimated that it took 19.2 years of linear Aβ accumulation, 4.2 years of hippocampal atrophy, and 3.3 years of memory impairment to reach AD clinical diagnostic levels.

The basis of these predictions is that humans with certain genetic mutations in presenilin one (PS1) and presenilin two (PS2) inevitably develop AD. The reason is that they express abnormally high levels of $A\beta_{42}$. Fibroblasts from these cases express such high levels. Transgenic cells, as well as transgenic mouse models that express these mutations, also have an overproduction of $A\beta_{42}$ levels in brain. These are detectable as early as 2–4 months of age [13].

In contrast to this overexpression of $A\beta_{42}$, a mutation in the AβPP gene (A673T), identified in some Icelandic individuals, reduces $A\beta_{42}$ production and protects against AD. The substitution is adjacent to the β-secretase cleavage site and results in a 40% decrease in the formation of amyloidogenic peptides *in vitro*. This provides proof of principle for the hypothesis that reducing the β-cleavage of AβPP may protect against the disease [14].

The window of opportunity between biomarker diagnosis of AD and clinical onset can be exploited to eliminate AD. Intervention with anti-inflammatory (NSAIDs), complement inhibitors, or other anti-inflammatory agents, in the decade or more of preclinical development should successfully prevent AD onset. To take advantage of this opportunity, a simple, non-invasive pre-AD diagnostic test that can be widely utilized by the general population needs to be available.

A test based on salivary $A\beta_{42}$ levels may meet these criteria. Bermelo-Pareija et al. [15] have broken ground in this area with their report that saliva levels of $A\beta_{42}$ are a potential marker of AD. They reported a small but statistically significant increase in AD patients compared with healthy controls (AD cases 6.81 ± 20.04 pg/ml; controls 2.89 ± 4.96 pg/ml). However, they found no differences in $A\beta_{40}$ levels between AD cases, PD cases, and controls. As a measuring device, they used a commercial ELISA kit supplied by Invitrogen. In view of this preliminary data, we undertook a more detailed examination of salivary $A\beta_{42}$ levels in control and AD cases to determine if a more accurate test might prove to be of practical value.

As a first step, we established that $A\beta_{42}$ is being continuously produced by all organs of the body. It has been assumed by some that it is only produced in brain and that its appearance in blood and urine is merely the result of brain clearance. We conducted experiments to illustrate that all organs of the body are continuously producing $A\beta_{42}$.

MATERIALS AND METHODS

$A\beta_{42}$ messenger RNA (mRNA) expression in human organs by RT-PCR and real-time quantitative PCR (qPCR)

We first showed that the messenger RNA for AβPP is present in all human organs tested (Fig. 1). Frozen samples were utilized as the starting material. The samples were homogenized by a sonic dismembrator (Fisher Scientific, Ottawa, ON). They were then treated with 100 microliters of TRIzol (GIBCO-BRL, Gathersburg, MD). After 1 h, the lysates were centrifuged at 10,000 rpm for 10 min. The supernatants were transferred to new tubes. The

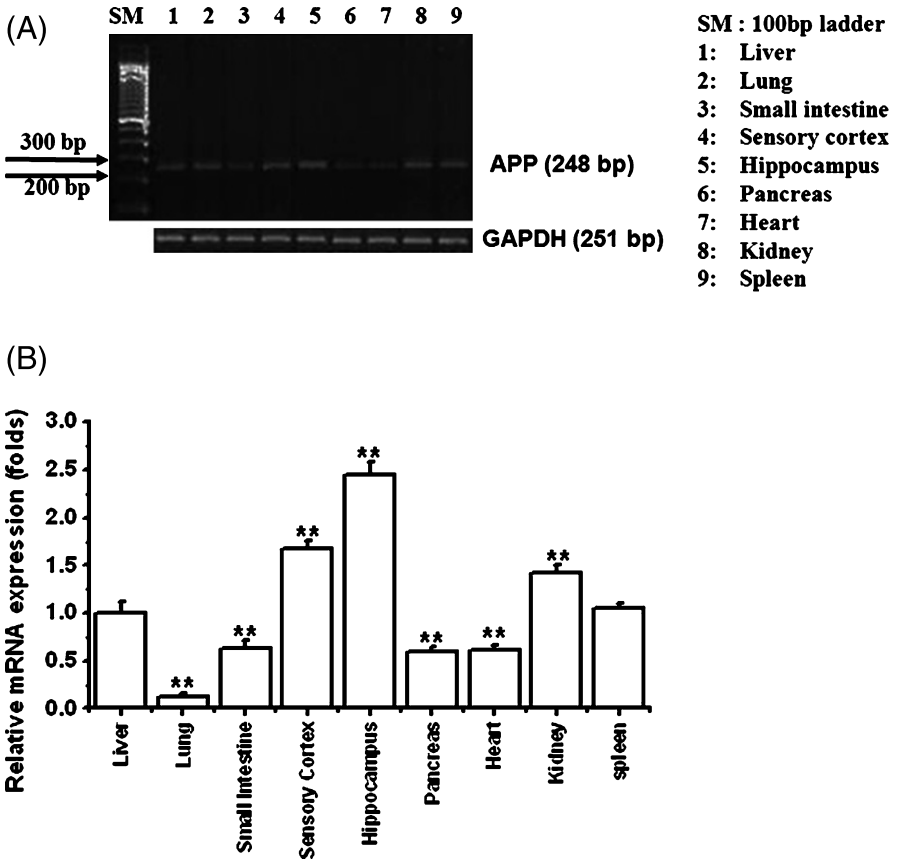

Fig. 1. Expression of amyloid-β protein precursor (AβPP) messenger ribonucleic acids (mRNAs) in various organs of human by (A) RT-PCR and (B) quantitative PCR (Q-PCR). Expression of GAPDH loading controls shown in the lower panels were used as a standard (A). Note that AβPP mRNAs, which can be translated into $A\beta_{42}$ proteins, were expressed in all human organs studied. B) Values are mean±SEM, $n = 3$. One-way ANOVA was carried out to test significance. $^{**}p < 0.01$ for other groups compared with liver group.

purity and amount of the RNA was then measured spectrophotometrically. A total RNA aliquot (20 μg) was used to synthesize the first strand complementary DNA (cDNA) using Moloney murine leukemia virus (M-MLV) reverse transcriptase (GIBCO-BRL). The cDNA product was then amplified by PCR using a GeneAmp thermal cycler (Applied Biosystems, Foster City, CA). Specific primers for human AβPP and GAPDH were for AβPP: Forward 5′-CGG AATTCCCTTGGTGTTCTTTGCAGAAG-3′ and Reverse 5′-CGGAATTCCGTTCTGCATCTGCTCA AAG-3′ (248 bp) [16]. For GAPDH: Forward 5′-CCATGTTCGTCATGGGTGTGAACCA-3′ and Reverse 5′-GCCAGTAGAGGCAGGGATGATG TTC-3′ (251 bp) [17]. PCR conditions were an initial denaturation step at 95°C for 6 min followed by a 30-cycle amplification program consisting of denaturation at 95°C for 45 s, annealing at 55–60°C for 1 min, and extension at 72°C for 1 min. A final extension was carried out at 72°C for 10 min. The amplified PCR products were identified using 1.5% agarose gels containing ethidium bromide (final concentration 0.5 μg/ml) and visualized under ultraviolet light.

qPCR was also performed. Total RNA (5 μg) and the primers listed above were mixed with Fast SYBR® Green Master Mix (Life Technologies, Carlsbad, CA). PCR was performed using a Quantstudio 6 Flex (Applied Biosystems, Foster City, CA). Comparative C_T (Delta delta C_T, $\Delta\Delta C_T$) values were used to determine the relative expression of AβPP [18]. The relative amount of mRNA in each organ was calculated with respect to the values of liver.

For measuring $A\beta_{42}$ levels in the PBS insoluble fraction, the precipitated parts were solubilized with 1% guanidine HCl. After the solution was centrifuged at 10,000 g for 30 min at 4°C, a sample (10 μL) was mixed with 90 μL PBS to do the ELISA test.

Measurements of $A\beta_{42}$ levels by ELISA assays

We next tested whether these mRNAs were being translated into proteins. Frozen tissues from human liver, lung, small intestine, sensory cortex, hippocampus, pancreas, heart, kidney, and spleen were thawed and dissected into small pieces. The dissected pieces were added to chilled tubes with a tenfold excess of 0.01 M phosphate buffered saline (PBS, pH 7.4). The mixture was sonicated ten times at 20% amplitude for 25 s (Branson Digital Sonifier, USA). The mixture was then centrifuged at 10,000 g for 30 min at 4°C.

For determining the $A\beta_{42}$ levels in these and other samples, two ELISA type tests were developed. The first steps in each were identical. They involved adding an $A\beta_{42}$ capture antibody to microwell plates, followed by blocking of any non-specific binding. This was achieved by adding 100 μL of the rabbit polyclonal anti-$A\beta_{42}$ antibody from Novus Biochemicals (NBP2-44113, Littleton, CO) diluted 1/1,000 in PBS to the plates. This antibody detects selectively the C-terminal aspect of $A\beta_{42}$. The plates were left overnight at 4°C to allow the antibody to bind. The supernatants were then discarded. Next, to block non-specific binding to the capture antibody, 400 μL of 5% bovine serum albumin (BSA, Sigma, St. Louis, MO) was added to the plates, followed by incubation at 37°C for 1 h. The BSA solution was discarded and the wells were washed twice with PBS.

The indirect method (M1) involved utilizing the Anaspec $A\beta_{42}$ monoclonal antibody (Cat #: 29547, Anaspec, Fremont, CA) to detect the N-terminal region of $A\beta_{42}$. This method required further treatment with a third, biotin-linked rabbit anti-mouse IgG antibody (DAKO, Missisauga, ON, Canada). A more direct method (M2) utilized a horseradish peroxidise (HRP)-linked monoclonal anti-$A\beta_{42}$ antibody (HRP-linked optimAb β-amyloid 1–16 monoclonal Ab, Eurogentec, Fremont, CA), which does not require a third antibody treatment.

For M1, the Anaspec antibody was added as 100 μL of a 1/200 dilution of 5% BSA in PBS. Incubation was carried out at 37°C for 2 h. The plates were washed twice with PBS-T. They were next incubated at 37°C for 1 h with 100 μL of a biotin-linked rabbit anti-mouse IgG antibody (DAKO, Missisauga, ON, Canada) diluted 1/200 in PBS containing 5% BSA. The supernatants were discarded. A streptavidin-HRP solution (ABC solution, 1/1,000 dilution, Vector Laboratories) was then added and incubation carried out at 37°C for 30 min. After the plate was washed twice with PBS-T, 100 μL of a tetramethylbenzidine (TMB) solution (Invitrogen, Carlsbad, CA) was added and incubation carried out at room temperature for 5–10 min. A stop solution of 100 μL of 0.2 N sulfuric acid was added to the well plates. Optical density was measured at 450 nm. The $A\beta_{42}$ protein concentration was calculated from a standard curve that was made with Human $A\beta_{42}$ peptides (Anaspec, Fremont, CA). It is of importance that the reaction should be performed in the laboratory at 20°C and the TMB solution kept at 4°C until it is added for color reaction.

For M2, the plates were incubated for 2 h at 37°C with 100 μL of the monoclonal anti-$A\beta_{42}$ antibody HRP-linked optimAb β-amyloid 1–16 (Eurogentec, Fremont, CA). After the plate was washed twice, 100 μL, PBS-T (TMB solution, Invitrogen, Carlsbad, CA) was added and incubation carried out at 37°C for 5–10 min. The stop solution (100 μl) was then added to the well plates. Optical density was measured at 450 nm. The $A\beta_{42}$ protein concentration was calculated from a standard curve as described above.

For determining the $A\beta_{42}$ levels in saliva samples, 2-3 ml of fresh saliva were first added to tubes containing thioflavin S (0.5 mg, Sigma St. Louis MO) to prevent $A\beta_{42}$ aggregation as well as sodium azide (0.5 mg, Fischer Scientific, Suwanee GA) to prevent bacterial growth. In typical assays, amounts varying from 0 (control) to 300 μL were added to the microwells and assays carried out as previously described.

To verify that all of the $A\beta_{42}$ in saliva samples was being captured and correctly assayed, recovery experiments were carried out.

For these recovery studies, human saliva (50 μL) from three normal and three AD cases, with or without the addition of $A\beta_{42}$ peptides (25 pg in 50 μL PBS, Anaspec, Fremont, CA), was incubated at 37°C for 4 h with the capture antibody. The $A\beta_{42}$ depleted saliva solution was discarded. The plate was washed two times with PBS and incubated at 37°C for 2 h with 100 μL of the detecting monoclonal anti-$A\beta_{42}$ antibody (Cat#: 29547, Anaspec, Fremont CA). The plate was washed twice with PBS and incubated with the biotin-linked rabbit anti-mouse IgG antibody (1/200 in 5% BSA including PBS, 100 μL, DAKO, Missisauga, ON, Canada) in 3% BSA, at 37°C for 1 h. Streptavidin-HRP solution (ABC solution, 1/1000 dilution, Vector Laboratories) was added and incubation carried out at 37°C for 30 min. After the plate was washed twice with PBS, a TMB solution (100 μL, Invitrogen, Carlsbad, CA) was added and incubation carried out at 37°C for 30 min. The stop solution was

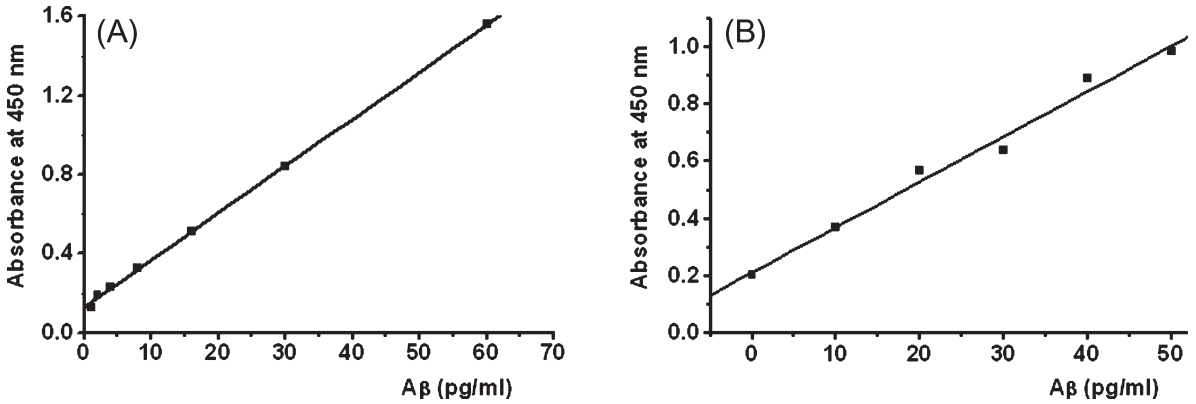

Fig. 2. Standard graphs of absorbance at 450 nm versus $A\beta_{42}$ capture with a rabbit polyclonal $A\beta_{42}$ antibody (Novus biological) in ELISA assays at 1/1,000 dilution. The detection antibody was (A) a mouse monoclonal $A\beta_{42}$ antibody 1/200 dilution (Anaspec) and (B) 1/500 dilution (Eurogentec). Notice the linear relationship between absorbance and $A\beta_{42}$ concentration at these dilutions in both antibodies. See Materials and Methods for details.

Table 1
$A\beta_{42}$ levels of human organs (both PBS soluble and insoluble fractions) measured by our ELISA type method and a kit from Invitrogen

Human tissue	$A\beta_{42}$ levels in our methods (pg/ml)		$A\beta_{42}$ levels in Invitrogen kit (pg/ml)	
	PBS soluble fraction	PBS insoluble fraction	PBS soluble fraction	PBS insoluble fraction
Lung	83.68	6.89	17.28	1.35
Heart	88.59	7.23	18.29	1.53
Kidney	122.65	10.44	29.22	1.69
Hippocampus	102.45	9.56	18.28	1.33
Sensory cortex	97.83	7.43	18.24	1.42
Liver	90.56	6.89	17.59	1.44
Spleen	134.38	9.93	29.49	2.01
Small intestine	80.58	6.44	18.02	1.52
Pancreas	128.22	8.94	40.1	2.26

Table 2
Stability of salivary $A\beta_{42}$ with and without thioflavin S and sodium azide as preservatives

Time Time		Amount of $A\beta_{42}$ in pg/ml			Amount of protein in mg/ml		
		Case 1	Case 2	Case 3	Case 1	Case 2	Case 3
Day 0	With preservatives	23.94	24.56	25.89	2.31	2.095	2.36
	Without preservatives	23.69	24.51	25.57	2.38	2.08	2.36
Day 1	With preservatives	23.33	23.95	24.88	2.42	2.16	2.27
	Without preservatives	18.53	16.52	16.94	2.73	2.41	2.71
Day 2	With preservatives	22.93	23.12	24.25	2.31	2.08	2.23
	Without preservatives	7.93	7.59	6.92	2.96	2.79	2.93
Day 3	With preservatives	22.94	23.46	23.86	2.38	2.14	2.42
	Without preservatives	2.94	2.18	2.09	3.04	3.60	3.34
Day 5	With preservatives	22.12	23.45	23.58	2.36	2.28	2.22
	Without preservatives	0.12	0.15	0.15	3.44	3.79	3.63
Day 7	With preservatives	21.55	22.84	22.69	2.32	2.21	2.24
	Without preservatives	0	0	0	3.49	3.91	3.58

added to the plates and optical density measured at 450 nm. The $A\beta_{42}$ protein concentration was calculated from a standard curve.

To compensate for any salivary dilution, total protein in the samples was measured using an assay kit provided by Thermo Scientific Inc (Rockford,

IL). The kit is based on reactions with bicinchoninic acid. Bovine serum albumin is the reference standard. Saliva samples or PBS as controls (10 microliters) were added to microwell plates. Incubation was carried out at 37°C for 30 min. The optical density was then read at 562 nm.

Data analysis

The significance of differences between data sets was analyzed by one-way ANOVA tests.

RESULTS

Figure 1 shows a band corresponding to the expected 249 base pair segment of AβPP for lung, heart, kidney, hippocampus, sensory cortex, liver, spleen, small intestine, and pancreas as determined by RT-PCR (Fig. 1A). Expression of GAPDH demonstrating equality of the loading control is shown in the lower panel. These data establish that AβPP mRNA is being expressed at easily detectable levels by all organs of the body and not just brain. Both cortex and hippocampus AβPP mRNA levels were higher than in any other regions (Fig. 1B). This was confirmed by data from Q-PCR.

To determine if the mRNAs are being translated into proteins that eventually lead to generation of $A\beta_{42}$, a reliable method of detecting this fraction needed to be developed. The results of our ELISA method are shown in Fig. 2. There is a linear relationship between absorbance at 450 nm and the $A\beta_{42}$ content. This makes it possible to determine if the mRNAs shown in Fig. 1 are being translated into $A\beta_{42}$ protein in various body organs. The results are shown in Table 1. Each organ contained a significant level of $A\beta_{42}$. The levels varied from a low of 80.58 pg/g protein in the small intestine to highs of 122.6 pg/g protein in the kidney, 128.2 pg/g protein in the pancreas, and 134.3 pg/g protein in the spleen. The hippocampus and sensory cortex of brain expressed intermediate levels of 102.4 pg/g protein and 97.8 pg/g protein, respectively.

As can be seen from Table 1, the Invitrogen kit picks up only about 20% of the $A\beta_{42}$ present in the organs, which could account for the high variability in the salivary levels reported by Bermelo-Pareija et al. [15]. Moreover, for a salivary $A\beta_{42}$ test to be practical, some method of stabilizing the levels needs to be established.

We tested whether adding thioflavin S to inhibit $A\beta_{42}$ aggregation and sodium azide to prevent bacterial growth would be effective. The results are shown in Table 2. $A\beta_{42}$ without preservatives deteriorates rapidly. Within 3 days at ambient temperature, the values had deteriorated to well below 1% of those originally present. Meanwhile total protein was increasing, indicative of bacterial growth. However with thioflavin S and sodium azide added, there was complete stability after 7 days at ambient temperature. This stabilization makes it practical to transport individual saliva samples for analysis over distances that would take many days to complete.

Table 3
$A\beta_{42}$ levels in both normal and AD cases measured by our ELISA-type assays

Case	Age	Gender	Diagnosis	$A\beta_{42}$ (pg/ml)	Family history
1	46	Male	Normal	25.32	+
2	53	Male	Normal	24.44	–
3	89	Male	Normal	22.35	–
4	16	Male	Normal	21.47	–
5	19	Male	Normal	20.27	–
6	19	Female	Normal	19.73	–
7	22	Male	Normal	22.27	–
8	24	Male	Normal	21.53	–
9	39	Male	Normal	22.22	–
10	42	Male	Normal	20.58	+
11	47	Female	Normal	20.15	+
12	52	Female	Normal	23.78	–
13	54	Male	Normal	20.05	+
14	54	Female	Normal	20.56	+
15	55	Female	Normal	29.66	+
16	54	Male	PD	22.32	–
17	57	Female	Normal	19.61	+
18	57	Female	Normal	23.07	–
19	59	Male	Normal	21.55	+
20	60	Male	Normal	22.02	–
21	62	Male	Normal	21.37	–
22	67	Male	Normal	24.63	–
23	78	Male	Normal	21.44	–
24	83	Female	Normal	20.56	–
25	86	Male	Normal	21.37	–
26	88	Male	Normal	22.46	+
27	92	Female	Normal	20.89	–
28	51	Female	PS-I	60.90	+
29	60	Female	Pre-AD	47.96	+
30	52	Female	Pre-AD	59.57	+
31	57	Male	AD	41.58	–
32	72	Female	AD	41.69	–
33	77	Female	AD	84.97	+
34	78	Male	AD	48.89	–
35	84	Female	AD	64.93	+
36	86	Female	AD	75.20	–
37	84	Male	AD	56.22	+

AD, Alzheimer's disease; PD, Parkinson's disease. 37 cases were tested. Controls (including 1 PD case): 27, AD: 7 and pre-AD: 3 cases. Mean ± SEM: 22.06 ± 0.41 (controls) and 59.07 ± 6.33 (AD). One-way ANOVA was carried out to test significance for data we investigated with our kit; $p < 0.001$ between controls and AD.

Table 4
$A\beta_{42}$ recovery study data from three normal and three AD cases

	$A\beta_{42}$ amount (pg/ml)					
Cases	CON 1	CON 2	CON 3	AD 1	AD 2	AD 3
Aβ (25 pg/ml)	25.33	24.89	25.12	25.33	24.94	25.04
Saliva	24.56	24.17	23.22	85.01	65.07	75.17
Saliva + Aβ (25 pg/ml)	49.35	49.02	48.85	109.72	91.46	100.04

CON, normal; AD, Alzheimer's disease. Note that mean and SEM of Aβ (25 pg/ml) is 25.11 ± 0.08 pg/ml.

Table 3 shows the results of salivary Aβ levels in a series of 37 volunteer donors according to their clinical state. As the Table illustrates, the non-AD cases (controls) expressed levels that were remarkably constant over ages from 16 to 92 with no differences being observed between males and females. The 27 non-AD cases, including one with Parkinson's disease, showed a mean of 22.06 ± 0.41 pg/ml. The 7 AD cases showed a mean of 59.07 ± 6.33 pg/ml. One-way ANOVA was carried out to test the significance of differences between the control and AD cases ($p < 0.001$).

Three non-AD cases were exceptional. Case 28 had a presenilin 1 (PS-1) mutation, which carries with it a 100% certainty of developing AD. The saliva test predicted this known certainty, with a value of 60.90 pg/ml. The level was slightly higher than the average for all AD donors, thus validating the predictive capacity of the saliva test. Cases 29 and 30 had values more than double those of normal controls. These two females had mothers and other relatives affected by AD, so they were known to be at a high risk to develop the disease. The high salivary $A\beta_{42}$ levels confirm that the risk in these cases is definite rather than possible. Overall, the data demonstrate that AD can be diagnosed as well as predicted on the basis of salivary $A\beta_{42}$ levels. Values in the range of 20 pg/ml indicate no risk, while values over 40 pg/ml indicate AD or pre-AD.

Family histories of AD in parents or siblings were obtained for all salivary contributors. The results are shown in Table 3. Of the 27 controls, 9 had a known history of AD. However, it can be anticipated that there will be false negatives in the control cases based on early deaths of parents from other causes.

To be certain that all of the $A\beta_{42}$ in the samples was being detected, recovery experiments were carried out in which representative samples were spiked with 25 pg of $A\beta_{42}$. The results are shown in Table 4 for 3 control and 3 AD cases. As can be seen from the Table, there was 100% recovery of the added Aβ (25.11 ± 0.08 pg/ml). We also measured $A\beta_{42}$ levels in PBS insoluble fractions. The results are shown in Table 1. $A\beta_{42}$ levels in PBS insoluble fractions were less than 10% of ones in PBS soluble fractions.

We also performed experiments to compare $A\beta_{42}$ values obtained using the monoclonal antibodies from Anaspec and Neurogentec. We selected 11 normal and 7 AD cases. Standard values are shown in Fig. 2B. The results are demonstrated in Table 5. The $A\beta_{42}$ levels using the Neurogentec antibody for detection were slightly lower than those with Anaspec antibody, but they were nevertheless closely similar.

DISCUSSION

The results presented here describe a method for accurate determination of $A\beta_{42}$. The results show that $A\beta_{42}$ is produced by all organs of the body and is a fundamental molecule that is continuously being generated throughout the body. Secretion of $A\beta_{42}$ in the saliva is a manifestation of this production. Measurement of saliva $A\beta_{42}$ levels in a series of volunteers demonstrated some remarkable results. Typical normal cases secreted low levels of $A\beta_{42}$ in

Table 5
Comparison of $A\beta_{42}$ levels in detection antibodies from two companies (Anaspec and Eurogentec)

Case	Age	Gender	Diagnosis	Aβ (pg/ml) Anaspec Ab	Aβ (pg/ml) Eurogentec Ab
1	46	Male	Normal	23.32	18.31
2	53	Male	Normal	24.44	19.55
3	89	Male	Normal	22.35	18.56
4	16	Male	Normal	21.47	18.66
5	19	Male	Normal	20.27	18.12
7	22	Male	Normal	22.27	19.23
12	52	Female	Normal	23.78	19.84
13	54	Male	Normal	20.05	19.58
14	54	Female	Normal	20.56	18.11
22	67	Male	Normal	24.63	20.34
24	88	Male	Normal	22.46	18.45
31	57	Male	AD	41.58	38.93
32	72	Female	AD	41.69	42.33
33	77	Female	AD	84.97	78.66
34	78	Male	AD	48.89	44.22
35	84	Female	AD	64.93	62.93
36	86	Female	AD	75.20	69.83
37	84	Male	AD	56.22	54.11

their saliva, which were almost identical regardless of sex or age. AD cases and some abnormal control cases secreted levels of $A\beta_{42}$ that were more than double those of typical non-AD cases. The data indicate that high salivary levels of $A\beta_{42}$ can be used to diagnose AD as well as to predict the risk of future onset.

The ELISA test is highly sensitive to experimental conditions, particularly those related to the time of exposure to the TMB solution. For verification, a linear relationship between added $A\beta_{42}$ and the OD reading at 450 nm up to 80 pg/ml, as shown in Fig. 2 is required. For saliva samples, at least 2 controls and 2 AD cases should be analyzed, to verify the expected large differences.

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Authors' disclosures available online (http://j-alz.com/manuscript-disclosures/16-0748r1).

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