

Serum p97 levels as an aid to identifying Alzheimer's disease

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Background: The application of formal clinical diagnostic criteria for the identification of Alzheimer's Disease (AD) has improved diagnostic sensitivity. However, there remains a need for non-invasive biological markers and laboratory tests, which can facilitate case identification, and the assessment of treatment response. The p97 protein is a secreted protein specifically expressed by amyloid plaque associated reactive microglia that may have AD diagnostic ability.

Methods: A quantitative radioimmunoassay was developed to measure serum p97. This study, under a double blind protocol, evaluated the utility of serum p97 as diagnostic test for AD. All subjects were referred to the UBC Clinic for Alzheimer's Disease and Related Disorders (CADRD) for clinical assessment of dementia. A serum p97 sample was obtained at the time of assessment but diagnosis of disease was determined independently of p97 examination.

Results: "Possible" and "probable" AD cases ($n = 41$) and cognitively normal controls ($n = 64$) showed a highly significant difference in mean p97 concentration (41 vs. 20 ng/ml, $p < 0.001$). There was some overlap in p97 distributions between AD cases and control subjects. The area under the curve (AUC) for the receiver operator curve (ROC) was 0.812.

Conclusions: These results further support the specificity of high serum p97 levels in AD and its potential utility as a biological marker in AD. The reproducible elevation of serum

p97 in AD underlines the need to further determine its role as a biological marker and diagnostic adjunct for AD.

1. Introduction

Alzheimer's Disease (AD) is a progressive neurodegenerative disease that affects cognition, behavior and function. The 'gold standard' for definite AD currently resides with post-mortem neuropathological examination despite the significant differences that exist between the sets of neuropathological criteria that have been used in longitudinal research studies [4,34,35]. Clinically, the diagnosis of AD is established through neurological, neurobehavioral, and neuroimaging assessments. The phenotype of AD is recognized as most typically having a core amnesic disorder coupled to other areas of higher cortical dysfunction and with a clinical course that begins gradually and is progressive. To date, laboratory testing has been largely directed at ruling out modifiable non-degenerative causes of dementia [2]. The application of clinical diagnostic criteria, such as those of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA), has allowed an improved clinicopathological diagnostic accuracy of 80% or greater. However, a recent large clinical and neuropathological study of Mayeux et al. reported that, although the sensitivity for AD diagnosis was very high (> 90%) with the NINCDS-ADRDA criteria, specificity is still lacking (55%). Progress in finding ante-mortem laboratory confirmatory or diagnostic tests for AD has been slow, though numerous biological markers and diagnostic tests have been proposed. These include measuring the CSF levels of tau, A β 1-40 and 1-

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42, α 1-antichymotrypsin and neural thread protein [3, 10,26,41]. A consensus report of the Working Group on Molecular and Biochemical Markers of Alzheimer's Disease [8] proposed steps that should be taken in the development of an AD biomarker. They noted that a non- or minimally invasive laboratory test for the detection of AD, such as a blood test, would have a great advantage over CSF measured biomarkers. The Working Group suggested that an ideal biomarker for AD should have a sensitivity of $> 80\%$ for detecting AD and a specificity of $> 80\%$ [8].

Potentially hindering the development of a diagnostic test for AD is the increasing recognition of the coexistence of multiple brain disorders that can comorbidly produce dementia. For example, vascular lesions coexist with senile neuritic plaques and neurofibrillary tangles in 10–20% of AD [6] while Lewy bodies coexist with plaque pathology in 15–25% [11]. The effects of these pathological comorbidities on diagnostic markers for AD are not clarified [19,25,32,37].

The 'gold standard' of AD diagnosis, i.e. post mortem examination of the brain for tangles and plaques, has been recently described as 'probabilistic' with recognition that clinical correlation to pathology is required [20]. In addition, it is becoming increasingly evident that AD may not be a single disease but rather a number of disorders whose symptoms converge to the established clinical and pathological definition of AD.

The p97 antigen, also known as melanotransferrin, is a sialoglycoprotein that belongs to the group of iron-binding proteins that include transferrin (Tf), lactoferrin, and ovotransferrin. p97 binds iron and is involved in cellular iron uptake, providing a transferrin-independent cellular iron-uptake pathway [16,23]. p97 exists in two forms, one of which is attached to the cell surface by a glycosyl-phosphatidylinositol anchor and the other is actively secreted. In the CNS, p97 and the transferrin receptors (TR) are highly localized to the capillary endothelium of human brain, whereas Tf itself is found mainly localized to glial cells [40]. In addition, p97 is specifically expressed on reactive microglial cells associated with amyloid plaques in post-mortem AD brain tissue. All other microglia not associated with senile AD plaques and those found in brain tissue from other neurodegenerative diseases, including Parkinson's disease, progressive supranuclear palsy, Huntington disease and amyotrophic lateral sclerosis, do not express detectable levels of p97 [21].

A quantitative serum and cerebrospinal fluid (CSF) p97 assay has been developed based on the radioimmunoassay (RIA) technique, which employs capture

antibodies bound to sub-micron polystyrene beads and radio-labelled secondary antibodies. In a prior study, which used a less sensitive (Pandex) immunofluorescence assay, the levels of p97 in a small sample of AD subjects were shown to be significantly elevated in CSF and serum compared to neurologically normal controls without overlapping values [24]. Additionally, extrapolation from this published data suggested that the p97 concentration might begin to increase an estimated two years prior to first observed symptoms of AD [24]. The current study was undertaken to reproduce the results from the initial Pandex pilot study and was based on a RIA technique that was developed. It was also carried out to further investigate the diagnostic utility of serum p97 in a larger sample of newly referred subjects using a strict double blinded technique where the clinical assessment and the laboratory assessment of p97 were performed independently and concurrently.

2. Methods

The current study was a cross-sectional evaluation of consecutive newly referred subjects to the UBC Clinic for Alzheimer's Disease and Related Disorders (CADRD) for the assessment of dementia during an 8 month recruitment period. Serum samples were obtained from these patients at the time of their cognitive and neurological assessment for dementia. The primary study objectives were to evaluate the utility of serum p97 as a diagnostic for AD by determining its sensitivity and specificity as well as a dichotomous cut-point where both sensitivity and specificity were simultaneously maximized [9,13,15,18,43].

2.1. Selection of Cases

There were 119 subjects in this study, 48 subjects with "probable" or "possible" AD according to the NINCDS-ADRDA criteria and 71 controls. All AD subjects had a complete neurological/medical examination, neuroimaging with CT or MRI, and selected laboratory studies. Formal neuropsychological assessment was conducted in selected AD cases, particularly when cognitive deficits were mild or when additional cognitive assessment was required for diagnostic clarification. Control subjects were derived from caregivers or other informants who had accompanied referred AD subjects to the CADRD and who consented to screening cognitive testing and blood drawing for p97 assay. All control subjects had a Modified Mini Mental State

Exam (3MS) score of > 88 . In the Canadian Study of Health and Aging (CSHA) a 3MS value of < 88 was sensitive at identifying all dementia cases [31,43]. Each consenting subject provided a sample of blood for the p97 assay on their initial visit to the CADRD. On the basis of the clinical assessment, without access to the p97 results, a provisional diagnosis was entered into the database for each subject by one of the clinical investigators (HF, DF, BLB) while the laboratory was similarly blinded to clinical information until the results were analyzed [7,12,33,39].

2.2. Serum p97 detection methods

The p97 assays were run on aliquoted sera that had been prepared according to the following protocol. Following the blood draw, the sample was allowed to clot at room temperature for at least 60 min. The serum was then recovered by centrifugation at 4°C and immediately frozen at -80°C and stored for up to 1 week. Samples were then thawed only once at 4°C overnight prior to carrying out the p97 assay. The p97 radioimmunoassay (RIA) used in this study utilized purified, secreted recombinant p97 as the standard. This p97 was recovered from the media used to culture Chinese hamster ovary (CHO) cells, which had been transfected with the human p97 gene, and purified using an affinity column containing immobilized anti-p97 monoclonal antibodies (mAb) [14,22]. The assay was based on creating a sandwich between the mAb Hyb C immobilized onto carboxypolystyrene beads ($3\ \mu\text{m}$), the sample p97 and secondary mAb, L235 labeled with ^{125}I . The serum sample was diluted 1:4 in dilution buffer (PBS, 1% BSA and 0.1% sodium azide) [22]. $100\ \mu\text{L}$ diluted serum samples and recombinant human secreted p97 spiked heat inactivated (HI) serum samples, which acted as standards, were added to a 5 mL tube; $20\ \mu\text{L}$ of Hyb C coated beads were added and incubated for 1 hour at room temperature. $100\ \mu\text{L}$ of ^{125}I labeled mAb L235 at $10\ \mu\text{g}/\text{ml}$ was next added in dilution buffer and incubated for 1 hour at room temperature. After centrifugation, the solution was removed from the pellet, which was then washed once with 1 mL washing buffer (PBS, 1% BSA and 0.1% sodium azide). The tube plus pellet was then placed in a Gamma counter. p97 standards were tested in duplicate; serum samples were tested in quadruplicate. The determination of p97 concentration was made through a calculation of the average of all CPM (counts per minute) for each sample. A calibration curve was prepared based on the HI serum sample

secreted p97. The slope, intercept, and R^2 were determined by linear regression. HI denatures p97 and renders it undetectable by the p97 RIA. The p97 concentration of the undoped sample was then determined from this calibration curve according to the following formula:

$$\begin{aligned} & \text{p97 concentration} \\ &= \text{dilution} \times (\text{Sample mean CPM} \\ & \quad - \text{HI sample mean CPM}) / \\ & \quad \text{Slope of calibration curve} \end{aligned}$$

All serum samples were assayed by a single laboratory. Blood samples were considered as not being evaluable if they were coagulated, or if they had intercepts greater than 10,000 CPM (high background). Evaluable samples were generally in the range 2,000–3,000 CPM (low background). If one of the quadruplicate CPM readings differed from the average by 3 standard deviations then the reading was ignored and the sample mean CPM determined from the remaining three readings. Sample data would be ignored and the sample re-tested for serum p97 concentration, if: 1) two of the quadruplicate CPM readings differed from the average by 3 standard deviations; 2) the measured p97 concentration was greater than the range of the standards (i.e., $> 120\ \text{ng}/\text{ml}$); 3) the HI sample CPM was greater than the "0" doped sample CPM or 4) the R^2 value of the calibration curve was < 0.9 . If after re-testing the sample data was still unacceptable, the subject was deleted from the trial.

2.3. Statistical methods

Descriptive statistics of means and standard deviations which, were calculated for continuous variables such as the serum p97 concentrations and percentages presented for categorical study variables. Continuous variables were compared between cases and controls using the t-test and Mann Whitney test. Spearman and Pearson correlations, along with their statistical significance, were calculated between p97 and other continuous variables. Sensitivity, specificity, and positive predictive value were calculated for the various cut-points of p97 that were used to classify cases as AD. The Kolmogorov-Smirnov test was used to compare distributions of p97 to the normal distribution.

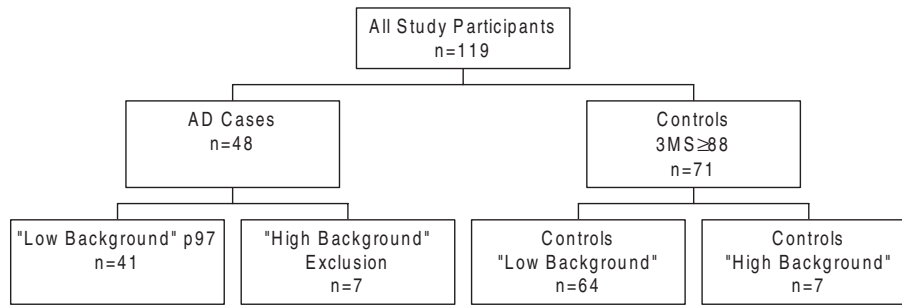


Fig. 1. Case classification of samples.

Table 1
Descriptive characteristics of AD cases and controls with serum p97 results

Variable	AD cases		Controls		p-value	
	N	Mean	N	Mean	(Significance measurement)	
					t-test	Mann-Whitney
Age	41	72.5 ± 7.0	64	61.0 ± 15.7	0.000	0.000
p97 concentration	41	41.0 ± 20.0	64	19.5 ± 8.3	0.000	0.000
Replication SD of p97 (ng/ml)	41	2.34 ± 1.28	64	1.79 ± 1.08	0.02	0.008
3MS	35	58.1 ± 19.8	64	97.1 ± 3.0	0.000	0.000
MMSE	39	17.5 ± 6.0	64	29.4 ± 0.9	0.000	0.000
DAD (%)	34	65 ± 26			NA	
FRS (sum of boxes)	37	26.3 ± 5.9			NA	

3. Results

Figure 1 illustrates the case classification process according to the study protocol. There were 48 AD subjects and 71 control subjects who participated in the study. Seven AD and 7 control subjects were excluded from the analysis for reason of having high background activity. The analysis of evaluable AD cases ($n = 41$) compared to cognitively normal controls ($n = 64$) is presented in Table 1. The mean age of the AD cases (mean 72.5 ± 7.0 yr., range 50–84 yr.) was significantly older than the control group (mean 61.0 ± 15.7 yr., range 30–90 yr.) which included some family members of AD cases ($p < 0.001$ t-test). There were more women than men in the AD group (66%) compared to controls (55%) though the difference was not statistically significant ($p = 0.3$). The mean values in AD cases of 3MS (58.1 ± 19.8), MMSE (17.5 ± 6.0), DAD (65 ± 26), and FRS (26.3 ± 5.9) fall within the mild to moderate disease severity range.

Mean values of serum p97 were increased twofold in AD subjects and were highly significantly different from controls ($p = 0.0001$ t-test; $p = 0.0001$ Mann Whitney). The mean for highly possible/probable AD cases (41.0 ± 20.0 ng/ml) was over double that of the mean for the control group (19.5 ± 8.3 ng/ml). For those individuals > 60 yr., the mean for the AD

group was again over double that of the controls (AD 40.9 ± 19.1 ng/ml and controls 19.2 ± 8.5 ng/ml; t-test $p < 0.001$, Mann Whitney $p < 0.001$) indicating that age did not appear to have an effect of the observed differences. For the full sample it was noted that the standard deviations in serum p97 levels were substantially larger in AD cases (20.0) than in controls (8.3) $p < 0.001$, F-test. The p97 replication standard deviation additionally was larger in AD cases than controls, indicating greater variability across repeat assays.

The scatter plot shown in Fig. 2 illustrates the difference between the mean serum p97 value of the AD group compared to controls. The controls were split into two groups, old controls > 60 yr. and young controls < 60 yr., in order to illustrate the lack of variation in p97 serum levels with age in control groups. Within the AD case population the relative lack of p97 concentrations around 30 ng/ml for AD cases suggested the possibility of a bimodal distribution. This was examined further with the Kolmogorov-Smirnov test of the null hypothesis of normality which yielded $p = 0.4$, a result consistent with normality and a single population, rather than two. However, in another study this bimodal distribution was noted and was statistically significant [36].

The upward shift of the AD p97 distribution and the limited overlap between cases and controls, as seen in Fig. 2, served as a basis for using serum p97 cut points

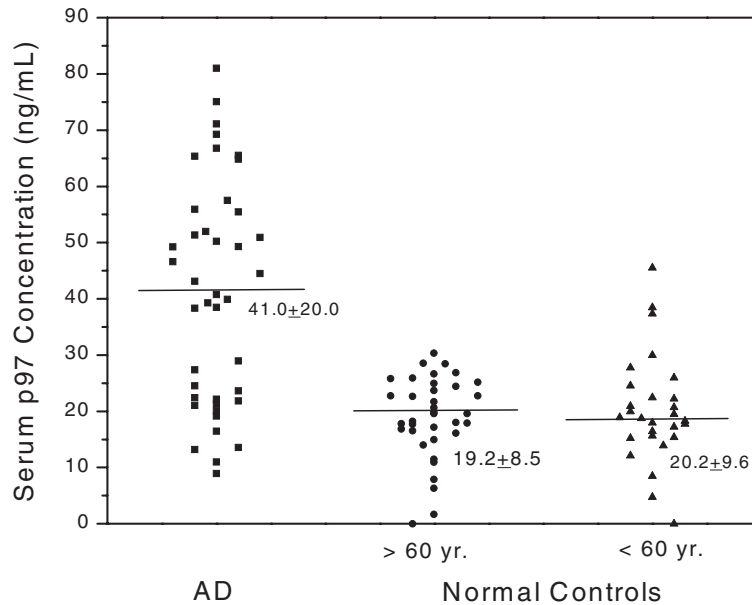


Fig. 2. Mean p97 concentration for AD cases and controls.

to classify subjects suffering from AD. To determine the ability of using serum p97 concentration as a diagnostic of AD, the data was assessed by the statistics of sensitivity, specificity and positive predictive value calculated for specified p97 concentrations. Table 2 presents the statistics for a range of cut points for serum p97 values.

A cut-point of 23 ng/ml approximately equalized sensitivity and specificity (71 and 72%, respectively), although the confidence intervals (CI) were fairly wide (for a sensitivity of 71% the 95% CI was 54–84% and for a specificity of 72% the 95% CI was 59–82%). No controls had values of > 46 ng/ml where specificity was 100%. For $k = 1, 2$ and 10, the concentrations that yielded PPV of 80% or larger were 26, 29, and 46 ng/ml respectively. The sensitivities, specificities, and positive predictive values for subjects aged 60 yr. and over were very similar to those found for the full sample (at a cut point of 24 ng/mL the sensitivity and specificity were both 70% with a PPV of 70%). Figure 3 presents an empiric receiver operator curve (ROC) for the AD and control samples with an area under the curve (AUC) of 0.812 for all subjects and improves to 0.831 for subjects over the age of 60 yr.

Age had no correlation with p97 for either cases or controls. A fitted regression model for p97 versus age including both linear and quadratic age terms was not significant ($p = 0.1$). There were no significant associations in AD cases between p97 and duration disease

as estimated by the caregiver, or gender. There were no significant correlations between p97 and measures of global disease severity (FRS), functional disability (DAD total score) or cognitive measures (3-MS total scores) (Table 3). There was a weak correlation of p97 with the SD of the p97 values in cases while in controls there was a significant negative correlation.

There were a total of 7 AD cases and 7 controls that were excluded from the analysis due to high background levels of p97 (see Fig. 1). The 7 AD cases with high background activity had mean p97 assay values of 118.4 ± 85.7 ng/ml compared to mean of 41 ± 20.0 ng/ml in low background cases ($p < 0.001$ Mann-Whitney test). Similarly the 7 high background control cases had mean p97 assay values of 56.2 ± 23.1 ng/ml, threefold higher than low background cases 19.5 ± 8.3 ng/ml ($p = 0.0001$ Mann-Whitney; 56 vs. 20, $p < 0.001$). Spearman correlations between the dichotomous background indicator (high vs. low) for all of the continuous and dichotomous variables were determined. These variables included demographic and clinical factors as well as p97 results. All cases ($n = 48$) and controls ($n = 71$) were included, regardless of background level or diagnosis. The only significant correlations between background level and other factors occurred for p97 concentration ($\rho = 0.46$, $p < 0.001$) and the p97 replication standard deviation ($\rho = 0.33$, $p < 0.001$). These correlations are a consequence of the case-control dif-

Table 2
Empirical sensitivity and specificity for specified p97 concentrations

Cutpoint (p97 Conc.)	Sensitivity		95% CI*	Specificity		95% CI*	PPV		
							$k = 1$	$k = 2$	$k = 10$
10	0.98	40/41	(0.87, 0.99)	0.11	7/64	(0.05, 0.21)	0.52	0.35	0.10
20	0.85	35/41	(0.71, 0.94)	0.58	37/64	(0.45, 0.70)	0.67	0.50	0.17
23	0.71	29/41	(0.54, 0.84)	0.72	46/64	(0.59, 0.82)	0.72	0.56	0.20
27	0.66	27/41	(0.49, 0.80)	0.88	56/64	(0.77, 0.94)	0.84	0.72	0.35
30	0.61	25/41	(0.45, 0.76)	0.94	60/64	(0.85, 0.98)	0.91	0.83	0.49
40	0.51	21/41	(0.35, 0.67)	0.98	63/64	(0.92, 0.99)	0.97	0.94	0.77
46	0.44	18/41	(0.28, 0.60)	1.00	64/64	(0.94, 0.99)	1.00	1.00	1.00

*CI = confidence interval. Sensitivity refers to the percentage of AD cases that are correctly classified as cases, and specificity to the percentage of controls that are correctly classified as controls, calculated for specified cut points on the p97 scale. Positive predictive value (PPV) refers to the percentage of true AD cases among the subjects who (correctly or incorrectly) are classified by the cut point rule as having AD. Specifically, $PPV = \{sens./[sens. + k(1-spec.)]\}$, where sensitivity and specificity are expressed as proportions between one and zero and k refers to the ratio of controls to AD subjects among those screened.

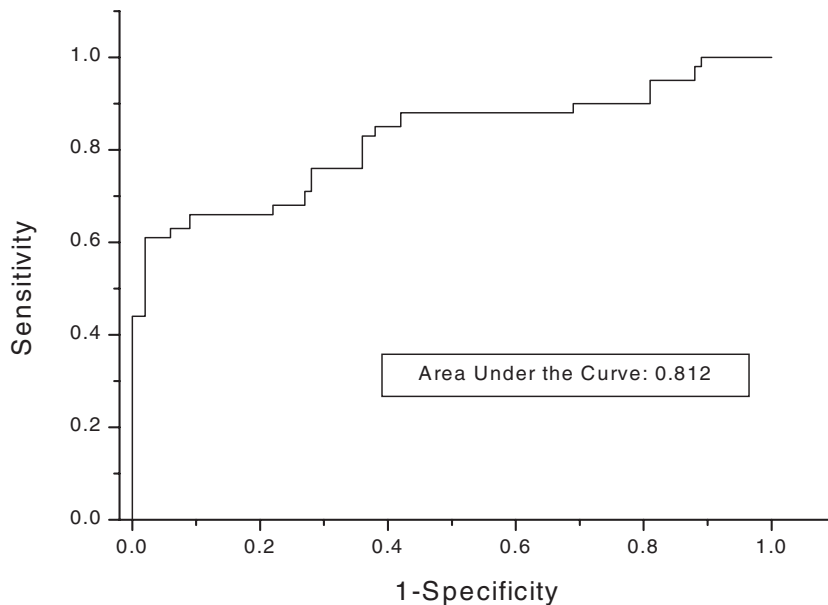


Fig. 3. Empiric receiver operator curve (ROC) for AD and control samples.

ferences in p97 concentrations. All other correlations between background and other factors were less than ± 0.1 . Thus, the occurrence of high background seems to be associated primarily with higher p97 assay values although the cause of the high background remains uncertain.

4. Discussion

To date the development of non-invasive biological markers for AD has had limited success. There have been a number of confirmed familial autosomal dominant mutations on chromosomes 21, 12 and 1. How-

ever, they account for only a small percentage of the total number of AD cases. Genetic risk markers including Apolipoprotein E genotyping may account for an estimated 50% of late onset sporadic disease. There is still a need to develop biological markers with diagnostic utility both for research studies as well as for clinical practice. Recent reports of the utility of measured APP fragments in blood and heme oxygenase levels hold promise but still require replication. The most widely investigated biological markers for AD including measured levels of $A\beta_{1-42}$; $A\beta_{1-40}$, and tau all involve cerebrospinal fluid analysis, which limits their applicability and acceptance.

In this study, conducted under a strict double-

Table 3
Association of p97 concentration with study characteristics for AD cases and controls

Variable	Cases			p-value		Controls			p-value	
	N	Pears. or mean \pm SD	Spear.			N	Pears. or	Spear. mean \pm SD		
SD of [p97]	41	0.360 p	0.252 s	0.02 p	0.11 s	64	-0.392 p	-0.409 s	0.001 p	0.001 s
SD of Age	41	0.002 p	0.084 s	0.99 p	0.60 s	64	-0.165 p	-0.120 s	0.19 p	0.34 s
Age: 30-59	4	41.4 \pm 30.7		0.96 t	0.97 mw	27	19.8 \pm 8.1		0.78 t	0.94 mw
60-90	37	40.9 \pm 19.1				37	19.2 \pm 8.5			
Duration of AD (months)	41	-0.241 p	-0.219 s	0.13 p	0.17 s	0				
Gender: Male	14	40.7 \pm 22.1		0.95 t	0.97 mw	29	19.0 \pm 6.4		0.71 t	0.90 mw
Female	27	41.1 \pm 19.3				35	19.8 \pm 9.6			
3MS	35	-0.042 p	-0.095 s	0.81 p	0.59 s	64	-0.032 p	-0.039 s	0.80 p	0.76 s
DAD	34	0.134 p	0.132 s	0.45 p	0.46 s	0				
FRS	37	-0.002 p	0.084 s	0.99 p	0.62 s	0				

Abbreviations: Pears. or p = Pearson correlation coefficient, Spear. or s = Spearman correlation coefficient, t = t-test, mw = Mann-Whitney test, NA = not applicable.

blinded procedure, there is a highly significant difference demonstrated between the mean concentrations of serum p97 in AD cases and cognitively normal controls. These findings confirm similar results from a previous study which utilized blood to measure p97 levels in AD and controls [24,36]. In the current study there is an overlap seen in this study in p97 levels between the AD cases and the cognitively normal controls, that has not been previously noted [24]. The presence of low or normal p97 levels in some clinically diagnosed AD cases raises the possibility that not all AD cases express high p97 levels. We postulate that this may result from the heterogeneity of Alzheimer pathology or from other factors such as medications or genetic polymorphisms that may interact with the underlying disease process. In previous studies, it has been reported that microglia associated with the senile neuritic plaques (SNP) overexpress p97 on their cell surface [21,40]. There are however AD cases where SNP pathology is less prominent than neurofibrillary tangle pathology [29]. It is possible that in such instances where subjects have lower SNP scores they will also have lower p97 levels due to low plaque activation of the microglial cells. Recently there have also been described gene polymorphisms of Interleukin 1, a pro-cytokine that seems to influence both the risk and expression of AD through inflammatory control mechanisms. This too might have some effect on the levels of expressed p97 relating to microglial activation. Additionally other co-morbid pathologies in the brains of AD subjects such as vascular lesions might influence p97 expression. Neuropathological evaluation of our study participants will be important in trying to draw correlations of p97 levels with plaque scores and with measures of microglial activation.

The gradual decline of sensitivity seen with increasing p97 concentrations in this study reflects on the wide distribution of values in the AD case group. On the other hand, the rapid rise of specificity is due to the much narrower distribution of the control p97 values where the specificity at values of > 30 ng/mL is upwards of 90%, reaching 98% at 40 ng/mL. Approximately equalized sensitivity and specificity (71-72%) was reached with a cut-point of 23 ng/mL. This cut point from the current study must be treated with some caution as the 95% confidence intervals for both the sensitivity (54-84%) and specificity (59-82%) at this value were quite wide, and the sample size relatively small. If, however, such a cut-point were confirmed in larger study samples it is possible that such a paradigm might allow subjects with a p97 score above a specified level to be most reliably classified as AD. For the balance of AD subjects with below cut point p97 values, it would not be implied that they did not have AD rather that it was simply less likely. While such a single cut-point might be of interest in clinical practice, other cut-points would have different utility in other settings. If there were a need for a screening test that had a high degree of sensitivity that would miss very few AD cases at the expense of specificity, then a relatively low cut-point could be used. On the other hand, if avoiding false positive diagnoses were desired for purposes of a proof of principal clinical trial for AD with leading edge technology, then a high p97 cut-point could be used to yield a high specificity at the expense of reduced sensitivity. The ROC curve representation of sensitivity and specificity reveals an area under the curve (AUC) of 0.81 (0.831 for > 60 yr.), which suggests that overall the p97 serum assay does have some utility in identifying AD.

The interpretation of the positive predictive value results of the serum p97 assay requires consideration of the k constant, which is determined based on the population of individuals for whom the test is intended. Clearly, if the population to be tested has few AD cases and large numbers of cognitively normal individuals, as might be seen in a population over the age of 65 (3.3% prevalence of AD in the Canadian Study of Health and Aging) [5] a positive predictive value of $> 80\%$ would be obtained at only the very highest concentrations of p97. However, if the population to be tested were over the age of 85 where the AD prevalence rates were 35–40% a much lower k constant would be applicable and 80% PPV might be reached at much lower p97 concentrations e.g. ~ 30 ng/mL. It can be concluded that the utility of elevated concentrations of p97 will depend on the target population.

In this study, there appeared to be no significant correlation between the clinical measures of disease severity and serum p97 levels for AD cases. This observation has also been noted with other AD biological markers including CSF tau levels, and A β 1-42 levels [1,17,30,38,42]. Importantly, it is again noted that p97 does not have a correlation with age or gender in cognitively normal individuals, so it is likely that p97 results can be interpreted without regard to age and gender.

In previous studies it has also been shown that non-disease factors including medications can influence brain microglial activation with potential influence downstream on p97 expression. The long-term use of non-steroidal antiinflammatory drugs (NSAIDs) in cognitively normal subjects is associated with a reduced number of activated microglia [27,28]. Although the difference is most noticeable in-patients with age-related senile plaques, patients with no plaques have also been shown to have a significant reduction in the baseline level of microglial activation. A similar effect might then be expected in AD with resultant effect on p97 expression. Unfortunately the small number of subjects in this study using anti-inflammatory drugs in this study did not allow us to evaluate the effect of such medications on the expression of p97 and these subjects were excluded from our analyses.

The current study used a serum radioimmunoassay that was developed to overcome some of the limitations of the Pandex immunofluorescence assay which had been used in previous studies [24]. In the RIA format, the fluorescein used to label the secondary antibody in the Pandex format was replaced with ^{125}I and the resulting complex was read in a gamma counter. With the RIA, the standard deviation of test values of AD

cases was noted to be significantly greater than that of cognitively normal control values with increasing SDs correlated to increasing p97 levels. The reason for this increasing variability in test results at higher p97 levels is presently uncertain but was observed both in subjects and controls. Some of the sera of both AD and control subjects had high background p97 levels measured which lead to their a priori exclusion as evaluable subjects. This exclusion involved 13% of AD cases, and 10% of cognitively normal controls. The results of analyzing these samples showed that their mean p97 values were significantly higher (\sim three times) than those that were considered as evaluable in the primary study analysis. The high background p97 levels appeared to be significantly correlated with larger replication standard deviations. Furthermore, the slope of the calibration curve developed from samples with high background were significantly less than evaluable samples. The reduced slope increased the error and uncertainty in determining the p97 concentrations. It is notable that the significant difference between the mean serum p97 levels of the AD and control groups was maintained in these samples with high background. We are also interested in examining if p97 is susceptible to serum proteases and it has been suggested that examining other bodily fluids may provide a more accurate determination of p97 levels.

5. Conclusion

The current study supports the role of p97 as a biological marker for AD where AD subjects have serum levels, which are increased compared to cognitively normal controls. This reproduces a major finding from the initial study of p97 in AD. The current study does find significant overlap in lower p97 levels between AD cases and cognitively normal controls possibly relating to the underlying biology of microglial activation and expression of p97 in AD. The sample size in the current study was not adequate to fully determine a stable optimal cut-point for sensitivity and specificity due to the wide confidence intervals around this point. As with other biological markers of AD, including tau and apolipoprotein E genotype, in this study serum p97 does not correlate with clinical measures of AD severity. Future studies with longitudinal and neuropathological assessment are in progress as are cohort studies to further test the above observations in larger sample sizes. The reproducibility of the elevations of serum p97 in AD underlines the importance of further studies

to determine its role as a biological marker or diagnostic adjunct in AD. Future work will address whether it is also useful for the differential diagnosis of AD against other dementias/neurodegenerative diseases or agonal state.

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