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Pittsburgh Compound B and the Postmortem Diagnosis of Alzheimer's Disease

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Abstract

Objective—Deposition of the amyloid- β (A β) peptide in neuritic plaques is a requirement for the diagnosis of Alzheimer's disease (AD). Although the continued development of *in vivo* imaging agents such as Pittsburgh Compound B (PiB) is promising, the diagnosis of AD is still challenging. This can be partially attributed to our lack of a detailed understanding of the interrelationship between the various pools and species of A β and other common indices of AD pathology. We hypothesized that recent advances in our ability to accurately measure A β postmortem (for example, using PiB), could form the basis of a simple means to deliver an accurate AD diagnosis.

Methods—We conducted a comprehensive analysis of the amount of A β 40 and A β 42 in increasingly insoluble fractions, oligomeric A β , and fibrillar A β (as defined by PiB binding), as well as plaques (diffuse and neuritic), and neurofibrillary tangles (NFTs) in autopsy specimens from age-matched, cognitively normal controls (N=23) and AD (N=22) cases, across multiple brain regions.

Results—Both PiB binding and the amount of SDS soluble A β were able to predict disease status; however, SDS soluble A β was a better measure. Oligomeric A β was not a predictor of disease status. PiB binding was strongly related to plaque count, although diffuse plaques were a stronger correlate than neuritic plaques.

Interpretation—Although postmortem PiB binding was somewhat useful in distinguishing AD from control cases, SDS soluble A β measured by standard immunoassay was substantially better. These findings have important implications for the development of imaging based biomarkers of AD.

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Introduction

The amyloid- β peptide ($A\beta$) is widely believed to be a critical factor in the development of Alzheimer's disease (AD) ^{1, 2}. The $A\beta$ found in the brain is actually a mixture of different peptides, with varying modifications, and with considerable N- and C-terminal heterogeneity; many of these forms of $A\beta$ are, to some extent, present in the neuritic plaques that help define the disease ^{3, 4}. Understanding the contribution of $A\beta$ to AD has advanced considerably in the last 20+ years, based on the identification and characterization of distinct species and pools of $A\beta$. This has allowed us to understand the potential role of $A\beta$ in AD beyond the context of plaques. In recent years, this has led to a conceptual shift in focus towards soluble, oligomeric forms of the peptide as a primary toxic species in AD pathogenesis ^{2, 5, 6}. However, various pools of $A\beta$ exist in equilibrium in the AD brain, ranging from the highly soluble to the nearly insoluble ⁷. A critical question that remains unanswered is the relative contribution of each of these pools of $A\beta$ to the overall burden of pathology in the AD brain, including how the different forms of $A\beta$ relate to one another, and how these relate to the major pathologic hallmarks of AD ⁸.

Significant technological advances in recent years now allow imaging of amyloid pathology *in vivo*. These methods evaluate $A\beta$ burden in a living person, and could potentially serve as both a biomarker, and as a diagnostic tool to detect incipient disease ⁹. A derivative of the amyloid dye Thioflavin T, Pittsburgh Compound B (PiB, 2-[4'-(Methylamino)phenyl]-6-hydroxybenzothiazole), ¹⁰ is currently the best studied of these imaging agents. After labeling with ¹¹C for PET imaging, increased PiB retention can be quantified in brain regions known to accumulate $A\beta$ deposits in AD patients ¹¹. The utility of PiB and other probes for determining how mild cognitive impairment (MCI) progresses to AD is being evaluated in a large, multicenter effort ^{11–13}.

Despite progress in understanding the contribution of $A\beta$ to neuronal dysfunction and neurodegeneration, the lack of a detailed analysis of the interrelationship between $A\beta$ and the other common indices of AD pathology has hampered our understanding of the development and progression of the disease. For instance, our current knowledge of the quantitative relationship between PiB binding and amyloid pathology in the brain is limited ^{14–18}. A better understanding of how these variables relate to one another is essential for the continued development of reliable diagnostic biomarkers for AD. In the present study, we analyzed increasingly insoluble pools of biochemically distinct species of $A\beta$ to quantify their relative contributions to the overall $A\beta$ burden, and to determine if any of these measures could be used to predict disease status.

Methods

Subjects and Neuropathological Assessment

Samples were obtained from the Alzheimer's Disease Center tissue repository at the University of Kentucky ^{8, 19, 20}. Controls (N = 23; 87.0 ± 6.5 years) had no history of ante-mortem cognitive impairment and were age-matched to AD cases (N = 22; 85.8 ± 7.6 years). The average postmortem interval (PMI) was similar for both groups (Control: 3.0 ± 0.8 ; AD: 2.9 ± 0.7 , hours). The details of the recruitment, inclusion criteria, and mental status test battery for our normal control group have been described previously ²¹. Cause of death exhibited no distinct pattern in any group. Human tissue collection and handling followed PHS guidelines and the University of Kentucky IRB.

Details of our autopsy procedures have been described elsewhere ^{8, 20, 22}. Briefly, brain weights were determined and a gross neuropathologic evaluation carried out at the time of autopsy. We used four disease affected brain regions (inferior parietal lobule [BA40];

hippocampal formation [CA1 and subiculum]; midfrontal gyrus [BA9]; superior and middle temporal gyri [SMTG, BA21 and 38]), and one unaffected region (cerebellum). For some analyses across brain regions, due to limited tissue availability, only a subset of cases was used. Tissue samples were dissected and frozen or fixed in 4% paraformaldehyde. For histology, paraffin-embedded specimens were cut at 8- μ m and stained with hematoxylin-eosin, the modified Bielschowsky method, or Gallyas silver method. Some sections were also immunostained with 10D5 (for A β). Braak staging²³ used both Gallyas and Bielschowsky-stained sections. Neurofibrillary tangles (NFTs), diffuse plaques (DPs), and neuritic plaques (NPs) were counted as described^{8, 20, 22}. Briefly, plaques were counted using a 10x objective (field size: 2.35 mm²) in the 5 most involved fields in each section. The most involved fields were determined by studying the whole section and marking it. Plaques were classified as DPs (plaques without surrounding dystrophic, argyrophilic neurites) and NPs (plaques surrounded by dystrophic, argyrophilic neurites) in each region. NFTs were counted with a 20x objective (field size: 0.586 mm²), also in the 5 most involved fields. An arithmetic mean was calculated for each count.

Measurement of A β

The amount of A β in tissue samples was determined using a three-step serial extraction procedure. This approach takes advantage of increasingly more denaturing conditions to serially extract A β that is progressively more insoluble, and is followed by the quantitative measurement of A β by ELISA. This is a standard procedure in our laboratory, and details of the methodology and antibodies used have been published^{7, 24–27}. Briefly, tissue was homogenized in standard PBS buffer (pH = 7.4, 1.0 ml/200 mg of tissue, with complete protease inhibitor cocktail, PIC; Amresco), and the supernatant collected following centrifugation at 20,800 $\times g$ for 30 min at 4°C. The pellet was serially re-extracted by brief sonication in 2% SDS (w/v, with PIC) followed by 70% (v/v) formic acid (FA). Sample extracts were stored frozen at –80°C until time of assay. Prior to ELISA, extracted material was neutralized and diluted²⁸. Standard curves of synthetic peptides were prepared in dilution buffer, and all standards and samples were run at least in triplicate. Capture was conducted using antibody Ab9 (human sequence A β 1–16); A β 40 was detected with Ab13.1.1 and A β 42 was detected with 12F4 (Covance)^{7, 25, 26}. Single-site assays for SDS-soluble, oligomeric A β were performed in a similar manner, except using antibody 4G8 (against A β 17–24; Covance) for capture and biotinylated-4G8 for detection, and comparing signals against synthetic oligomeric A β ^{24, 29}.

³H-PiB binding to brain homogenates was carried out in a 96-well version of the filtration assay of Klunk *et al.*¹⁵, as per Rosen *et al.*³⁰. Briefly, PBS homogenate was diluted into a 96-well polypropylene plate in triplicate. Two hundred μ l of 1nM ³H-PiB was added to each well; 1 μ M of unlabeled competitor (BTA-1) was added to the third well to determine nonspecific binding (by subtraction). Femtomoles of ³H-PIB bound were calculated per wet weight of tissue after correcting for counting efficiency. Prior to analysis, PiB binding values were standardized between data sets to internal controls that were cases common to each set.

For examination of A β by Western blot, 10 μ l of the FA fractions were dried under vacuum (Labconco Centrifugal Concentrator), then reconstituted in standard loading buffer. These samples, as well as 10 μ l of the PBS and SDS extracts, were separated on 12% Bis-Tris SDS-PAGE with MES XT running buffer (BioRad). The proteins were then transferred to nitrocellulose. After transfer, the membranes were boiled in PBS for 5 minutes and blocked overnight in 1% BSA and 2% BlockAce (Serotec) in PBS. The membranes were probed with antibody 6E10 (Covance; 2 μ g/mL in PBS with 5% nonfat dry milk), followed by rabbit anti-mouse IgG (Rockland). Reactive bands were visualized with Super Signal West Dura HRP Substrate (Pierce) and exposed to film.

Statistical Analyses

We recently described a similar analytical strategy in a subset of these cases used to evaluate RNA oxidation in AD²⁷. Data were analyzed using the SPSS®. Multivariate analyses (MANCOVA) were adjusted for age, postmortem interval, and gender. Group comparisons were made using Student's *t*-test and adjusted where necessary for multiple comparisons using the Holm-Bonferroni Method³¹. To control for inflation of the type I error rate, significance of correlations was similarly corrected. Curve fitting was performed using Sigmaplot®.

Results

As expected, the AD cases had lower MMSE scores; lower brain weight; and more DPs, NPs, and NFTs (Table 1). Neuropathology was significantly increased in all four affected brain regions (Supplementary Figure 1). The number of NFTs was the strongest negative correlate ($R^2 = 0.73$, $p < 0.0001$) of MMSE scores (NPs, $R^2 = 0.47$; DPs $R^2 = 0.32$). The total amount of extractable A β and PiB binding (essentially fibrillar A β) was significantly higher in the AD cases. A multivariate analysis across all four brain regions confirmed that both total A β ($p < 0.04$) and PiB binding ($p < 0.01$) were significantly elevated in AD cases relative to controls (Figure 1). Using a strategy similar to that used for the study of *in vivo* PiB binding, we standardized postmortem PiB binding in the disease affected regions to the cerebellum, which is considered to be somewhat spared in AD; the differences were no longer significant when analyzed using this method (Supplementary Figure 2A), indicating that it is not necessarily useful to standardize PiB binding data to this region. However, we did detect increases in PBS and SDS soluble A β in the cerebellum, as well as a small increase in PiB binding (Supplementary Figure 2B), indicating that there are disease related changes in this brain region in AD. Although what these changes mean for the AD brain are unknown, it is worth noting that recent guidelines for the neuropathologic assessment of AD take into account cerebellar pathology^{32, 33}.

Oligomeric A β failed to reach overall statistical significance ($p < 0.14$) in the multivariate model, and distinct A β oligomers from the late stage AD brain were difficult to resolve by immunoblot (Supplementary Figure 3). Although multiple bands of various molecular weights can be detected in the AD brain, we did not see consistent evidence of any prominent species that might contribute disproportionately to the pathology, such as A β *56³⁴. The amount of oligomeric A β appeared less in AD cases using the single site ELISA approach. Although these data do not rule out the influence of some form of oligomeric A β in AD, given these inconclusive results, we subsequently excluded oligomeric A β as a study variable. Overall, the majority of the A β peptide in the brain was relatively insoluble, and was found in the FA fraction (Supplementary Figure 4).

Postmortem PiB binding was strongly correlated with the total number of plaques (Figure 2). This relationship still held if zero values were included or excluded from the analysis. Although PiB binding could be used to predict the number of either DPs ($R^2 = 0.68$, $p < 0.001$) or NPs ($R^2 = 0.37$, $p < 0.001$), the relationship was stronger for DPs. Postmortem PiB binding was not related to the number of NFTs ($R^2 = 0.04$, $p < 0.2$). However, both NPs ($R^2 = 0.46$, $p < 0.0001$) and NFTs ($R^2 = 0.50$, $p < 0.0001$) were robustly predicted by FA soluble A β 42. A cross validation study, where the cohort was divided into two approximately equal sized groups, yielded similar results. Finally, we used a stepwise multiple regression procedure to select the best subset of variables (DPs, NPs or NFTs; PBS, SDS or FA soluble A β) that explained the amount of postmortem PiB binding in the brain, as measured by R^2 ($p < 0.05$ to enter, $p < 0.10$ to remove). In this analysis, PiB binding was best explained by a function combining the number of DPs with the amount of PBS soluble A β ($R^2 = 0.50$, $F[2, 42] = 20.70$, $p < 0.001$).

In the final part of the data analysis, we determined which combination of A β measurements best discriminated AD cases from controls. To this end, we used a logistic regression model for the presence of disease. We then used a stepwise procedure to identify the subset of measurements that best predicted that a randomly selected subject was an AD case (by log odds ratio). A simple solution was obtained, as there was only one variable in the best subset: the total amount of SDS soluble A β , with an odds ratio of 1.06 ± 0.04 (95 % C.I.). A box plot of the same data showed minimal overlap between AD cases and controls (Figure 3). While informative, the overall sample size was too small to conduct a training-validation approach to selecting the best predictor of disease status. Although values between 90 and 95 could be used as a cut-off point to declare an individual to be an AD case, the data set was not large enough to identify a more precise number. PiB binding offered no discernible advantage as a diagnostic tool in this context.

Discussion

Although earlier studies reported an association between PiB binding and amyloid deposits (as defined primarily by immunohistochemistry)^{16–18}, little is known as to how PiB correlates with either NPs or DPs in the postmortem brain. We could detect a moderate to strong relationship between PiB binding and both types of plaques in the brain. Interestingly, the relationship was stronger between PiB binding and the number of DPs. In fact, it is likely that we are underestimating the strength of the relationship between PiB binding and DPs. For example, the relationship between total area occupied by A β (as detected by immunohistochemistry) and PiB binding¹⁷ is approximately linear. The plaque count data available for this study are prone to ceiling effects, and do not take plaque size into account. It is possible that the relationship may actually be stronger than what we observed in this data set.

We detected an approximate 11-fold elevation in extractable A β in the AD brain relative to controls, compared to more modest increases in the amount of PiB binding (~4 fold). We did not detect a robust elevation in oligomeric A β using the single site immunoassay method in this study. While these data do not rule out the possibility that oligomeric and fibrillar A β contribute to AD^{35,36}, they do indicate that any role these two species play in mediating AD pathogenesis occurs in the background of a tremendous amount of A β in other pools. It is possible that oligomeric and fibrillar species of A β contribute to AD via their synergy with other A β pools. It is also possible that the largest contribution of oligomeric A β to neuronal dysfunction and degeneration is of far greater importance earlier in the disease process (such as in preclinical AD³⁷ or in cases of amnesic MCI³⁸). Alternatively, the location or local concentration of oligomeric species may be the key to their ability to promote disease onset and progression. Finally, the immunoassay approach that we used in this case series detects only relatively large forms of oligomeric A β ; it is likely that smaller forms of oligomeric A β (*e.g.*, dimers, trimers, etc.) escape detection by this method. It is possible that these smaller forms of oligomeric A β may be more important for the disease process³⁹. Nevertheless, the lack of a clear increase in oligomeric A β in AD cases highlights that, in spite of recent advances, there are still limitations on our understanding of the disease process.

It is noteworthy that PiB binding in the postmortem brain was unable to discriminate between cases and controls as well as SDS soluble A β . Postmortem PiB binding is evaluated under highly favorable experimental conditions, while binding *in vivo* occurs under less optimal conditions. Blood flow and sequestration on the time scale of the imaging session are significant *in vivo* variables, whereas PiB has greater access to amyloid binding sites *ex vivo*. It is well known that there is considerable variability and heterogeneity in PiB retention in the human brain, with PiB retention increasing in a non-linear manner during the progression of disease and unable to uniformly discriminate AD from non-AD cases⁴⁰.

Postmortem PiB binding appears to involve only a small fraction of the total amount of A β in the brain, and it is likely that some portion of the unlabeled amyloid is significant to the disease process⁴¹. It is also likely that differentially soluble pools of A β in the brain deposit at different rates⁴², and it is unclear which of these pools PiB retention represents. The study of PiB binding *ex vivo* could shed light on these issues.

Although these data do not diminish the potential clinical utility of PiB as an agent for detecting the deposition of A β in the brain of living patients, they nevertheless raise the possibility that *in vivo* imaging using PiB is largely detecting deposits of A β that are considered by neuropathologists as less important to the AD clinical phenotype than either NPs or NFTs. For instance, in this study and many others, the strongest relationship between MMSE and neuropathology is with neocortical NFTs^{8, 20}, which are not related to the amount of PiB binding. This finding is in line with some of the earliest studies of PiB *in vivo*, which reported no relationship between PiB binding and MMSE scores¹¹. However, recent work in defining the preclinical state of AD has raised the intriguing possibility that DPs are being overly discounted as a factor in pathology³⁷. There are currently relatively few individuals that have come to autopsy following PiB neuroimaging. Further study of these individuals, and integration with other biomarker data¹³, will be essential for developing a reliable clinical screening procedure for the detection of AD and monitoring its progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Post-mortem PiB binding (*top panel*: $F[1,16] = 5.08$, $p < 0.04$) and the total amount of extractable Aβ (*bottom panel*: $F[1,16] = 5.08$, $p < 0.04$) were significantly higher in AD cases versus controls across multiple brain regions. * = $p < 0.05$; t -test, adjusted for multiple comparisons.

Figure 2.

Postmortem PiB binding is strongly correlated to the number of plaques as defined by standard neuropathologic criteria. PiB binding was a strong predictor ($F[1,30] = 26.21$, $p < 0.001$) of the total number of plaques; the relationship was stronger if zero values were included ($R^2 = 0.68$, $F[1,43] = 93.91$, $p < 0.001$). PiB binding was predictive for both the number of neuritic ($R^2 = 0.37$, $F[1,43] = 27.10$, $p < 0.001$) and diffuse plaques ($R^2 = 0.68$, $F[1,43] = 95.62$, $p < 0.001$).

Figure 3.

SDS soluble A β was identified as an exceptionally strong indicator of disease state. The area under the receiver operator curve was 0.978 ($p = 0.0016$), with a sensitivity of 100% and a specificity of 95.7%. The Hosmer-Lemeshow goodness of fit statistic ($p = 0.51$) indicated no evidence of a lack of fit. A boxplot of SDS soluble A β , segregated by disease state, shows almost no overlap between cases and controls (A); PiB binding, in contrast, shows considerable overlap (B).

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Table 1

Case Demographics[#]

		Pathologic Variables										
	N	Age (y)	Brain Weight (g)	PMI (h)	Braak Stage	MMSE (Final)	Diffuse Plaques	Neuritic Plaques	NFTs	Total Extractable Aβ	Oligomeric Aβ	PIB Binding
Control Cases	16 F / 7 M	87.0 ± 6.5	1192 ± 139	3.0 ± 0.8	1.3 ± 1.1	28.6 ± 1.4	11.2 ± 3.7	5.2 ± 1.1	0.1 ± 0.0	178 ± 37	137.5 ± 59.8	18.1 ± 5.8
AD Cases	14 F / 8 M	85.8 ± 7.6	1072 ± 108 *	2.9 ± 0.7	5.4 ± 1.1 *	12.1 ± 8.1 *	38.0 ± 3.3 *	9.9 ± 2.1 *	11.4 ± 2.4 *	1981 ± 535 *	44.0 ± 12.6	79.1 ± 13.6 *

[#] Values given are ± standard deviation for single value variables (age, brain weight, PMI, Braak Stage, and final MMSE), or ± standard error of the mean for variables based on the average of multiple measurements (pathologic variables). Aβ values (and PIB binding) are expressed in pmol/g of tissue; data were obtained from the inferior parietal lobule, but were similar in other affected brain regions.

^{*} Significantly different (p<0.05) between AD and control cases; all initial between group comparisons were performed using Student's t-test (2-tailed, equal variances not assumed) with a Holm-Bonferroni correction for multiple comparisons.