

Association between Amylin and Amyloid- β Peptides in Plasma in the Context of Apolipoprotein E4 Allele

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Abstract

Amylin, a pancreatic peptide that readily crosses the blood brain barrier (BBB), and amyloid-beta peptide (A β), the main component of amyloid plaques and a major component of Alzheimer's disease (AD) pathology in the brain, share several features. These include having similar β -sheet secondary structures, binding to the same receptor, and being degraded by the same protease. Thus, amylin may be associated with A β , but the nature of their relationship remains unclear. In this study, we used human samples to study the relationship between plasma amylin and A β in the context of the apolipoprotein E alleles (ApoE). We found that concentrations of A β 1-42 ($P < 0.0001$) and A β 1-40 ($P < 0.0001$) increased with each quartile increase of amylin. Using multivariate regression analysis, the study sample showed that plasma amylin was associated with A β 1-42 ($\beta = +0.149$, $SE = 0.025$, $P < 0.0001$) and A β 1-40 ($\beta = +0.034$, $SE = 0.016$, $P = 0.04$) as an outcome after adjusting for age, gender, ethnicity, ApoE4, BMI, diabetes, stroke, kidney function and lipid profile. This positive association between amylin and A β 1-42 in plasma was found regardless of the ApoE genotype. In contrast, the relationship between amylin and A β 1-40 in plasma seen in ApoE4 non-carriers disappeared in the presence of ApoE4. Using AD mouse models, our recent study demonstrates that intraperitoneal (i.p.) injection of synthetic amylin enhances the removal of A β from the brain into blood, thus resulting in increased blood levels of both amylin and A β . The positive association between amylin and A β , especially A β 1-42, in human blood samples is probably relevant to the findings in the AD mouse models. The presence of ApoE4 may attenuate amylin's capacity to remove A β , especially A β 1-40, from the AD brain.

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Competing Interests: The finding on amylin diagnosis and treatment for AD has been filed for the patent application. The patent is titled "Compositions, Methods and Assays Comprising Amylin or Amylin Analogs for Abeta Peptide Mediated Disorders" and numbered WO2013/151729 A1. However, the authors' patent application does not alter the authors' adherence to PLOS ONE policies on sharing data and material.

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Introduction

Amylin is a short peptide of 37 amino acids produced and secreted by the pancreas. Amylin and amyloid-beta peptide (A β), the main component of amyloid plaques and a major component of brain Alzheimer's disease (AD) pathology, share several features, including similar β -sheet secondary structures [1], binding to the same amylin receptor [2], and being degraded by the same protease insulin-degrading enzyme (IDE) [3–5]. They appear to affect each other in complex ways. A recent study found an accumulation of amylin amyloid in the cerebrovascular system in the AD brain, resulting in impaired vascular functioning [6]. Amylin readily penetrates the blood brain barrier (BBB) [7,8] and mediates important brain functions including inhibiting appetite thereby improving glucose metabolism [9,10], relaxing cerebrovascular structure [11,12], and, in all likelihood, enhancing neural regeneration [13]. High levels of A β in the AD brain may block amylin's ability to bind to its receptor, thus hindering normal amylin functions that are essential to the brain [10].

Our recent study using two AD mouse models demonstrates another important function of amylin in the brain. Chronic

treatment with intraperitoneal (i.p.) injection of amylin or its clinical analog, pramlintide, enhanced removal of A β from the brain and improved their cognitive impairment (submitted and under review). Through efflux, A β can pass through the BBB into blood [14]. BBB dysfunction, decreased cerebral blood flow, and impaired vascular clearance of A β from the brain are all thought to contribute to AD pathogenesis [15]. It is well known that the concentration of A β in blood is much lower than the concentration of A β in the brain [16], suggesting that only a small portion of A β in the brain can be removed from the brain. As A β is a key element of AD pathogenesis in the brain [17], if a drug or substance like amylin or its analogs can enhance the removal of A β from the AD brain into the blood, it might prove an effective treatment for the disease. The use of solanezumab is an example of this treatment strategy. This immune drug that removes A β from the AD brain into blood has been shown to delay cognitive decline in those in an early stage of AD [18].

In humans the relationship between amylin and A β in plasma is unclear. It will be important to determine whether this naturally occurring peptide derived from the pancreas has any role in regulating A β in the brain. If our mouse finding indicating that

peripheral amylin passes through the BBB and removes A β from the brain is relevant to humans, we anticipate that amylin will be positively associated with A β in human plasma samples. Apolipoprotein E4 (ApoE4) is the major risk factor for AD with late onset [19]. The ApoE4 allele is associated with BBB damage [20], which likely adversely affects removal of A β from brain into blood. Using a large, homebound elderly population we aimed to examine the relationship between amylin and A β in blood in the context of the ApoE alleles.

Materials and Methods

Study Population and Recruitment

We studied a group of 1092 subjects, all of whom had measurements of plasma amylin and A β , as well as ApoE genotyping as part of a population-based study, the *Nutrition, Aging and Memory in the Elderly (NAME) study* [21]. Subjects included homebound elderly clients who were enrolled in one of four homecare agencies in the Boston area between 2002 and 2007. Anyone receiving homecare services was registered with one of these agencies if he/she lived in the city of Boston, had an annual income <\$18,890, and needed homecare service. All homebound elders aged 60 and older at each of the four agencies were invited to participate in the study. All enrolled subjects gave written informed consent. The protocol, consent form and consent procedure were approved by the Institutional Review Boards of Tufts University New England Medical Center and Boston University School of Medicine. All the signed consent forms have been kept and locked in the research area.

Eligibility for enrollment required that the participants spoke English, were physically able to participate in the study home visits, and had sufficient vision and hearing to read and hear the content of the neuropsychological tests. Those with Mini-Mental State Examination (MMSE) ≤ 10 or verbal IQ <75 were not eligible to continue in the study. Of all eligible subjects, 66% enrolled in the study, and gave informed consent [22]. The subjects were screened for cognitive impairment using the Mini Mental State Examination (MMSE) [23].

Measurements

Plasma Amylin and A β . Fasting blood draws were conducted. Blood samples were centrifuged immediately following blood draw to isolate plasma. We used ELISA assay to measure amylin concentration in plasma according to the manufacturer's instructions (LINCO Research, St. Charles, Missouri). All samples were assayed in duplicate and averaged to give final values.

To measure A β a sandwich A β ELISA was used, as described previously [24]. Briefly, plates were coated with 2G3 (anti-A β 40) and 21F12 (anti-A β 42) antibodies overnight at 4°C. Samples were then loaded and incubated overnight at 4°C followed by incubation with a biotinylated monoclonal anti-N terminus A β antibody (3D6B) for 2 hrs. Finally, streptavidin-conjugated alkaline phosphatase (Promega, USA) was added and incubated, and the signal was amplified by adding alkaline phosphatase fluorescent substrate (Promega, USA), which was then measured.

ApoE genotyping. A 244 bp fragment of the apoE gene including the two polymorphic sites was amplified by PCR using a robotic Thermal Cycler (ABI 877, Perkin-Elmer/Applied Biosystems), using oligonucleotide primers F4 (5'-ACAGAATT-CGCCCCGGCTGGTACAC-3') and F6 (5'-TAAGCTTGG-CACGGCTGTC AAGGA-3'). The PCR products were digested with 5 units of Hha I and the fragments separated by electrophoresis on 8% polyacrylamide non-denaturing gel. The

specific allelic fragments were: E2; E3; and E4. ApoE4 was defined by E4/4, E3/4 or E2/4 [25].

Other blood tests. Serum lipid profiles, including cholesterol, LDL and HDL, and serum creatinine, were measured by the clinical laboratory according to the standard protocols at Jean Mayer USDA Human Nutrition Research Center on Aging (HNRCA), Tufts University.

Other Clinical Evaluation

Weight and height were measured twice using standardized instruments for weight and height, and the average of two measurements was used to calculate BMI (kg/m²). Diabetes was defined by the use of anti-diabetic medication or fasting glucose greater than 126 mg/dl, parameters widely used in population-based studies [26]. Subjects were asked to show all the medications they were taking, and research assistants documented the medication names according to the labels.

Statistical Analysis

Statistical analysis was performed using SAS (version 9.1). Normally distributed variables, such as age, were presented as mean \pm SD and compared using t-tests for the ApoE4 subgroups or using ANOVA test across the quartiles. Variables with skewed distributions (plasma amylin, A β 1-42, A β 1-40, and A β 1-40/A β 1-42 ratio) were presented as median (25th, 75th percentiles) and compared using Wilcoxon rank sum test for ApoE4 subgroups or using Kruskal-Wallis test across quartiles [24]. The Chi-Square test was used to compare proportions for binary endpoints. Amylin (LogAmylin), A β 1-42 (Log A β 1-42), and A β 1-40 (Log A β 1-40) were transformed to log₁₀ for multivariate regression due to skewed distributions. Univariate and multivariate linear regression were used to examine associations between Log Amylin and Log A β 1-42 or Log A β 1-40 while adjusting for age, ApoE4, depression, creatinine and other confounders. For all analyses, the two-sided significance level of 0.05 was used.

Results

Study Population

From the completed NAME study, 1092 subjects with ApoE genotyping and measurements of plasma amylin and A β were used for the study analysis (Table 1); 24% of them carried at least one ApoE4 allele. The average age (mean \pm SD) of this study sample was 75.0 \pm 8.0 years old, and 76% were female. The population was multi-ethnic, with 61% Caucasian, 35% African American and 4% other ethnicities. Most subjects (67%) had high school education or above. The average BMI was 31.6 \pm 8.6, and 37% had a history of diabetes (Table 1). The distributions of all amylin, A β 1-42 and A β 1-40 in plasma were skewed (Table 1). For plasma amylin (pM/L): median = 22.3, Q₁ = 11.8, Q₃ = 40.0; for A β 1-42 (pg/ml): median = 17.4, Q₁ = 11.8, Q₃ = 22.6, and for A β 1-40 (pg/ml): median = 133.4, Q₁ = 99.5, Q₃ = 172.7.

We divided subjects into ApoE4 non-carriers (n = 834) and ApoE4 carriers (n = 258) (Table 1). There were no differences in demographic variables between the two ApoE subgroups with the exception that African Americans were more likely to be ApoE4 carriers than ApoE4 non-carriers (48% vs. 31%, p = 0.03). There were no differences in lipid profiles between the two ApoE4 subgroups. Compared to the ApoE4 non-carriers, ApoE4 carriers had a slightly lower concentration of A β 1-42 in plasma (p = 0.03) and a higher A β 1-40/A β 1-42 ratio (p = 0.007). There were no differences in the concentrations of amylin and A β 1-40 in the ApoE4 subgroups.

Table 1. General information and univariate correlations of amylin and A β stratified by ApoE4 status.

	All subjects n = 1092	ApoE4 Non-carriers n = 834	ApoE4 Carriers n = 258
General Information			
Age, year, mean \pm SD	75.0 \pm 8.0	75.2 \pm 8.5	74.3 \pm 8.4
Female, n/total (%)	831/1092 (76%)	628/834 (75%)	203/258 (79%)
African Americans, n/total (%)	383/1087 (35%)	258/829 (31%)	125/258 (48%)***
High School Graduate and above, n/total (%)	726/1086 (67%)	555/832 (67%)	171/254 (67%)
History of stroke, n/total (%)	216/1062 (20%)	170/813 (21%)	46/249 (18%)
BMI, mean \pm SD	31.6 \pm 8.6	31.7 \pm 8.9	31.3 \pm 8.0
Diabetes, n/total (%)	387/1059 (37%)	293/810 (36%)	94/249 (38%)
Creatinin, mean \pm SD	1.1 \pm 1.0	1.0 \pm 0.8	1.2 \pm 1.5
Cholesterol, mg/dL, Mean \pm SD	184.9 \pm 43.2	184.4 \pm 43.5	187.0 \pm 42.3
LDL, mg/dL, Mean \pm SD	107.3 \pm 35.9	106.4 \pm 36.1	110.4 \pm 35.4
HDL, mg/dL, Mean \pm SD	49.6 \pm 14.8	49.4 \pm 15.0	50.4 \pm 14.1
Amylin and Aβ in Plasma			
Amylin, pM/L, Median (Q1, Q3)	22.3 (11.8, 40.0)	20.4 (11.8, 38.7)	22.5 (11.5, 41.2)
A β 1- 42, pg/ml, Median (Q1, Q3)	17.4 (11.8, 22.6)	18.0 (12.2, 27.7)	17.1 (11.4, 23.5)*
A β 1- 40, pg/ml, Median (Q1, Q3)	133.4 (99.5, 172.7)	134.0 (99.7, 174.1)	135.2 (100.3, 183.8)
A β 1- 40/A β 1- 42 ratio, Median (Q1, Q3)	7.6 (4.9, 11.0)	7.4 (4.7, 10.7)	8.8 (5.3, 11.9)**
Univariate Spearman Correlations			
Amylin with A β 1- 42	r = +0.20, p < 0.0001	r = +0.21, p < 0.0001	r = +0.22, p = 0.0003
Amylin with A β 1- 40	r = +0.12, p < 0.0001	r = +0.15, p < 0.0001	r = +0.04, p = 0.57

Mean \pm SD or n/total (%) and the comparisons between ApoE4 non-carriers and ApoE4 carriers are presented. P values for the statistical significance are shown.

*p = 0.03;

**p = 0.007;

***p < 0.0001.

Univariate Spearman Correlations on the relationships between amylin and A β are shown for the whole sample and the ApoE4 subgroups.

BMI = Body Mass Index; LDL = low density lipoprotein; HDL = high density lipoprotein.

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A Positive Relationship between Plasma Amylin and A β

Using Spearman rank correlation analysis, the study sample showed that amylin was positively and moderately associated with A β 1-42 (r = +0.20, p < 0.0001) and A β 1-40 (r = +0.12, p < 0.0001) in plasma (Table 1). Subjects were divided into quartiles based on the concentration of plasma amylin (Table 2). In plasma with increasing quartile of amylin the concentrations of A β 1-42 (p < 0.0001) increased in a linear pattern; the concentrations of A β 1-40 (p < 0.0001) also increased, but in a non-linear pattern

with a U shape; and the ratios of A β 1-40/A β 1-42 (p < 0.0001) decreased in a linear pattern.

While there was no difference in age across the four amylin quartiles, average BMI (p < 0.0001), kidney function as assessed by creatinine levels (p < 0.0001), and the rate of diabetes (p < 0.05) all increased with increasing quartile of amylin (Table 3). Cholesterol and LDL levels had a positive relationship with increasing 1st to 3rd quartile of amylin, but their levels were lower in the 4th quartile of amylin, indicating a nonlinear relationship. HDL concentration

Table 2. Comparisons of A β 1-42, A β 1-40 and A β 1-40/A β 1-42 ratio across the amylin quartiles.

Amylin Quartiles	Quartile 1	Quartile 2	Quartile 3	Quartile 4	P values
A β 1- 42, Median	16.0	16.3	17.1	21.4	
(Q1, Q3)	(11.4, 22.3)	(11.4, 24.2)	(12.2, 26.1)	(13.7, 39.7)	<0.0001
A β 1- 40, Median	134.4	126.8	127.0	150.2	
(Q1, Q3)	(101.9, 176.1)	(91.0, 166.8)	(91.4, 160.5)	(117.2, 160.5)	<0.0001
A β 1-40/A β 1- 42, Median	9.0	7.8	7.1	6.9	
(Q1, Q3)	(6.03, 12.6)	(5.1, 11.2)	(4.6, 10.3)	(4.3, 10.0)	<0.0001

Median (Q1, Q3) is used to describe the distributions of plasma A β 1-42, A β 1-40 and the A β 1-40/A β 1-42 ratio. Using Kruskal-Wallis test, the comparisons are shown across the amylin quartiles. P values for the statistical significance are shown.

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Table 3. Comparisons of age, metabolic diseases and biomarkers across the amylin quartiles.

Amylin Quartiles	Quartile 1	Quartile 2	Quartile 3	Quartile 4
Age, year, Mean \pm SD	75.5 \pm 8.7	75.0 \pm 8.4	75.1 \pm 8.5	74.2 \pm 8.5
History of stroke, n/total (%)	54/265 (20%)	61/272 (22%)	53/270 (20%)	50/268 (19%)
BMI, Mean \pm SD **	30.0 \pm 8.4	31.4 \pm 9.0	32.0 \pm 7.6	32.9 \pm 9.1
Diabetes, n/total (%) *	97/265 (37%)	87/269 (32%)	92/268 (34%)	117/265 (44%)
Creatinine, mg/dL, Mean \pm SD***	0.90 \pm 0.68	0.93 \pm 0.92	1.05 \pm 0.74	1.39 \pm 1.53
Cholesterol, mg/dL, Mean \pm SD **	183.8 \pm 46.3	183.8 \pm 41.3	192.1 \pm 43.9	179.0 \pm 40.2
LDL, mg/dL, Mean \pm SD **	106.7 \pm 38.1	108.1 \pm 35.1	112.4 \pm 35.6	100.9 \pm 33.5
HDL, mg/dL, Mean \pm SD ***	53.6 \pm 16.6	49.3 \pm 13.7	49.2 \pm 14.7	46.6 \pm 13.4

Mean \pm SD with ANOVA test or n/total with Chi-Square test is used to describe the distributions and comparisons of age, diseases and the biomarkers across the amylin quartiles.

* $p \leq .05$.

** $p \leq .001$,

*** $p \leq .0001$ for the statistical significance are shown.

BMI = Body Mass Index; LDL = low density lipoprotein; HDL = high density lipoprotein.

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was inversely associated with increasing quartile of amylin in plasma ($P < 0.0001$).

Because of their skewed distributions, amylin, A β 1-42 and A β 1-40 were transformed to \log_{10} for multivariate linear regression. Using multivariate regression (Table 4), \log_{10} of amylin remained positively associated with both \log_{10} of plasma A β 1-42 ($\beta = +0.166$, $SE = 0.024$, $P < 0.0001$) and \log_{10} of plasma A β 1-40 ($\beta = +0.053$, $SE = 0.016$, $P = 0.001$) respectively after adjusting for age, gender, ethnicity, BMI and ApoE4 (Model I). After adding kidney function as assessed by concentration of creatinine, \log_{10} of amylin was still associated with either \log_{10} of plasma A β 1-42 ($\beta = +0.150$, $SE = 0.024$, $P < 0.0001$) or \log_{10} of plasma A β 1-40 ($\beta = +0.031$, $SE = 0.016$, $P = 0.05$) (Model II), but the relationships were attenuated. Finally, the relationship between \log_{10} of amylin and either \log_{10} of plasma A β 1-42 ($\beta = +0.146$, $SE = 0.025$, $P < 0.0001$) or \log_{10} of plasma A β 1-40 ($\beta = +0.034$, $SE = 0.016$, $P = 0.04$) as an outcome persisted after adjusting for age, gender, ethnicity, ApoE4, BMI, diabetes, stroke, creatinine and lipid profile including cholesterol, LDL and HDL (Model III). Adding education and MMSE to the model did not change the relationships between amylin and A β (data not shown). The concentration of creatinine was positively associated with both \log_{10} of plasma A β 1-40 and \log_{10} of plasma A β 1-42. ApoE4 was negatively associated with plasma A β 1-42, but not with plasma A β 1-40 in this model.

The Relationship between Amylin and A β in the Context of ApoE Allele

Subjects were divided into ApoE4 non-carriers and carriers (Table 1). In the absence of ApoE4, the relationships between amylin and A β 1-42 ($\beta = +0.158$, $SE = 0.031$, $P < 0.0001$) or A β 1-40 ($\beta = +0.044$, $SE = 0.020$, $P = 0.03$) in plasma still remained after adjusting for age, gender, ethnicity, BMI, diabetes, stroke, creatinine and the lipid profile (Tables 1 and 5). With increasing quartile of amylin, the concentrations of A β 1-42 ($p < 0.0001$) (Figure 1A) and A β 1-40 ($p < 0.0001$) (Figure 1C) increased in plasma in ApoE4 non-carriers. Since increased amylin was more associated with A β 1-42 than with A β 1-40, the ratio of A β 1-40/A β 1-42 was decreased with increasing quartile of amylin in the absence (median: Q1 = 8.7; Q2 = 7.7; Q3 = 7.0 and Q4 = 6.6, $p = 0.0001$).

Table 4. Effects of amylin on A β 42 or A β 42 in plasma in multivariate regression analyses.

Whole sample	Log A β 1-42		Log A β 1-40	
	Estimate β (SE)	P value	Estimate β (SE)	P value
Model I: Log Amylin	+0.166 (0.024)	<0.0001	+0.053 (0.016)	0.001
Model II: Log Amylin	+0.150 (0.024)	<0.0001	+0.031 (0.016)	0.05
Model III: Log Amylin	+0.149 (0.025)	<0.0001	+0.034 (0.016)	0.04

Model I: adjusting for age, gender, ethnicity, BMI and ApoE4; n = 1000 for A β 1-42; n = 1001 for A β 1-40.

Model II: Model I plus creatinine; n = 983 for A β 1-42; n = 984 for A β 1-40.

Model III: Model II plus diabetes, stroke, cholesterol, LDL and HDL; n = 951 for A β 1-42; n = 952 for A β 1-40.

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In contrast to ApoE4 non-carriers, ApoE4 carriers had a different pattern of the relationship between amylin and A β . In the presence of ApoE4, while the relationship between amylin and A β 1-42 ($\beta = +0.112$, $SE = 0.042$, $P = 0.008$) remained but attenuated, the relationship between amylin and A β 1-40 disappeared after adjusting for the confounders (Tables 1 and 5). In ApoE4 carriers, with increasing quartile of amylin, the concentrations of A β 1-42 increased in plasma ($p = 0.0004$) (Figure 1B), but the relationship between amylin quartiles and A β 1-40 in plasma was weak and presented with a U shape (Figure 1D). The ratio of A β 1-40/A β 1-42 was decreased with increasing quartile of amylin in the presence of ApoE4 allele (median: Q1 = 10.1; Q2 = 9.0; Q3 = 8.5 and Q4 = 7.3, $p = 0.006$). ApoE4 carriers had a higher level of A β 1-40/A β 1-42 ratio than ApoE4 non-carriers in amylin quartiles 1 and 3 with statistical significance ($p < 0.05$).

Discussion

Our recent study using AD mouse models demonstrated that i.p. injection of synthetic amylin or its analog, pramlintide, enhanced the removal of A β from the brain into blood (submitted and under review). In light of this effect, we hypothesized that

Table 5. Effects of amylin on A β 42 or A β 42 in plasma in the absence and the presence of ApoE4 allele.

ApoE4 non-carriers	Log A β 1-42 (n = 746)		Log A β 1-40 (n = 746)	
	Estimate β (SE)	P value	Estimate β (SE)	P value
Log Amylin	+0.158 (0.031)	<0.0001	+0.044 (0.020)	0.03
ApoE4 carriers	Log A β 1-42 (n = 229)		Log A β 1-40 (n = 230)	
	Estimate β (SE)	P value	Estimate β (SE)	P value
Log Amylin	+0.112 (0.042)	0.008	-0.001 (0.031)	0.98

Adjusted for age, gender, ethnicity, BMI, diabetes, stroke, creatinine, cholesterol, LDL and HDL for the ApoE subgroups.

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endogenous amylin in blood would enhance the removal of A β from the brain, especially in elderly with amyloid pathology in the brain. This would lead to a positive relationship between these two peptides in blood. Our current human study did indeed show a positive association between naturally occurring amylin and A β in

plasma, likely due to a mechanism similar to that seen in the mouse model. Thus it is possible that endogenous and synthetic amylin have similar effects on A β in the brain.

It is intriguing that amylin (Tables 2 and 3) and A β , especially A β 1-42, were positively associated in plasma, suggesting that

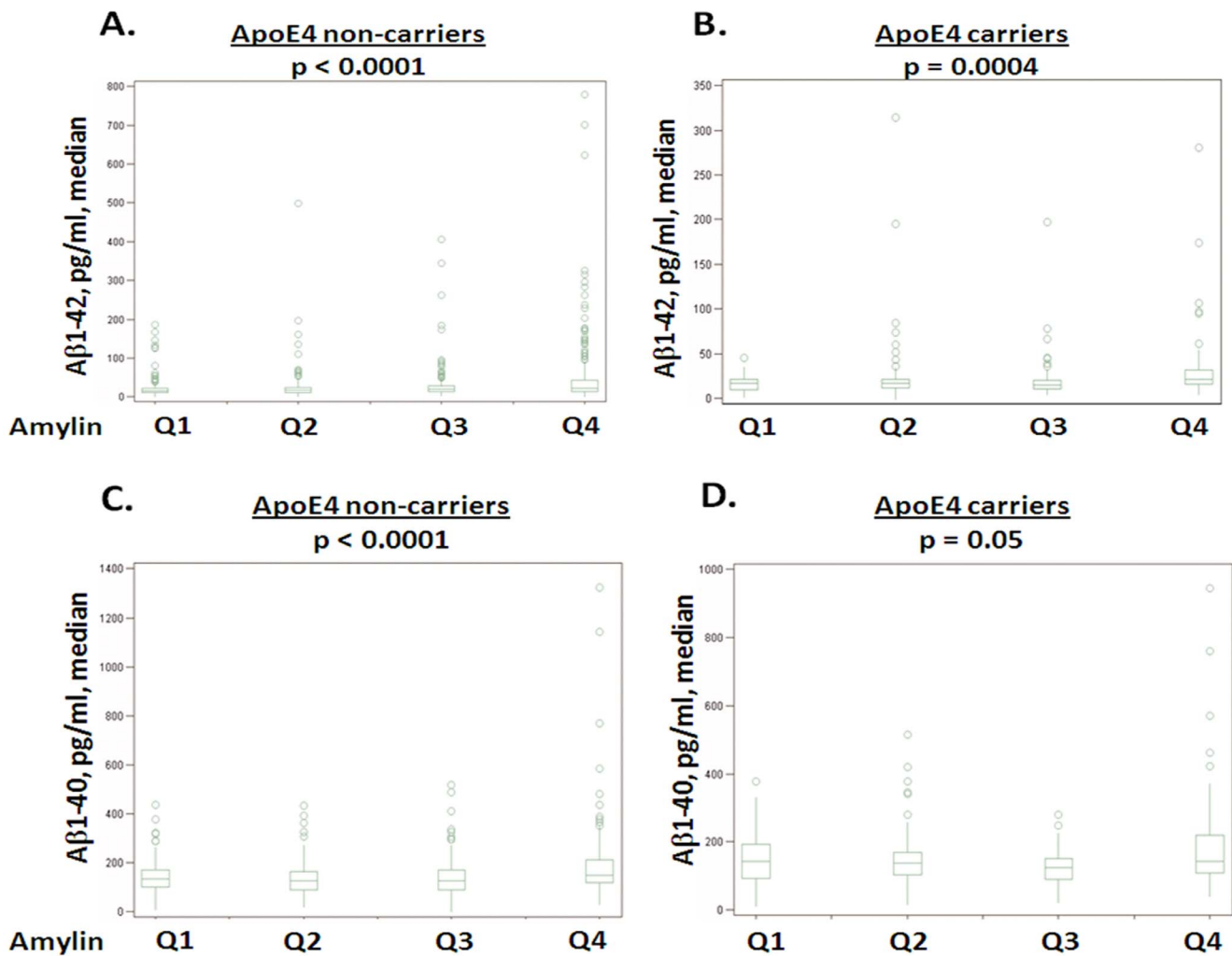


Figure 1. Characterization of A β with increasing amylin quartiles. Subjects are divided into four quartiles based on the concentration of amylin in plasma. Box plots were used to illustrate the median concentration with 25% (Q1) and 75% (Q3) range of plasma A β . Plasma A β 1-42 (A and B) and A β 1-40 (C and D) in the absence (A and C) and presence (B and D) of ApoE4 allele in quartile 1 (Q1), quartile 2 (Q2), quartile 3 (Q3) and quartile 4 (Q4) of plasma amylin are shown.

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naturally occurring amylin may also enhance removal of A β from the brain. In general, a positive association between two peptides in a compartment in the body can occur by three mechanisms, e.g. 1) co-production/co-secretion, 2) competitive degradation/clearance, or 3) one peptide moving another to the same location. It is logical to anticipate that if two molecules do not encounter each other in the same tissue regions, they generally will not compete for the same protease degradation or bind the same receptor to be cleared or influence each other, and thus will not have a positive association. Although amylin is a peripheral peptide produced and secreted by the pancreas and A β occurs primarily in the brain, especially the AD brain [16], amylin does readily cross the BBB [7,8] and thus amylin and A β may therefore encounter each other in the brain. Another pancreatic peptide, insulin, is much less likely than amylin to be transported into the brain via the BBB [7,8]. It is not surprising that in the same plasma samples we did not find any association between insulin and A β (data not shown). Note that when using cell cultures, insulin and A β encounter each other in the cell media and a significantly positive relationship between insulin and A β is observed [3–5].

Amylin levels were inversely associated with the A β 1-40/A β 1-42 ratio in plasma (Table 2 and Figure 1). Two large, prospective population studies have shown that a high plasma A β 40/A β 42 ratio, determined by both low A β 42 and high A β 40, increases the risk of AD [27,28]. While A β 42 is a major component of AD pathology in the brain [29], A β 40 is a component of cerebral amyloid angiopathy (CAA) [30]. High levels of plasma A β 40 are associated with cerebral microvascular pathology, white matter hyperintensities (WHI) and lacunar infarcts [31,32]. The plasma A β 42 decline seen in the pre-clinical stage of AD [33,34], indicating the formation of AD pathology. Thus a high A β 40/A β 42 ratio in plasma may be a biomarker of cerebral microvascular pathology, which is associated with high plasma A β 40, co-existing AD pathology, which is associated with low plasma A β 42. Since plasma amylin levels were found to be inversely associated with A β 40/A β 42 ratio, it is possible that higher plasma amylin is a protecting factor for the development of AD.

ApoE4 is a major risk factor for late-onset AD as well as for cerebrovascular disease [35]. The positive association between amylin and A β 1-40 in blood disappeared in the presence of ApoE4 (Table 5). Although the effect of ApoE4 on the relationship between amylin and A β is unknown, we hypothesized that ApoE4 may attenuate amylin's activity in removing A β , especially A β 40, out of the brain via the BBB. A β 40 is the primary peptide that is deposited in the cerebrovasculature of the AD brain under the influence of the ApoE4 allele [36]. BBB dysfunction, decreased cerebral blood flow, and impaired vascular clearance of A β from brain are all thought to contribute to AD pathogenesis [15]. Amylin has been shown to have a vasorelaxant effect [37] that may result in enhanced removal of A β from the brain. A recent study found an accumulation of amylin amyloid in the

cerebrovasculature of the AD brain [6]; the resulting microvascular dysfunction may interfere with amylin's ability to relax cerebrovasculature. Since some ApoE4 carriers do not develop AD even at a great age [38], other factors, such as amylin, may interact with ApoE4 to influence AD development.

High plasma levels of amylin were associated with obesity and type 2 diabetes, as well as with other biomarkers of metabolic syndrome and cerebrovascular disease including low HDL levels, high creatinine levels, and non-linear increased levels of cholesterol and LDL (Table 2). These data suggest a relationship between amylin resistance, obesity, and type 2 diabetes, which is consistent with findings in other studies [39] [40] [41,42]. Amylin was independently associated with A β even after adjusting for these biomarkers of metabolic syndrome (Table 3). Since amylin is cleared by the kidney [43], the relationship between plasma amylin and A β , especially A β 1-40, was influenced by adding creatinine to the models.

Amylin's major role in the brain is to reduce food intake thereby controlling body weight and regulating glucose metabolism [44]. Administering exogenous amylin, either peripherally or intracerebroventricularly, results in reduced appetite and food intake [8]. Pramlintide, an amylin analog differing by three amino acids, is an effective and well-tolerated drug in clinical use for the treatment of diabetes [45] [46]. Given the effectiveness of the amylin class of peptides in reducing amyloid pathology in the brain in the preclinical study and the relationship between amylin and A β in the context of ApoE allele seen in this human study, pramlintide may have potential as a treatment in AD. A clinical trial of pramlintide in AD, an off-label use, may be warranted. Limitations of our study are its cross-sectional design and lack of brain imaging. Longitudinal studies are needed to confirm the causal relationship between high levels of plasma amylin and decreased A β deposition in the brain. There were no diagnoses of AD and mild cognitive impairment (MCI) for this population based study. Future studies are necessary to examine the concentrations of amylin and its relationship to A β in specific diagnostic groups.

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Author Contributions

Conceived and designed the experiments: WQQ HZ. Performed the experiments: MW HZ. Analyzed the data: MD MM EL WQQ. Contributed reagents/materials/analysis tools: WQQ MM. Wrote the paper: WQQ MW EL.

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