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Featured Articles

Amyloid beta peptides in human plasma and tissues and their significance for Alzheimer's disease

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Abstract

Background: We evaluated the amounts of amyloid beta (Aβ)) peptides in the central nervous system (CNS) and in reservoirs outside the CNS and their potential impact on Aβ plasma levels and Alzheimer's disease (AD) pathology.

Methods: Amyloid β levels were measured in (1) the plasma of AD and nondemented (ND) controls in a longitudinal study, (2) the plasma of a cohort of AD patients receiving a cholinesterase inhibitor, and (3) the skeletal muscle, liver, aorta, platelets, leptomeningeal arteries, and in gray and white matter of AD and ND control subjects.

Results: Plasma Aβ levels fluctuated over time and among individuals, suggesting continuous contributions from brain and peripheral tissues and associations with reactive circulating proteins. Arteries with atherosclerosis had larger amounts of Aβ40 than disease-free vessels. Inactivated platelets contained more Aβ peptides than activated ones. Substantially more Aβ was present in liver samples from ND patients. Overall, AD brain and skeletal muscle contained increased levels of Aβ.

Conclusions: Efforts to use plasma levels of Aβ peptides as AD biomarkers or disease-staging scales have failed. Peripheral tissues might contribute to both the circulating amyloid pool and AD pathology within the brain and its vasculature. The wide spread of plasma Aβ values is also due in part to the ability of Aβ to bind to a variety of plasma and membrane proteins. Sources outside the CNS must be accounted for because pharmacologic interventions to reduce cerebral amyloid are assessed by monitoring Aβ plasma levels. Furthermore, the long-range impact of Aβ immunotherapy on peripheral Aβ sources should also be considered.

Keywords: Plasma Aβ; Alzheimer's disease; Peripheral Aβ; Atherosclerotic vascular disease; Aβ immunotherapy

1. Background

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by dementia and an abundance of amyloid beta (Aβ) peptides in the brain parenchyma and cerebral vasculature that are derived from the Aβ precursor protein (AβPP).

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Genetic investigations strongly suggest that Aβ peptides have a central role in AD pathogenesis. However, genetically determined AD is rare, whereas the sporadic form of the disease accounts for up to 98% of patients. The cause of sporadic AD is complex and multifactorial, with contributions from genetic as well as environmental factors. Amyloid β peptides are considered an important pathologic marker of AD because of their profuse extracellular deposition in senile and diffuse plaques and vascular walls. In addition, Aβ is present in neurons and glial cells and in soluble oligomeric forms that diffuse along the narrow extracellular space of the brain. During the last three decades enormous resources have been expended to determine the chemical structure of the Aβ peptides in their diverse physical forms and to elucidate the multiple functional roles of Aβ. Most important, vast investments have been made to create therapeutic agents that either interfere with Aβ production or are capable of dispersing amyloid deposits.

Because of the omnipresence of Aβ peptides in familial and sporadic AD, the levels of these molecules in physiologic fluids have been measured and characterized to establish their potential utility as reliable disease biomarkers. Unfortunately, despite the seemingly obvious association of Aβ with AD pathology, the efforts to validate plasma Aβ peptides as dementia biomarkers and correlate threshold concentration levels with disease stage have been fraught with frustration [1]. In the brain, Aβ peptides exist both within defined deposits (plaques and vascular) and as a diverse array of other forms including soluble, membrane-associated, and intracellular species that might play far more significant roles in the production of dementia than the molecules sequestered in extracellular plaques. In addition, the critical role that circulating Aβ peptides play in AD pathology cannot be ignored. Besides the brain, Aβ peptides are generated outside of the central nervous system (CNS) in appreciable quantities by the skeletal muscle, platelets, and vascular walls [2–4]. Other non-neural tissues expressing AβPP include pancreas, kidney, spleen, heart, liver, testis, aorta, lung, intestines, skin, as well as the adrenal, salivary, and thyroid glands [5–7]. These distinct reservoirs might allow for an active and dynamic interchange of Aβ peptides between the brain and periphery. These sources undoubtedly contribute to the pool of circulating Aβ and must be considered when the success or failure of AD pharmacologic interventions, intended to reduce cerebral amyloid, is assessed by monitoring Aβ plasma levels. Interestingly, Aβ peptides in the periphery fail to generate filamentous structures, probably as a result of the presence of albumin, erythrocyte membrane proteins, and the other multiple circulating molecules that bind Aβ peptides avidly and thereby alter their apparent free plasma levels [8–10].

In the present study, the potential significance of the different sources and pools of Aβ on the plasma levels of these peptides and on the general pathology of AD was evaluated in several complementary ways. A comprehensive, longitudinal assessment of plasma Aβ levels in AD and age-matched nondemented (ND) control individuals was performed at baseline and 3, 6, and 12 months. An additional longitudinal study determined the potential effects of the cholinesterase inhibitor donepezil hydrochloride (Aricept) on plasma Aβ levels in AD patients and compared them with a control group. Amyloid β levels were also estimated in inactivated platelets and collagen/thrombin activated platelets. Finally, in an autopsy cohort, the levels of Aβ peptides were determined in skeletal muscle, in human aorta and leptomeningeal arteries with and without atherosclerotic vascular disease (AVD), human liver, and independently in brain gray and white matter in AD and in ND matching age controls.

2. Materials and methods

2.1. Human subjects

All clinical protocols and experiments were carried out under the guidelines of Sun Health Corporation Institutional Review Board and Oregon Health and Science University Institutional Review Board. All living specimen donors signed a consent agreement for participation in the present investigation. Postmortem specimens were obtained from the Brain and Body Donation Program of Sun Health Research Institute (SHRI) [11].

2.2. Quantification of Aβ peptides in plasma

The inclusion criteria were as follows: subjects must be at least 65 years, fluent in English, accompanied by a collateral informant, educated (at least a 6th grade level), able to provide a work history, able to sign and date the informed consent, and able to meet one of the diagnostic criteria (ND control, AD, mild cognitive impairment, vascular dementia, or non-AD dementia). Subjects were not restricted by gender, race, or ethnic background. Subjects were excluded if they were diagnosed with delirium (Diagnostic and Statistical Manual [of Mental Disorders], Fourth Edition [DSM-IV]), were not able to be assessed as a result of conditions such as blindness or deafness, had a history of alcohol or substance abuse/dependence (DSM-IV) within the past 10 years, were unable to undergo brain magnetic resonance scanning, or could not provide a collateral informant. The diagnosis was based on the National Institute of Neurological Disorders and Strokes/Alzheimer's Disease and Related Disorders (NINDS/ADRDA) criteria. Blood samples were obtained at baseline and 3, 6, and 12 months under fasting conditions from 17 individuals with clinically diagnosed AD (average age, 81.4 years; 10 women and seven men) and 21 ND subjects (average age, 75.8 years;14 women and seven men). As a result of subject attrition, in the total of 38 enrolled individuals, 28 cases had their plasma analyzed at four time points and 10 cases at three time points. The average Mini-Mental State Examination (MMSE) score for the ND control group was 29.0 (range, 24 to 30) and 22.3 (range, 15 to 30) for the AD group (Table 1).

Table 1
Longitudinal study of plasma individual Aβ values and patient demographics

Patient no.	Age (y)	Gender	*APOE* genotype	MMSE	Aβ40 (pg/mL)	Aβ42 (pg/mL)	Total Aβ (pg/mL)	Ratio Aβ42/Aβ40
ND controls (n = 21)								
1	82	F	3/3	30	628.66	76.92	705.58	0.12
2	62	F	4/4	28	338.75	61.02	399.77	0.18
3	78	M	3/3	28	356.23	79.86	436.09	0.22
5	78	M	3/3	30	311.11	79.54	390.65	0.26
9	78	F	3/4	29	484.36	109.24	593.60	0.23
12	77	M	3/3	30	259.56	98.70	358.26	0.38
13	60	F	2/4	29	184.82	108.37	293.19	0.59
14	63	M	3/3	28	221.54	119.71	341.25	0.54
16	79	F	3/4	30	380.87	176.98	557.85	0.47
21	81	F	3/3	29	252.95	157.79	410.74	0.62
23	83	F	3/4	28	145.03	105.10	250.13	0.73
25	71	F	3/4	29	320.26	192.81	513.07	0.60
29	79	F	3/3	30	94.63	142.73	237.36	1.51
30	75	F	4/4	30	372.68	200.44	573.12	0.54
39	86	M	2/3	29	466.19	124.60	590.79	0.27
40	83	F	3/4	29	419.18	185.65	604.82	0.44
41	77	F	3/3	28	402.03	97.36	499.39	0.24
45	81	F	2/3	30	235.55	105.24	340.79	0.48
49	70	F	3/3	30	429.33	108.53	537.86	0.25
50	73	M	3/4	30	555.23	185.90	741.13	0.34
56	76	M	3/4	24	373.72	102.33	476.05	0.27
Average	75.81	14F/7M		28.95	344.41	124.71	469.12	0.44
SD	7.10			1.40	132.43	42.34	141.09	0.30
AD (n = 17)								
4	77	F	3/4	19	236.43	92.13	328.56	0.39
8	80	M	3/3	24	565.46	136.05	701.51	0.24
11	73	F	3/4	26	554.35	123.57	677.92	0.22
15	89	M	3/4	18	365.17	168.33	533.50	0.46
24	90	M	3/3	23	531.23	189.63	720.86	0.36
26	76	M	3/3	23	328.39	121.47	449.86	0.37
28	74	F	4/4	17	340.11	99.33	439.44	0.29
32	75	M	3/4	30	505.17	406.03	911.20	0.80
37	82	F	3/4	25	486.52	212.39	698.91	0.44
42	83	M	3/4	22	257.69	133.92	391.62	0.52
43	88	F	3/3	24	563.54	108.70	672.24	0.19
44	81	F	3/4	21	235.55	100.31	335.86	0.43
48	87	M	3/4	27	434.55	120.26	554.81	0.28
53	83	F	3/3	22	411.23	83.44	494.67	0.20
55	79	F	3/4	15	756.84	74.32	831.16	0.10
57	85	F	3/3	22	219.99	103.25	323.24	0.47
59	81	F	3/4	21	416.86	105.38	522.23	0.25
Average	81.35	10F/7M		22.29	424.06	139.91	563.98	0.35
SD	5.29			3.74	147.73	77.82	178.47	0.16
ND versus AD, *P* value	.011			<.001	.088	.448	.075	.292

NOTE. The Aβ amounts represent the average of those individuals completing three and four time points: baseline, 3, 6, and 12 months.

The plasma levels of Aβ1-40 and Aβ1-42 peptides were immunoassayed in duplicate by enzyme-linked immunosorbent assays (ELISAs) that were obtained from Immunobiological Laboratories (Minneapolis, MN) and from Innogenetics (Gent, Belgium), respectively. The ELISAs were carried out following the manufacturers' instructions and executed by the same investigator who was blinded to the identity of the specimens. The high sensitivity method was used for the Aβ1-42 kit. Aβ1-40 had a measurement range of 7.81 to 500 pg/mL and a sensitivity of 5.00 pg/mL. The coefficient of variation (CV) values for interassay measurements were <7%. The CV values for intra-assay measurements were <8%. With the high sensitivity method, the Aβ1-42 ELISA had a measurement range of 7.81 to 1,000 pg/mL, and the sensitivity was 5.00 pg/mL. The CV values for interassay and intra-assay measurements were <10% and <5%, respectively [12].

In a second study, the potential disease-modifying effects of donepezil, a cholinesterase inhibitor, were evaluated by quantifying the levels of Aβ in plasma. The inclusion criteria for this study were diagnosis of probable AD according to NINDS/ADRDA criteria, MMSE >10, and willingness to

undergo serial blood draws. The donepezil-initiation and the stable-donepezil groups were well-matched in age (Table 2). The Aβ peptide levels were determined in a population of 28 individuals at baseline and 12 weeks later. All subjects had a diagnosis of probable AD. Twenty subjects had never been exposed to cholinesterase inhibitors at baseline. After the baseline plasma collections, these subjects initiated donepezil at a dose of 5 mg orally per day for 1 month and then increased to 10 mg orally per day and remained on this dose until the follow-up plasma sampling. This group was compared with a second group of 8 subjects who were on stable doses of donepezil 10 mg per day for at least 6 months before baseline, and who remained on donepezil through the time of follow-up to plasma collection. At both time stages, the plasma Aβ levels were quantified by ELISA by taking three plasma samples per individual on Monday, Wednesday, and Friday under fasting conditions at base time and 12 weeks afterward. The donepezil-initiation cohort was composed of 15 women and 5 men, average age 76.4 years, and the stable-donepezil cohort was composed of 6 women and 2 men, average age 75.8 years. At baseline, the average MMSE of the donepezil-initiation cohort was 23.5 (range, 14 to 28), and the average MMSE of the stable-donepezil group was 18.5 (range, 11 to 25). All individuals completed the study. All immunoassay evaluations of Aβ40 and Aβ42 were performed by using the same ELISA techniques as described above.

2.3. Quantification of Aβ peptides in brain gray matter

Amyloid β peptides were quantified in the superior frontal gyrus of 23 AD neuropathologically diagnosed cases (average age, 85.6 years; 11 women and 12 men) and 20 ND individuals (average age, 77.7 years; five women and 15 men). All brain specimens were obtained from the rapid autopsy Brain Bank of SHRI and had an average postmortem delay

Table 2
Therapeutic study of plasma Aβ values and patient demographics

Patient no.	Age (y)	Gender	MMSE	Aβ40 (pg/mL)	Aβ42 (pg/mL)	Total Aβ (pg/mL)	Ratio Aβ42/Aβ40	Aβ40 (pg/mL)	Aβ42 (pg/mL)	Total Aβ (pg/mL)	Ratio Aβ42/Aβ40
Baseline stable-donepezil (n = 8)								Follow-up stable-donepezil (n = 8)			
1	85	M	11	701.76	121.46	823.22	0.173	667.35	114.92	782.27	0.17
2	84	F	15	307.98	310.00	617.98	1.007	294.73	262.14	556.87	0.89
3	70	F	22	249.25	153.13	402.39	0.614	258.74	132.07	390.81	0.51
4	78	F	19	218.32	107.05	325.37	0.490	208.62	117.33	325.95	0.56
5	76	F	21	294.78	111.31	406.09	0.378	199.18	109.86	309.04	0.55
6	57	M	18	238.95	139.09	378.04	0.582	343.57	131.89	475.45	0.38
7	77	F	25	168.23	141.66	309.90	0.842	221.38	156.49	377.87	0.71
8	79	F	17	284.72	171.83	456.55	0.604	242.81	167.59	410.39	0.69
Average	75.75	6F/2M	18.50	308.00	156.94	464.94	0.59	304.55	149.04	453.58	0.56
SD	8.91		4.34	165.43	65.51	173.33	0.26	154.17	49.97	154.88	0.22
Baseline versus follow-up, *P* value				.966	.790	.892	.819				
Baseline donepezil-initiation (n = 20)								Follow-up donepezil-initiation (n = 20)			
10	82	F	23	390.82	209.63	600.45	0.536	385.39	193.45	578.84	0.50
11	75	M	28	314.91	144.20	459.11	0.458	326.65	153.49	480.14	0.47
12	73	F	28	290.91	150.43	441.35	0.517	303.57	125.99	429.56	0.42
13	75	F	27	231.84	129.83	361.67	0.560	305.57	125.44	431.00	0.41
14	80	F	25	271.05	575.01	846.06	2.121	302.41	543.31	845.71	1.80
15	77	F	14	143.75	125.48	269.23	0.873	171.99	135.00	306.99	0.79
16	79	F	27	221.14	219.88	441.02	0.994	293.23	219.31	512.53	0.75
17	56	F	22	212.57	141.17	353.74	0.664	215.08	130.99	346.07	0.61
18	83	M	24	237.59	107.95	345.54	0.454	247.17	112.53	359.69	0.46
19	81	F	23	696.84	175.94	872.79	0.252	721.14	147.71	868.84	0.21
20	75	F	25	353.42	214.65	568.06	0.607	433.12	177.48	610.60	0.41
21	76	F	28	203.20	99.18	302.38	0.488	252.69	104.93	357.62	0.42
22	81	M	26	227.14	240.34	467.48	1.058	212.57	222.79	435.36	1.05
23	59	M	20	257.08	700.09	957.18	2.723	294.58	593.55	888.13	2.02
24	70	F	26	326.79	126.61	453.40	0.387	324.16	132.69	456.85	0.41
25	76	F	25	433.26	92.64	525.90	0.214	385.99	94.44	480.44	0.25
26	83	F	15	307.96	307.77	615.73	0.999	573.54	266.84	840.39	0.47
27	77	M	25	267.80	131.55	399.35	0.491	240.79	130.64	371.43	0.54
28	78	F	21	314.03	238.54	552.57	0.760	324.45	183.84	508.28	0.57
29	92	F	18	546.82	145.97	692.79	0.267	658.09	124.91	783.00	0.19
Average	76.4	15F/5M	23.5	312.45	213.84	526.29	0.771	348.61	195.97	544.57	0.64
SD	7.96		4.11	127.87	156.54	192.12	0.62	146.42	135.01	194.00	0.48
Baseline versus follow-up, *P* value				.411	.701	.766	.433				

NOTE. All cases are probable AD. These values represent the average of three readings per week.

time of 2.5 hours [11]. All AD and ND brains were rated following the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) criteria and the Braak stage classification. All 43 AD and ND cases were *apolipoprotein E* (*APOE*) genotyped. Briefly, the cerebral frontal gyrus cortices were homogenized with glass-distilled formic acid (GDFA), and the acid-soluble fraction was submitted to fast protein liquid chromatography (FPLC) on a Superose 12 column (Amersham/GE Healthcare, Chalfont St Giles, UK) with 80% GDFA as the mobile phase [13]. The fractions containing the Aβ peptides were collected and neutralized, and the Aβ40 and Aβ42 were quantified by europium immunoassay with antisera against Aβ40 (Aβ residues 34-40) and Aβ42 (Aβ residues 36-42) as capture antibodies and the europium-labeled anti Aβ 17-24 (4G8) as the reporter antibody (Zenetec, Maryland Heights, MO) and the europium enhancement solution (Wallac Inc, Gaithersburg MD), as previously described [2,10].

2.4. Quantification of Aβ peptides in white matter

The Aβ peptides were quantified in the white matter (WM) of 10 cases of neuropathologically diagnosed AD (average age, 84.0 years; six women and four men) with moderate to severe WM rarefaction and from 13 ND controls (average age, 82.8; seven women and six men) with none to mild WM rarefaction. The GDFA-soluble Aβ peptides were separated by FPLC and after neutralization were submitted to europium immunoassay as described in detail in a previous publication [13].

2.5. Quantification of Aβ peptides from inactivated and activated human platelets

Fresh human platelets were obtained from five Red Cross donors by plateletpheresis. The experimental processing of the platelets was performed following the protocols published by Li [3,14] with minor modifications. The platelets were suspended in a final plasma volume of 220 to 300 mL, which included 32 to 50 mL of anticoagulant citrate dextrose solution-formula A (ACD-A) and maintained at room temperature (∼25°C). The platelet suspensions were centrifuged (type 19 rotor; Beckman Coulter, Fullerton, CA) at 3,800*g* for 30 minutes at 25°C in 250-mL capacity polyallomer bottles. After removal of the plasma, the pelleted platelets were suspended in a total volume of 200 mL of 0.38% sodium citrate, 0.6% glucose, and 0.72% NaCl, pH 7.0 (washing buffer [WB]), and this washing and centrifugation step was repeated twice. The supernatants were eliminated, and each of the individual platelet preparations was divided into three equal fractions and washed once more with 32 mL of WB. The supernatants were discarded. Two milliliters of the inactivated pelleted platelets was lysed by the addition of 10 mL of 98% GDFA by using a 30-mL capacity glass homogenizer. The platelet homogenate were centrifuged in polyallomer tubes at 250,000*g* for 1 hour at 25°C in a SW41 rotor (Beckman Coulter). The top layer of lipids and small pellet of insoluble material were eliminated, and the intermediate supernatant fraction was collected and apportioned into 500-μL samples that were submitted to FPLC size-exclusion Superose 12 columns (Amersham Biosciences) with 80% GDFA as the mobile phase to isolate Aβ peptides, as previously described [13].

For the preparation of activated platelets, the platelets were separated from plasma as described above. A volume of 2 mL of the pelleted platelets was suspended in 20 mL of Tyrode's buffer (137 mmol/L NaCl, 2.68 mmol/L KCl, 11.9 mmol/L $NaHCO_3$, 0.42 mmol/L NaH_2PO_4, 2 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 5.5 mmol/L glucose, pH 7.4) containing 1 unit per milliliter of human thrombin (Calbiochem, San Diego, CA) and 20 μg/mL of human collagen (Sigma, St Louis, MO). The activated clumped platelets were dispersed, and after 30 minutes of stirring, the whole preparation was freeze-dried. To the recovered lyophilized powder, 10 mL of 80% GDFA was added, and the suspension was thoroughly homogenized (Tenbroeck glass homogenizer) and centrifuged at 250,000*g* (SW41 rotor) for 1 hour in polyallomer tubes. The top layer of lipids and insoluble pellet were discarded, and the supernatant was submitted in 500-μL aliquots to FPLC separation [13]. Both the activated and inactivated platelet fractions containing the Aβ peptides were submitted to ELISA as in Section 2.2.

2.6. Quantification of Aβ peptides from aorta

The amounts of Aβ peptides present in the aortic walls of six elderly individuals (mean age, 83 years) with severe AVD were quantified. The atherosclerotic specimens (∼9 g of tissue) had extensive zones of calcification and multiple complicated lesions, with ulceration and rupture of the fibrous caps showing areas of thrombosis. These complicated aortic atheromatous lesions also showed large crater-like morphology with hemorrhagic areas. For the control, we used a pool of two aortic specimens (∼1.5 g each) from individuals with a mean age of 82 years, without atherosclerotic lesions, and with minimal fatty streaks. All specimens were extensively rinsed with cold distilled water to remove all traces of blood. The adventitial connective tissue, easily dissected from the subjacent tunica media, was removed. The aortic tissues were pooled, minced, and pulverized in liquid nitrogen by using a mortar and pestle, suspended in 20 mL of 98% GDFA, and stirred overnight at 4°C. The suspension was centrifuged at 250,000*g* for 1 hour at 4°C by using a SW-41 Ti rotor (Beckman Coulter). The acid-soluble phase was collected and filtered (Whatman #1 paper; Whatman plc, Maidstone, Kent, UK). Five hundred microliter aliquots of GDFA samples were submitted to FPLC [13].

The fractions containing Aβ peptides were pooled, and the volume was reduced to 500 μL and dialyzed (Spectrapor #6 membrane 1,000-d cutoff; Spectrum Labs, Rancho Dominguez, CA) against deionized water followed by dialysis against 5 mol/L guanidine hydrochloride, 50 mmol/L

Tris-HCl, pH 8.0 at room temperature. For quantification, the dialyzed samples were submitted to Aβ40 and Aβ42 ELISAs as described in Section 2.2.

2.7. Quantification of Aβ peptides from leptomeningeal arteries

Two grams of leptomeningeal arteries, representing the anterior, middle, and posterior cerebral arteries showing advanced AVD lesions, was dissected and pooled from four individuals with AD (average age, 82.0 years; two men and two women) and from a group of five ND individuals without AVD (average age, 86.6 years; three women and two men). The two separate leptomeningeal arterial pools were minced, extensively rinsed with cold distilled water to remove blood cells, and thoroughly homogenized in 10 mL of GDFA. The leptomeningeal vessels, with and without AVD, were centrifuged, and the acid supernatant was collected and submitted to size-exclusion FPLC (Superose 12) in 80% GDFA [13]. The Aβ-containing fractions were pooled, the volume was reduced, and the samples were dialyzed as described for the aorta specimens. The Aβ peptides were quantified by ELISA as described in Section 2.2.

2.8. Quantification of Aβ peptides in skeletal muscle

Temporalis skeletal muscles were obtained from the same population in which brain Aβ was quantified in the superior frontal gyrus (see Section 2.3). A detailed extraction, purification, and europium immunoassay of Aβ peptides present in skeletal muscle are fully described elsewhere [2].

2.9. Quantification of Aβ peptides from liver

Liver specimens (1 g each) were obtained from six neuropathologically diagnosed AD cases (average age, 83 years; three women and three men) and from six ND individuals (average age, 87 years; two women and four men). The liver tissues were minced and thoroughly washed to eliminate entrapped blood as much as possible. Each liver specimen was homogenized in 90% GDFA and centrifuged (250,000*g*, 1 hour at 4°C), and the acid soluble fraction was collected and submitted to FPLC [13]. After volume reduction and dialysis (see aorta preparation), Aβ peptides were quantified by ELISA as described in Section 2.2.

3. Results

3.1. Plasma Aβ

All mean values in this study were compared by using two independent sample *t* tests. The differences in Aβ40 and Aβ42 levels, the total Aβ levels (Aβ40 + Aβ42), and the Aβ42/Aβ40 ratios between the AD and ND groups were not statistically significant (Table 1 and Figure 1). The AD (mean age, 81.4; standard deviation [SD], 5.3) and ND (mean age, 75.8; SD, 7.1) groups differed significantly in age ($P = .01$), but age did not correlate with plasma concentrations of Aβ40, Aβ42, or Aβ40/42 ratio within either the ND or AD groups (r^2 values ranging from 0.00006 to 0.04). The *t* tests were performed without correction for multiple comparisons (eg, Bonferroni), because this would have biased the results toward an increased rate of false-positive findings. Because the results with respect to plasma Aβ concentrations were all negative, we did not consider it necessary to apply a correction for multiple comparisons. The same situation was observed in the donepezil therapeutic study in which the high range of variability was confirmed, and no statistically significant differences were found between the donepezil-initiation and stable-donepezil populations (Table 2 and Figure 2), and no significant correlations were evident between Alzheimer's Disease Assessment Scale-cognitive subscale (ADAS-Cog), MMSE score, age, or gender and *APOE* genotype.

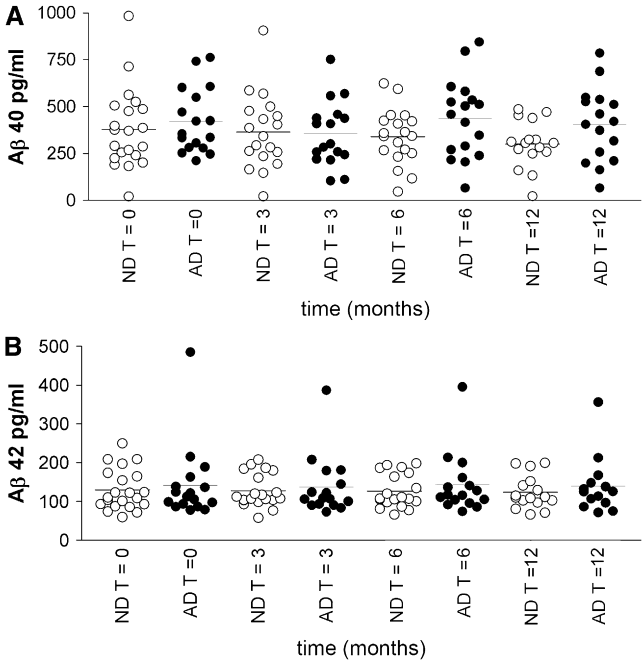

Fig. 1. Longitudinal comparison of AD (black) and ND control (white) plasma Aβ levels as measured by ELISA. Measurements were taken at baseline (T = 0) and at 3, 6, and 12 months. The horizontal bars represent the mean values. (A) Aβ40, pg/mL; (B) Aβ42, pg/mL.

Amyloid β40 and Aβ42 levels fluctuated widely among individuals over time in both AD subjects and ND controls (Figure 1). The amounts of Aβ40 among individuals during the year of the study fluctuated from 659 to 16 pg/mL, and Aβ42 levels varied between 149 and 4.0 pg/mL. The average Aβ42/40 ratios also varied substantially among individuals (Tables 1 and 2).

3.2. Brain Aβ (gray matter)

Quantifying Aβ peptides in the cerebral cortex demonstrated the prevalence of Aβ42 over Aβ40 in AD individuals

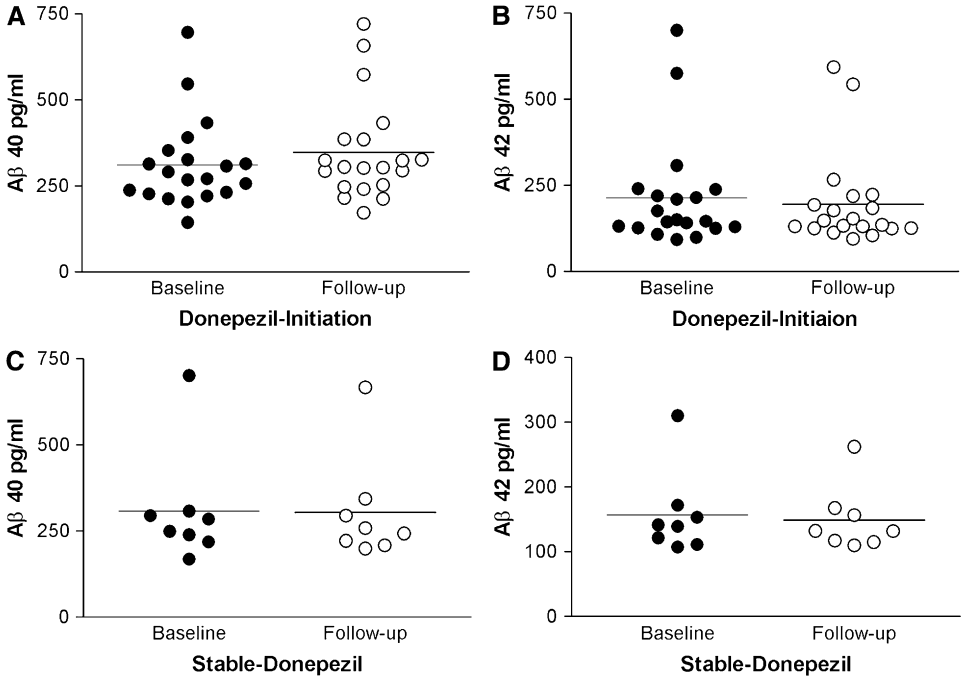

Fig. 2. Plasma Aβ levels measured by ELISA in the therapeutic study with donepezil. Measurements were taken at baseline and 12 weeks later (follow-up). (A) Aβ40, pg/mL, in donepezil-initiation group; (B) Aβ42, pg/mL, in donepezil-initiation group; (C) Aβ40, pg/mL, in stable-donepezil group; (D) Aβ42, pg/mL, in stable-donepezil group.

[2], with a high level of variability (Table 3). The mean value of Aβ42 was 6,096 ng/g tissue (range, 1,047 to 19,680 ng/g; SD, 1,099), with Aβ40 averaging 608 ng/g tissue (range, 26 to 3,434 ng/g; SD, 154). By contrast, in the ND control population the average amount of Aβ42 was 784 ng/g tissue (range, 42 to 3,402 ng/g; SD, 221) and for Aβ40 was 209 ng/g tissue (range, 0 to 731 ng/g; SD, 59). There were significant differences in the amounts of cortical Aβ peptides observed between AD and ND (Aβ42, $P < .001$; Aβ40, $P = .022$), as well as between total Aβ ($P < .001$). In those individuals with AD carrying the *APOE* ε4 allele, the mean value for total Aβ was 8,373 ng/g of cortex, whereas in those without the *APOE* ε4, the total Aβ mean was 5,631 ng/g ($P = .223$). In the case of the ND cohort, those individuals carrying the *APOE* ε4 had a total Aβ average of 2,178 ng/g of cortex, whereas those without the *APOE* ε4 allele had a mean of 783 ng/g of tissue ($P = .043$). However, this marginally significant value might reflect the fact that only three heterozygous individuals out of 20 harbored an *APOE* ε4 allele.

Table 3
Aβ peptide levels in human tissues

	Aβ40 (ng/g)	Aβ42 (ng/g)	Total Aβ (ng/g)
Brain gray matter			
AD (n = 23)	608	6096	6,704
ND (n = 20)	209	784	993
Brain WM			
AD (n = 10)	1,069	1,135	2,204
ND (n = 13)	111	418	529
Platelets			
Q (n = 5)	83.8	1.7	85.5
A (n = 5)	56.8	1.6	58.4
Aorta			
Severe AVD (n = 6)	75.3	0.7	76.0
Minimal AVD (n = 2)	31.4	1.0	32.4
Leptomeningeal arteries			
Severe AVD (n = 4)	113.1	28.0	141.1
Minimal AVD (n = 5)	17.3	5.3	22.6
Skeletal muscle			
AD (n = 23)	37.8	15.7	53.5
ND (n = 20)	29.8	10.2	40.0
Liver			
AD (n = 6)	8.6	1.7	10.3
ND (n = 6)	67.5	15.5	83.0

Abbreviations: Q, quiescent; A, activated.

3.3. Brain Aβ (white matter)

Investigation of the Aβ peptide levels in WM revealed that in AD the mean values for Aβ42 and Aβ40 were 1,135 ng/g of tissue (range, 323 to 3,313 ng/g; SD, 895) and 1,069 ng/g of tissue (range, 5.0 to 4,149 ng/g; SD, 1601) [13], respectively (Table 3). The highest values of Aβ among the 10 AD subjects were exhibited in two individuals carrying the *APOE* ε4/ε4 genotype with Aβ42 levels of 1,624 ng/g and 3,313 ng/g and Aβ40 4,149 ng/g and 4,030 ng/g of WM. The ND cohort, on the other hand, had an Aβ42 mean of 418 ng/g of tissue (range, 122 to 880 ng/g; SD, 233), and for Aβ40 it was 111 ng/g of tissue (range, 0 to 272 ng/g; SD, 77). The differences between the AD and ND groups were significant for both: Aβ42, $P = .05$ and Aβ40, $P = .02$. The difference

between the total Aβ values in AD and ND groups was also statistically significant ($P = .02$).

3.4. Platelet Aβ

The Aβ40 and Aβ42 peptides were quantified in a pool of platelets in their quiescent and activated forms from five healthy individuals. In the latter case, the levels of Aβ were measured in the platelets and their medium, because activation is followed by immediate secretion of by-products into the medium. The Aβ40 and Aβ42 peptides in the quiescent platelets yielded 83.8 and 1.7 ng/mL of packed platelets, respectively. In the case of the activated platelets, these peptides represented 56.8 and 1.6 ng/mL, respectively (Table 3).

3.5. Aortic Aβ

In the aortic walls with advanced atherosclerotic lesions, Aβ40 was present at 75.3 ng/g of tissue and Aβ42 at 0.7 ng/g of tissue, higher levels than those observed in aorta samples with minimal atherosclerotic lesions (fatty streaks) that contained 31.4 ng/g of tissue and 1.0 ng/g of tissue for Aβ40 and Aβ42, respectively (Table 3).

3.6. Leptomeningeal vascular Aβ

There were significant differences between the amounts of Aβ peptides present in the leptomeningeal arteries with and without atherosclerotic plaques. In the former, the Aβ40 levels were 113.1 ng/g of tissue and Aβ42 amounted to 28.0 ng/g of tissue, whereas in the latter these corresponded to 17.3 and 5.3 ng/g of tissue, respectively (Table 3).

3.7. Skeletal muscle Aβ

Amyloid β peptides in the AD and ND groups from skeletal muscle showed a lesser degree of variability [2]. The differences between AD and ND control regarding the Aβ40 were borderline significant ($P = .067$), whereas there was a statistically significant difference with respect to the Aβ42 ($P = .010$) and total Aβ ($P = .019$) (Table 3). However, values between the AD and ND groups and between individuals exhibited substantial variance. The *APOE* ε4 alleles, although more numerous in the AD group (10 of 46 alleles) than in the ND cohort (three of 40 alleles), were not directly correlated with the levels of Aβ40 or Aβ42 peptides in skeletal muscle.

3.8. Liver Aβ

Amyloid β40 and Aβ42 in the postmortem liver of ND individuals were, on the average, 67.5 ng/g of tissue (range, 18.8 to 94.6 ng/g) and 15.5 ng/g of tissue (range, 6.4 to 20.6 ng/g), respectively, whereas the Aβ40 and Aβ42 in AD subjects averaged 8.6 ng/g of tissue (range, 6.7 to 11.7) and 1.7 ng/g (range, 0 to 2.6) of tissue, respectively. The ND control patients' liver tissue contained eight-fold more total Aβ than AD liver, with a total mean value for the ND control group of 83 ng/g of tissue and 10.3 ng/g of tissue in the AD group (Table 3).

4. Discussion

Longitudinal studies of plasma Aβ40 and Aβ42 levels demonstrated wide temporal variation within and among the individuals involved in the present investigation. The overall mean values for Aβ40 and Aβ42 in the longitudinal study were 384 and 132 pg/mL, respectively. The overall mean values for Aβ40 and Aβ42 in the therapeutic study were 319 and 179 pg/mL, respectively. These two independent plasma analyses showed virtually equivalent levels for total Aβ peptides: longitudinal, 517 pg/mL and therapeutic, 497 pg/mL. In both investigations there were no statistical correlations noted between Aβ values and MMSE scores, diagnoses, age, or gender. In support of our observations are several previous biomarker studies in which the plasma Aβ levels were not correlated with the diagnosis, medications, or with *APOE* genotype [1,15,16].

The spread of plasma Aβ values might be due to the amphoteric and amphipathic structure of Aβ peptides that results in their avid binding to a variety of plasma and membrane proteins. At physiologic concentrations of human serum albumin (approximately 40 mg/mL), this molecule can bind more than 95% of Aβ40/42 peptides at a stochiometry of 1:1. Kinetic studies have demonstrated that 1 mL of fresh plasma from healthy individuals, spiked with 5 or 20 ng Aβ40 or Aβ42, results in the recovery of 36% of Aβ1-40 and 26% of Aβ1-42 after a 1-hour incubation [10]. When variable amounts of washed erythrocytes are diluted with phosphate-buffered saline to hematocrit concentrations of 10% to 50% and are incubated with 5 ng/mL of Aβ40 or Aβ42, the amount of free Aβ peptides decreased as the hematocrit values increased. A large number of plasma proteins including immunoglobulins, apolipoprotein J, apoE, complement C1q, C4, C3, C5, and C6, transthyretin, apoferritin, amyloid P component, and α2-macroglobulin bind and mask Aβ peptides [9,10,17]. Freshly purified plasma lipoproteins are capable of sequestering 94% of circulating Aβ peptides [9]. In particular, apoE, a ligand for various lipoprotein receptors bound to the surface of circulating low-density lipoprotein (LDL), has high affinity for Aβ peptides [18,19].

Plasma cholesterol appears to play a pivotal role in Aβ chemistry. The production of lipoproteins by liver and brain is likely to cause concomitant fluctuations in plasma levels of free and bound Aβ. Interestingly, individuals with higher midlife plasma cholesterol levels have an elevated risk of developing AD, and individuals with clinically or neuropathologically diagnosed AD have higher plasma cholesterol levels compared with ND controls [20,21]. Furthermore, in AβPP transgenic (Tg) mice it has been observed that hypercholesterolemia accelerates the evolution of amyloid pathology [22] and that cholesterol accumulates in senile plaques of AD patients as well as AβPP Tg mice [23]. In this context, a broad body of evidence derived from postmortem, epidemiologic,

correlative, and experimental studies links AVD with AD, and multiple established risk factors for AVD have now been recognized to be risk factors for the development of AD [24–28]. Our studies also revealed that aortas with either mild fatty streaks or advanced AVD contained Aβ peptides, predominately Aβ40. Likewise, leptomeningeal arteries with AVD pathology have larger amounts of Aβ peptides than lesion-free arteries. A feature common to both AVD and vascular amyloidosis is a persistent degenerative pathology associated with chronic arterial wall inflammation [27,29]. Amyloid β accumulation also induces microvascular inflammation mediated by proinflammatory molecules activated in glia, endothelium, smooth muscle cells, and pericytes [30]. Circulating markers of inflammation are also increased in AVD, among them C-reactive protein [31]. Some of these proinflammatory molecules also represent risk factors for AD [32,33]. Cognitive decline in AD is apparently associated with the degree of cerebral amyloid angiopathy, arteriosclerosis, and lipohyalinosis [34]. In particular, the capillary deposition of Aβ42 is highly correlated with AD pathology [35]. The severity of circle of Willis and leptomeningeal arterial stenosis caused by AVD significantly correlates with the neuropathologic lesions of AD [25–28]. Inferring a functional, pathologic role for the Aβ peptides in atherosclerotic plaques is reasonably justified by the multiple associations between hypercholesterolemia and AVD [31], hypercholesterolemia and AD [36], and AVD and AD [25,28].

Amyloid β in the brain is mostly synthesized by neurons, but other cells such as glia, vascular endothelia, and myocytes also have the means to produce AβPP and Aβ peptides. Although there are multiple mechanisms for uptake and clearance of Aβ peptides through the cerebrovasculature, the functionality of these pathways during the aging process has not been well-established [37]. The receptor for advanced glycation end products (RAGE) and the LDL receptor and LDL receptor–related protein (LRP) have been advocated as active mechanisms for uptake and clearance of Aβ, respectively [38–41]. Amyloid β is also eliminated from the brain through the periarterial spaces of the cerebral vasculature that shunt Aβ peptides into the lymphatics of the head and neck and finally back into the systemic venous circulation [42,43]. This suggests that the clogging of the periarterial spaces in cortical and leptomeningeal arteries by fibrillar Aβ deposition is probably one of the more devastating pathologic hemodynamic events in AD [44]. In AD, the WM contains four-fold more ''soluble'' total Aβ (mean, 2,200 ng/g tissue) than ND controls (mean, 520 ng/g tissue) [13]. It can be postulated that soluble Aβ in the WM normally drains through the periarterial spaces into the systemic circulation, as suggested by the gross dilation of the periarterial spaces observed in AD [44].

Platelets represent another significant source of Aβ peptides in the circulation. These structures mostly contain Aβ ending at residue 40, with a small amount of Aβ42, in both the quiescent and activated states. Our in vitro, thrombin-collagen–activated platelets released Aβ40 and Aβ42. If this phenomenon occurs in vivo, platelets contribute to the pool of circulating Aβ peptides [45,46]. Intriguingly, our activated platelets apparently contain ∼30% less Aβ40 than the quiescent ones. Although we do not have an explanation for this decay, it is possible that platelet activation and release of Aβ stimulate aggregation or sequestration of these peptides, thus escaping detection by the immunoassay. Alternatively, Aβ might intervene to facilitate platelet aggregation [47]. Platelets express $A\beta PP_{695}$, $A\beta PP_{751}$, and $A\beta PP_{770}$ isoforms, whose ratio appears to be altered in AD [48–50]. The latter two, also known as protease nexin-2 (PN2), have a serine (Kunitz) protease inhibitor (KPI) insert 56 amino acids long close to their N-termini [3,14,46,51]. This domain has an important role in the coagulation cascade, where it functions as an inhibitor of factors IXa, Xa, Xia, and tissue factor VIIa complex [52]. AβPP and Aβ peptides have been observed in atherosclerotic plaque macrophages that have engulfed platelets in areas of neovascularization [53,54]. Although platelets and circulating Aβ might be a significant source of these peptides in the atherosclerotic lesions, it is possible that additional Aβ peptides originate directly from the arterial tunica media myocytes and endothelial cells that also express the AβPP molecule [55]. During the process of aging, many aberrations occur in the vascular walls and endothelium, as well as in platelets [56], hemostatic functions, and fibrinolytic activity, that lead to increased atherosclerosis and thrombosis [57,58]. Several studies have suggested that platelets are an important link between vascular repair and vascular amyloidosis and between atherosclerosis and AD [54,59].

The cholinergic neuromuscular junctions of skeletal muscle are another potentially rich source of Aβ peptides [2,60,61]. The production of Aβ peptides is exacerbated in inclusion body myositis, the most common skeletal muscle inflammatory disease among the elderly [61–63]. Our studies revealed significant differences in Aβ42 and total Aβ between AD and ND control populations. This observation suggests that AD might have systemic manifestations. Skeletal muscle also generates longer Aβ peptides ending at residues 44, 45, and 46 [2]. The potential contribution of skeletal muscle Aβ to the circulating pool should be considered because the total muscle mass represents about one third of the body weight.

The AβPP/Aβ peptides produced in different tissues might also have different half-lives and degradation rates because these molecules are substrates for a large variety of proteolytic enzymes [64]. The liver is the major organ responsible for Aβ clearance from plasma, capable of capturing up to 90% of the circulating Aβ peptides, a fraction of which is degraded, with the remainder removed through the bile [65]. A comparatively smaller amount of Aβ is eliminated through the kidneys and secreted in the urine [66]. In addition, the liver endocytic uptake is mediated by the hepatocyte LRP-1 and enhanced by the presence of insulin [67]. The reduction in the amount of liver Aβ in the AD cases might be due to liver failure in the advanced stages of the disease.

In summary, Aβ peptides are produced by a wide variety of tissues in both the CNS and periphery. Several lines of evidence suggest that a global assessment of Aβ sources and sinks might help to understand AD pathology and dementia. It will also help to elucidate the relationship between Aβ and cholesterol metabolism, as well as the role of Aβ in AVD. Large individual fluctuations in plasma Aβ values are the most important observation in our longitudinal studies. When assessing Aβ therapeutic interventions, the potential multiple contributions to the plasma Aβ pool, as well as the time and conditions of sampling physiologic fluids for Aβ evaluation, need to be considered. For example, CSF specimens demonstrated wide-ranging Aβ levels within individuals during a period of 36 hours [68]. We are aware that investigations of this type have limitations including small sample size caused by difficulties in recruitment and attrition of participants and patient physical conditions. In addition, Aβ peptide fluctuations caused by diet, medications, stress, circadian rhythm [68], metabolic conditions, etc can affect the outcome.

Therapeutic vaccination trials in Tg animals and humans have revealed that senile plaques, a cardinal pathologic feature of AD, are dynamic structures [69] subject to dissolution by Aβ immunotherapy [42,70]. Peripheral Aβ production appears to contribute to brain amyloid through transport into the CNS, and correspondingly the brain Aβ contributes to the pool in circulation [37]. Therefore, brain as well as plasma Aβ levels are the consequence of the intricate relationships that exist among several interacting Aβ peptide sources that are tempered by the natural physiologic decline accompanying aging and associated morbidities. The ultimate pathology of AD is focused within the brain, but neither the brain nor AβPP/Aβ exists as neatly isolated entities. The long-term CNS and systemic consequences of Aβ immunization and secretase inhibitory treatments have the potential to disturb a wide range of cellular and systemic functions, in which AβPP metabolites and Aβ are essential [71–74]. As clinical trials advance, efforts should be undertaken to recognize adverse events both within and outside the brain proper. Although Aβ deposits are a logical therapeutic focus, it remains unclear whether the deposited or soluble forms of this molecule are the most toxic. Indeed, senile plaques might represent a mechanism of defense whereby excessive harmful levels of soluble Aβ peptides are inactivated into fibrillar core structures surrounded by glial cells [74–76]. Preventing or dissolving these deposits might be injurious to the brain. Understanding the dynamic balance between Aβ pools and their function might add clarity and suggest new routes to improve AD therapeutic strategies.

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