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Aβ 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 non-demented (ND) controls in a longitudinal study 2) The plasma of a cohort of AD patients receiving a cholinesterase inhibitor 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.

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Arteries with atherosclerosis had larger amounts of A β 40 than disease-free vessels. Inactivated platelets contained more A β peptides than activated ones. Substantially more A β was present in liver samples from ND patients. Overall, AD brain and skeletal muscle contained increased levels of A β .

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

Keywords

plasma A β ; Alzheimer's disease; peripheral A β ; atherosclerotic vascular disease; A β immunotherapy

1. Background

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by dementia and an abundance of amyloid-beta (A β) peptides in the brain parenchyma and cerebral vasculature that are derived from the amyloid-beta precursor protein (A β PP). Genetic investigations strongly suggest that A β peptides have a central role in AD pathogenesis. However, genetically-determined AD is rare, while 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 pathological marker of AD due to their profuse extracellular deposition in senile and diffuse plaques and vascular walls. In addition, A β 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 A β peptides in their diverse physical forms and to elucidate the multiple functional roles of A β . Most important, vast investments have been made to create therapeutic agents that either interfere with A β production or are capable of dispersing amyloid deposits.

Due to the omnipresence of A β peptides in familial and sporadic AD, the levels of these molecules in physiological fluids have been measured and characterized in order to establish their potential utility as reliable disease biomarkers. Unfortunately, despite the seemingly obvious association of A β with AD pathology, the efforts to validate plasma A β peptides as dementia biomarkers and correlate threshold concentration levels with disease stage have been fraught with frustration [1]. In the brain, A β 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 may play far more significant roles in the production of dementia than the molecules sequestered in extracellular plaques. In addition, the critical role that circulating A β peptides play in AD pathology cannot be ignored. Besides the brain, A β 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 A β PP 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 may allow for an active and dynamic interchange of A β peptides between the brain and periphery. These sources undoubtedly contribute to the pool of circulating A β , and must be considered when the success or failure of AD pharmacological interventions, intended to reduce cerebral amyloid, is assessed by monitoring A β plasma levels. Interestingly, A β peptides in the periphery fail to generate filamentous structures probably due to the presence of albumin, erythrocyte

membrane proteins and the other multiple circulating molecules that bind A β 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 A β on the plasma levels of these peptides and on the general pathology of AD were evaluated in several complimentary ways. A comprehensive, longitudinal assessment of plasma A β levels in AD and age-matched non-demented (ND) control individuals was performed at baseline, 3, 6 and 12 months. An additional longitudinal study determined the potential effects of the cholinesterase inhibitor donepezil hydrochloride (Aricept) on plasma A β levels in AD patients and compared them to 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 A β 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 A β peptides in plasma

The inclusion criteria is 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 are diagnosed with delirium (DSM-IV), were not able to be assessed due to 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 MR scanning or could not provide a collateral informant. The diagnosis was based on the NINDS-ADRDA criteria. Blood samples were obtained at baseline, 3 months, 6 months and 12 months, under fasting conditions, from 17 individuals with clinically-diagnosed AD (average age 81.4 years; 10 females and 7 males) and 21 ND subjects (average age 75.8 years; 14 females and 7 males). Due to subject attrition, in the total of 38 enrolled individuals, 28 cases had their plasma analyzed at 4 time points and 10 cases at 3 time points. The average mini-mental state examination (MMSE) score for the ND control group was 29.0 (range 24–30) and 22.3 (range 15–30) for the AD group (Table 1).

The plasma levels of A β 1-40 and A β 1-42 peptides were immunoassayed, in duplicate, by enzyme-linked immunosorbent assays (ELISA) that were obtained from Immunobiological Laboratories (Minneapolis, MN) and from Innogenetics (Gent, Belgium), respectively. The ELISAs were carried out following the manufacturer's instructions and executed by the same investigator who was blind to the identity of the specimens. The high sensitivity method was used for the A β 1-42 kit. A β 1-40 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 inter-assay measurements were < 7%. The CV values for intra-assay measurements were < 8%. Using the high sensitivity method, the A β 1-42 ELISA had a measurement range of 7.81 to 1000 pg/ml and the sensitivity

was 5.00 pg/ml. The CV for inter-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 A β in plasma. The inclusion criteria for this study were diagnosis of probable Alzheimer's disease 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 A β 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 PO per day for one month and then increased to 10 mg PO per day and remained on this dose until the follow-up plasma sampling. This group was compared to a second group of 8 subjects who were on stable doses of donepezil 10 mg per day for at least 6 months prior to baseline, and who remained on donepezil through the time of follow-up to plasma collection. At both time stages, the plasma A β levels were quantified by ELISA by taking 3 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 females and 5 males, average age 76.4 years and the "stable-donepezil" cohort was composed 6 females and 2 males, average age 75.8 years. At baseline, the average MMSE of the donepezil-initiation cohort was 23.5 (range 14–28) and the stable-donepezil group was 18.5 (range 11–25). All individuals completed the study. All immunoassay evaluations of A β 40 and A β 42 were performed using the same ELISA techniques as described above.

2.3 Quantification of A β 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 females and 12 males) and 20 ND individuals (average age 77.7 years; 5 females and 15 males). All brain specimens were obtained from the rapid autopsy Brain Bank of SHRI and had an average postmortem delay time of 2.5 h [11]. All AD and ND brains were rated following the CERAD criteria (Consortium to Establish a Registry for Alzheimer's Disease) and the Braak stage classification. All 43 AD and ND cases were apolipoprotein E (Apo E) genotyped. Briefly, the cerebral frontal gyrus cortices were homogenized with glass-distilled formic acid (GDFA) and the acid-soluble fraction submitted to fast protein liquid chromatography (FPLC) on a Superose 12 column (Amersham/GE Healthcare) using 80% GDFA as the mobile phase [13]. The fractions containing the A β peptides were collected and neutralized and the A β 40 and A β 42 quantified by Europium immunoassay using antisera against A β 40 (A β residues 34–40) and A β 42 (A β residues 36–42) as capture antibodies and the Europium labeled anti A β 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 A β peptides in white matter

The A β peptides were quantified in the white matter (WM) of 10 cases of neuropathologically-diagnosed AD (average age 84.0 years; 6 females and 4 males) with moderate to severe WM rarefaction and from 13 ND controls (average age 82.8; 7 females and 6 males) with none to mild WM rarefaction. The GDFA-soluble A β peptides were separated by FPLC and after neutralization submitted to europium immunoassay as described in detail in a previous publication [13].

2.5 Quantification of A β peptides from inactivated and activated human platelets

Fresh human platelets were obtained from 5 Red Cross donors by platelet-pheresis. The experimental processing of the platelets was performed following the protocols published by

Li QX, et al. [3;14] with minor modifications. The platelets were suspended in a final plasma volume of 220–300 ml, which included 32–50 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) at $3,800 \times g$ for 30 min at 25° C in 250 ml-capacity polyallomer bottles. Following 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 divided into 3 equal fractions and washed once more with 32 ml of WB. The supernatants were discarded. Two ml of the inactivated pelleted platelets were lysed by the addition of 10 ml of 98% GDFA using a 30 ml-capacity glass homogenizer. The platelet homogenate was centrifuged in polyallomer tubes at $250,000 \times g$ for 1 h at 25° C in a SW41 rotor (Beckman Coulter, Fullerton, CA). The top layer of lipids and small pellet of insoluble material were eliminated and the intermediate supernatant fraction collected and apportioned into 500 μ l samples that were submitted to FPLC size-exclusion Superose 12 columns (Amersham Biosciences) using 80% GDFA as the mobile phase to isolate A β 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 mM NaCl, 2.68 mM KCl, 11.9 mM NaHCO₃, 0.42 mM NaH₂PO₄, 2 mM CaCl₂, 1 mM MgCl₂, 5.5 mM glucose, pH 7.4) containing 1 unit per ml of human thrombin (Calbiochem, San Diego, CA) and 20 μ g/ml of human collagen (Sigma, St. Louis, MO). The activated clumped platelets were dispersed and after 30 min of stirring, the whole preparation was freeze-dried. To the recovered lyophilized powder, 10 ml of 80% GDFA was added and the suspension thoroughly homogenized (Tenbroeck glass homogenizer), centrifuged at $250,000 \times g$ (SW41 rotor) for 1 h using polyallomer tubes. The top layer of lipids and insoluble pellet were discarded and the supernatant submitted in 500 μ l aliquots to FPLC separation [13]. Both the activated and inactivated platelet fractions containing the A β peptides were submitted to ELISA as in Section 2.2.

2.6 Quantification of A β peptides from aorta

The amounts of A β peptides present in the aortic walls of 6 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 utilized a pool of two aortic specimens (~1.5 g each) from individuals with a mean age of 82 years, without atherosclerotic lesions and 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 using a mortar and pestle, suspended in 20 ml of 98% GDFA and stirred overnight at 4° C. The suspension was centrifuged at $250,000 \times g$ for 1 h at 4° C using a SW-41 Ti rotor (Beckman). The acid-soluble phase was collected and filtered (Whatman # 1 paper). Five hundred μ l aliquots of GDFA samples were submitted to FPLC [13].

The fractions containing A β peptides were pooled, the volume reduced to 500 μ l and dialyzed (Spectrapor # 6 membrane 1,000 Da cut off) against deionized water, followed by dialysis against 5 M guanidine-hydrochloride, 50 mM Tris-HCl, pH 8.0 at room temperature. For quantification the dialyzed samples were submitted to A β 40 and A β 42 ELISAs as described in Section 2.2.

2.7 Quantification of A β peptides from leptomeningeal arteries

Two grams of leptomeningeal arteries, representing the anterior, middle, and posterior cerebral arteries showing advanced AVD lesions were dissected and pooled from 4 individuals with AD (average age 82.0 years; 2 males and 2 females) and from a group of 5 ND individuals without AVD (average age 86.6 years; 3 females and 2 males). 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 collected and submitted to size-exclusion FPLC (Superose 12) in 80% GDFA [13]. The A β containing fractions were pooled, the volume reduced and the samples dialyzed as described for the aorta specimens. The A β peptides were quantified by ELISA as described in Section 2.2.

2.8 Quantification of A β peptides in skeletal muscle

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

2.9 Quantification of A β peptides from liver

Liver specimens (1 g each) were obtained from 6 neuropathologically diagnosed AD cases (average age 83 years; 3 females and 3 males) and from 6 ND individuals (average age 87 years; 2 females and 4 males). The liver tissues were minced and thoroughly washed to eliminate entrapped blood as much as possible. Each liver specimen was homogenized in 90% GDFA, centrifuged (250,000 $\times$ g, 1 h at 4°C) and the acid soluble fraction collected and submitted to FPLC [13]. After volume reduction and dialysis (see aorta preparation), A β peptides were quantified by ELISA as described in Section 2.2.

3. Results

3.1 Plasma A β

All mean values in this study were compared using two-independent sample t-tests. The differences in A β 40 and A β 42 levels, the total A β levels (A β 40+A β 42) and the A β 42/A β 40 ratios between the AD and ND groups were not statistically significant (Table 1 and Figure 1). The AD (mean age 81.4, SD = 5.3) and ND (mean age 75.8, SD = 7.1) groups differed significantly in age ($p = 0.01$), but age did not correlate with plasma concentrations of A β 40, A β 42 or A β 40/42 ratio, within either the ND or AD groups (r squared values ranging from 0.00006 to 0.04). T-tests were performed without correction for multiple comparisons (e.g. Bonferroni), as this would have biased the results towards an increased rate of false positive findings. As the results with respect to plasma A β 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 ADAS-Cog, MMSE score, age or gender and *Apo E* genotype.

Amyloid- β 40 and A β 42 levels fluctuated widely among individuals over time in both AD subjects and ND controls (Figure 1). The amounts of A β 40 among individuals over the year of the study fluctuated from 659 pg/ml to 16 pg/ml and A β 42 levels varied between 149 pg/ml and 4.0 pg/ml. The average A β 42/40 ratios also varied substantially among individuals (see Tables 1 and 2).

3.2 Brain A β (gray matter)

Quantifying A β peptides in the cerebral cortex demonstrated the prevalence of A β 42 over A β 40 in AD individuals [2], with a high level of variability (Table 3). The mean value of A β 42 was 6,096 ng/g tissue (range: 1,047–19,680 ng/g; SD: 1,099) with A β 40 averaging 608 ng/g tissue (range: 26–3,434 ng/g; SD: 154). By contrast, in the ND control population the average amount of A β 42 was 784 ng/g tissue (range: 42–3,402; ng/g; SD 221) and for A β 40 was 209 ng/g tissue (range: 0–731 ng/g; SD: 59). There were significant differences in the amounts of cortical A β peptides observed between AD and ND (A β 42 $p < 0.001$; A β 40 $p = 0.022$) as well as between total A β ($p = < 0.001$). In those individuals with AD carrying the *Apo E* ϵ 4 allele the mean value for total A β was 8,373 ng/g of cortex while in those without the *Apo E* ϵ 4 the total A β mean was 5,631 ng/g ($p = 0.223$). In the case of the ND cohort, those individuals carrying the *Apo E* ϵ 4 had a total A β average of 2,178 ng/g of cortex whereas those without the *Apo E* ϵ 4 allele had a mean of 783 ng/g of tissue ($p = 0.043$). However, this marginally significant value may reflect the fact that only 3 heterozygous individuals out of 20 harbored an *Apo E* ϵ 4 allele.

3.3 Brain A β (white matter)

Investigation of the A β peptide levels in WM revealed that in AD the mean values for A β 42 and A β 40 were 1,135 ng/g of tissue (range: 323–3,313 ng/g; SD: 895) and 1,069 ng/g of tissue (5.0–4,149 ng/g; SD: 1601) [13], respectively (Table 3). The highest values of A β among the 10 AD subjects were exhibited in two individuals carrying the *Apo E* ϵ 4/ ϵ 4 genotype with A β 42 levels of 1,624 ng/g and 3,313 ng/g and A β 40 4,149 ng/g and 4,030 ng/g of WM. The ND cohort, on the other hand, had an A β 42 mean of 418 ng/g of tissue (range: 122–880 ng/g; SD: 233) and for A β 40 111 ng/g of tissue (range: 0–272 ng/g; SD: 77). The differences between the AD and ND groups were significant for both: A β 42, $p = 0.05$ and A β 40, $p = 0.02$. The difference between the total A β values in AD and ND groups was also statistically significant ($p = 0.02$).

3.4 Platelet A β

The A β 40 and A β 42 peptides were quantified in a pool of platelets in their quiescent and activated forms from 5 healthy individuals. In the latter case, the levels of A β were measured in the platelets and their medium, since activation is followed by immediate secretion of by-products into the medium. The A β 40 and A β 42 peptides in the quiescent platelets yielded 83.8 ng/ml and 1.7 ng/ml of packed platelets, respectively. In the case of the activated platelets these peptides represented 56.8 ng/ml and 1.6 ng/ml, respectively (Table 3).

3.5 Aortic A β

In the aortic walls with advanced atherosclerotic lesions, A β 40 was present at 75.3 ng/g of tissue and A β 42 at 0.7 ng/g of tissue, higher levels than those observed in aorta samples with minimal atherosclerotic lesions (fatty streaks) which contained 31.4 ng/g of tissue and 1.0 ng/g of tissue for A β 40 and A β 42, respectively (Table 3).

3.6 Leptomeningeal vascular A β

There were significant differences between the amounts of A β peptides present in the leptomeningeal arteries with and without atherosclerotic plaques. In the former, the A β 40 levels were 113.1 ng/g of tissue and A β 42 amounted to 28.0 ng/g of tissue, while in the latter these corresponded to 17.3 ng/g and 5.3 ng/g of tissue, respectively (Table 3).

3.7 Skeletal muscle A β

Amyloid- β peptides in the AD and ND groups from skeletal muscle demonstrated a lesser degree of variability [2]. The differences between AD and ND control regarding the A β 40 were borderline significant ($p = 0.067$) while there was a statistically significant difference with respect to the A β 42 ($p = 0.010$) and total A β ($p = 0.019$) (Table 3). However, values between the AD and ND groups and between individuals exhibited substantial variance. The *Apo E* ϵ 4 alleles, although more numerous in the AD group (10 out of 46 alleles) than in the ND cohort (3 out of 40 alleles) were not directly correlated with the levels of A β 40 or A β 42 peptides in skeletal muscle.

3.8 Liver A β

Amyloid- β 40 and A β 42 in the postmortem liver of ND individuals were on the average 67.5 ng/g of tissue (range: 18.8–94.6 ng/g) and 15.5 ng/g of tissue (range: 6.4–20.6 ng/g) while the A β 40 and A β 42 in AD subjects averaged 8.6 ng/g of tissue (range: 6.7–11.7) and 1.7 ng/g (range: 0–2.6) of tissue, respectively. The ND control patients' liver tissue contained 8-fold more total A β 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 A β 40 and A β 42 levels demonstrated wide temporal variation within and among the individuals involved in the present investigation. The overall mean values for A β 40 and A β 42 in the longitudinal study were 384 pg/ml and 132 pg/ml, respectively. The overall mean values for A β 40 and A β 42 in the therapeutic study were 319 pg/ml and 179 pg/ml, respectively. These two independent plasma analyses showed virtually equivalent levels for total A β peptides: longitudinal = 517 pg/ml and therapeutic = 497 pg/ml. In both investigations there were no statistical correlations noted between A β values and MMSE scores, diagnoses, age or gender. In support of our observations are several previous biomarker studies in which the plasma A β levels were not correlated with the diagnosis, medications or with Apo E genotype [15;16] (reviewed in [1]).

The spread of plasma A β values may be due to the amphoteric and amphipathic structure of A β peptides that results in their avid binding to a variety of plasma and membrane proteins. At physiological concentrations of human serum albumin (approximately 40 mg/ml), this molecule can bind more than 95% of A β 40/42 peptides at a stoichiometry of 1:1. Kinetic studies have demonstrated that 1 ml of fresh plasma from healthy individuals, spiked with 5 ng or 20 ng A β 40 or A β 42 results in the recovery of 36% of A β 1-40 and 26% of A β 1-42 after a 1 h incubation [10]. When variable amounts of washed erythrocytes are diluted with PBS to hematocrit concentrations of 10% to 50% and are incubated with 5 ng/ml of A β 40 or A β 42, the amount of free A β peptides decreased as the hematocrit values increased. A large number of plasma proteins including immunoglobulins, apolipoprotein J, Apo E, complement C1q, C4, C3, C5 and C6, transthyretin, apoferritin, amyloid-P component and α 2-macroglobulin bind and mask A β peptides [9;10;17]. Freshly purified plasma lipoproteins are capable of sequestering 94% of circulating A β peptides [9]. In particular Apo E, a ligand for various lipoprotein receptors bound to the surface of circulating low density lipoprotein (LDL), has high affinity for A β peptides [18;19].

Plasma cholesterol appears to play a pivotal role in A β chemistry. The production of lipoproteins by liver and brain are likely to cause concomitant fluctuations in plasma levels of free and bound A β . 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 to ND controls [20;21].

Furthermore, in A β PP 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 A β PP 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 A β peptides, predominately A β 40. Likewise, leptomeningeal arteries with AVD pathology have larger amounts of A β 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 A β 42 is highly correlated with AD pathology [35]. The severity of circle of Willis and leptomeningeal arterial stenosis caused by AVD significantly correlates with the neuropathological lesions of AD [25–28]. Inferring a functional, pathologic role for the A β 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 A β PP and A β peptides. Although there are multiple mechanisms for uptake and clearance of A β 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 A β , respectively [38–41]. Amyloid- β is also eliminated from the brain through the periarterial spaces of the cerebral vasculature that shunt A β peptides into the lymphatics of the head and neck and finally back into the systemic venous circulation [42; 43]. This suggests the clogging of the periarterial spaces in cortical and leptomeningeal arteries by fibrillar A β deposition is probably one of the more devastating pathological hemodynamic events in AD [44]. In AD, the WM contains 4-fold more ‘soluble’ total A β (mean 2,200 ng/g tissue) than ND controls (mean 520 ng/g tissue) [13]. It can be postulated that soluble A β 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 A β peptides in the circulation. These structures mostly contain A β ending at residue 40, with a small amount of A β 42, in both the quiescent and activated states. Our *in vitro*, thrombin-collagen activated platelets released A β 40 and A β 42. If this phenomenon occurs *in vivo*, platelets contribute to the pool of circulating A β peptides [45;46]. Intriguingly, our activated platelets apparently contain ~30% less A β 40 than the quiescent ones. Although we do not have an explanation for this decay, it is possible that platelet activation and release of A β stimulates aggregation or sequestration of these peptides, thus escaping detection by the immunoassay. Alternatively, A β may intervene to facilitate platelet aggregation [47]. Platelets express A β PP₆₉₅, A β PP₇₅₁ and A β PP₇₇₀ 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]. A β PP and A β peptides have been observed in atherosclerotic plaque macrophages that have engulfed platelets in areas of neo-vascularization [53;54]. Although platelets and circulating A β may be

a significant source of these peptides in the atherosclerotic lesions, it is possible that additional A β peptides originate directly from the arterial tunica media myocytes and endothelial cells that also express the A β PP 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 A β peptides [2;60;61]. The production of A β 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 A β 42 and total A β between AD and ND control populations. This observation suggests that AD might have systemic manifestations. Skeletal muscle also generates longer A β peptides ending at residues 44, 45 and 45 [2]. The potential contribution of skeletal muscle A β to the circulating pool should be considered since the total muscle mass represents about one third of the body weight.

The A β PP/A β peptides produced in different tissues may also have different half lives and degradation rates since these molecules are substrates for a large variety of proteolytic enzymes (reviewed in [64]). The liver is the major organ responsible for A β clearance from plasma, capable of capturing up to 90% of the circulating A β peptides, a fraction of which is degraded with the remainder removed through the bile [65]. A comparatively smaller amount of A β is eliminated through the kidneys and secreted in the urine [66]. Additionally, 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 A β in the AD cases may be due to liver failure in the advanced stages of the disease.

In summary, A β 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 A β sources and sinks may help to understand AD pathology and dementia. It will also help to elucidate the relationship between A β and cholesterol metabolism as well as the role of A β in AVD. Large individual fluctuations in plasma A β values are the most important observation in our longitudinal studies. When assessing A β therapeutic interventions, the potential multiple contributions to the plasma A β pool as well as the time and conditions of sampling physiological fluids for A β evaluation need to be considered. For example, CSF specimens demonstrated wide ranging A β levels within individuals along a period of 36 h [68]. We are aware that investigations of this type have limitations including small sample size due to difficulties in recruitment and attrition of participants and patient physical conditions. In addition, A β peptides fluctuations due to 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 A β immunotherapy [42;70]. Peripheral A β production appears to contribute to brain amyloid through transport into the CNS and correspondingly the brain A β contributes to the pool in circulation [37]. Therefore, brain as well as plasma A β levels are the consequence of the intricate relationships that exist among several interacting A β peptides 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 A β PP/A β exist as neatly isolated entities. The long term CNS and systemic consequences of A β immunization and secretase inhibitory treatments have the potential to disturb a wide range of cellular and systemic functions, in which A β PP metabolites and A β are essential [71–74]. As clinical trials advance, efforts should be undertaken to recognize adverse events both within

and outside the brain proper. Although A β 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 may represent a mechanism of defense whereby excessive harmful levels of soluble A β peptides are inactivated into fibrillar core structures surrounded by glial cells [74–76]. Preventing or dissolving these deposits may be injurious to the brain. Understanding the dynamic balance between A β pools and their function may add clarity and suggest new routes to improve AD therapeutic strategies.

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Abbreviations

Aβ	amyloid-beta
AβPP	amyloid-beta precursor protein
ACD-A	anticoagulant citrate dextrose solution – formula A
AD	Alzheimer's disease
ADAS-Cog	Alzheimer's Disease Assessment Scale-cognitive subscale
Apo E	apolipoprotein E
AVD	atherosclerotic vascular disease
CNS	central nervous system
CERAD	consortium to establish a registry for Alzheimer's disease
ELISA	enzyme-linked immunosorbent assay
FPLC	fast protein liquid chromatography
GDFA	glass distilled formic acid
GM	gray matter
KPI	Kunitz protease inhibitor

LDL	low-density lipoprotein
LRP	low-density lipoprotein receptor related protein
MMSE	mini-mental state examination
ND	non-demented
NINDS-ADRDA	National Institute of Neurological Disorders and Strokes, and Alzheimer's Disease and Related Disorders
PN2	protease nexin-2
PO	per os, orally
RAGE	receptor for advanced glycation end products
SD	standard deviation
SHRI	Sun Health Research Institute
Tg	transgenic
WB	washing buffer
WM	white matter

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Figure 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.

Figure 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.

Table 1
Longitudinal study of plasma individual Aβ values and patient demographics
The Aβ amounts represent the average of those individuals completing 3 and 4 time points: baseline, 3, 6, 12 months.

ND controls (n = 21)	Age (years)	Gender	ApoE genotype	MMSE	Aβ40 pg/ml	Aβ42 pg/ml	Total Aβ pg/ml	ratio Aβ 42/Aβ 40
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
AVG	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)	Age (years)	Gender	ApoE genotype	MMSE	Aβ40 pg/ml	Aβ42 pg/ml	Total Aβpg/ml	ratio Aβ 42/Aβ 40
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

ND controls (n = 21)	Age (years)	Gender	ApoE genotype	MMSE	Aβ40 pg/ml	Aβ42 pg/ml	Total Aβ pg/ml	ratio Aβ 42/Aβ 40
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
AVG	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 vs AD <i>p</i> =	0.011			<0.001	0.088	0.448	0.075	0.292

ND = non-demented; ApoE = apolipoprotein E; MMSE = mini-mental state examination; avg = average; F = female; M = male; AD = Alzheimer's disease; SD = standard deviation; *p* = unpaired, 2-tailed, t-test

Table 2
Therapeutic study of plasma Aβ values and patient demographics
All cases are probable AD. These values represent the average of 3 readings per week.

Baseline Stable-Donepezil (n=8)							Follow-up Stable-Donepezil (n=8)				
	Age (years)	Gender	MMSE	Aβ 40 pg/ml	Aβ 42 pg/ml	Total Aβ pg/ml	ratio Aβ42/Aβ40	Aβ 40 pg/ml	Aβ 42 pg/ml	Total Aβ pg/ml	ratio Aβ42/Aβ40
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
AVG	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 vs follow-up p=				0.966	0.790	0.892	0.819				
Baseline Donepezil-Initiation (n=20)							Follow-up Donepezil-Initiation (n=20)				
	Age (years)	Gender	MMSE	Aβ 40 pg/ml	Aβ 42 pg/ml	Total Aβ pg/ml	ratio Aβ42/Aβ40	Aβ 40 pg/ml	Aβ 42 pg/ml	Total Aβ pg/ml	ratio Aβ42/Aβ40
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

Baseline Stable-Donepezil (n=8)				Follow-up Stable-Donepezil (n=8)							
	Age (years)	Gender	MMSE	Aβ 40 pg/ml	Aβ 42 pg/ml	Total Aβ pg/ml	ratio Aβ42/Aβ40	Aβ 40 pg/ml	Aβ 42 pg/ml	Total Aβ pg/ml	ratio Aβ42/Aβ40
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
AVG	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 vs follow-up <i>p</i> =				0.411	0.701	0.766	0.433				

MMSE = mini-mental state examination; avg = average; F = female; M = male; SD = standard deviation; *p* = unpaired, 2-tailed, t-test

Table 3
Aβ Peptide Levels in Human Tissues

	Aβ 40 ng/g	Aβ 42 ng/g	Total Aβng/g
Brain-gray matter			
AD (n = 23)	608	6096	6704
ND (n = 20)	209	784	993
Brain-white matter			
AD (n = 10)	1069	1135	2204
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

AD = Alzheimer's disease; ND = non-demented; Q = quiescent; A = activated; AVD = atherosclerotic vascular disease