CHAPTER – IV

Plasma and Red blood cells αβ 1-42 levels
4.1 Introduction

Aβ has been a central focus of many AD researchers since its initial purification from dense core plaque deposits in AD brain (Glenner and Wong, 1984). According to the amyloid cascade hypothesis, the pathogenesis of AD involves a chronic imbalance between the production and clearance of Aβ, especially Aβ1–42, which is most prone to aggregate (Hardy and Selkoe 2002). Evidence for disturbance in the Aβ metabolism, especially in the early or preclinical stages of the disease, would thus lend important support to the amyloid hypothesis in sporadic disease. To date, there are three possible methods to monitor Aβ production or deposition in vivo in cases of sporadic AD, including analysis of Aβ species in CSF (Blennow and Hampel 2003), live visualisation of amyloid plaques in AD brains with PET (Klunk et al., 2004) and measurement of Aβ in peripheral blood (Irizarry 2004). Determination of Aβ in biological fluids is a compelling candidate as biomarkers for AD; especially, blood-plasma would be preferable in clinical settings for the diagnosis of AD. The development of sensitive ELISAs for Aβ 1-40 and Aβ1-42 enabled the detection and quantitation of Aβ in human blood.

4.2 Plasma Aβ

The source of Aβ 1-40 and Aβ1-42 in plasma is unknown, brain has been considered the origin of deposited Aβ in the brain of patients with AD, but peripheral sources such as blood may also be important (Skovronsky et al, 2001), even though how Aβ deposition in the brain affects plasma concentrations of Aβ is still unclear. Aβ is derived from proteolytic cleavage of APP. APP is produced by a variety of cell types in the brain and elsewhere, but the origins of Aβ brain deposits in AD and deposits in the cerebral vessels are uncertain. Amyloid deposits in cerebral vessels may be derived from circulating Aβ (Chen et al., 1995). Some have
suggested that the source of parenchymal cerebral amyloid deposits may originate in the periphery (DeMattos et al., 2002). Platelets contain high levels of membrane-associated and soluble forms of APP, which, when cleaved, can release Aβ (Van Nostrand et al., 1990; Cole et al., 1990; Li QX et al., 1998). Platelets may also be the source of Aβ detected in whole blood (Urmoneit et al., 1997) because Aβ, like APP, is also released upon platelet activation (Smith 1997). If plasma Aβ originates from tissues other than brain, there may not be an association between plasma Aβ levels and Aβ deposited in the brain. However, investigators have shown that Aβ–Apo E and Aβ–Apo J complexes cross the blood brain barrier (Zlokovic et al., 1993; Zlokovic et al., 1994); thus, Aβ present in plasma may contribute to the development of Aβ deposits in the brain.

Recent studies demonstrate that Aβ is transported both ways across the blood brain barrier (BBB) (DeMattos et al., 2002), resulting in a dynamic equilibrium between brain Aβ and circulatory Aβ. The steady-state level of Aβ depends on the balance between production and clearance. The transport of Aβ across the BBB is mainly mediated by receptors i.e. receptor for advanced glycation end products (RAGE) and lipoprotein receptor-related protein (LRP) on endothelial cells. Aβ in the extra- and intra-cellular space can be degraded by enzymes [i.e. neprilysin (NEP) and insulin-degrading enzyme (IDE)]. Peripheral anti-Aβ antibodies and Aβ-bindable substances are able to enter the brain at low levels, where they prevent Aβ aggregation and resolve Aβ fibrils. By binding to peripheral Aβ they also exert as a peripheral sink to promote the efflux of Aβ from the brain and disrupt the Aβ equilibrium between the brain and the blood, resulting in the clearance of Aβ from the brain as seen in Figure 4.1. (Wang et al., 2006).
Previous studies report that plasma concentrations of both Aβ1–40 and Aβ1–42 are increased with age over 65 years (Mayeux et al., 2003) and are increased in familial AD with presenilin or APP mutations as well as in Down syndrome with APP triplication (Scheuner et al., 1996; Schupf et al., 2001). Also, plasma Aβ is increased in first degree relatives of people with AD, who are at an increased risk of developing the disease (Younkin 1998). It has also been reported that plasma Aβ 1-40 and Aβ1-42 levels represent a heritable trait that has been used in a linkage analysis of chromosome 10 to AD, suggesting that variant forms of a gene may be associated with elevated levels of Aβ1-42 (Ertekin–Taner et al., 2000; 2001), thus raising the possibility that sporadic cases of AD might be associated with detectable and diagnostic changes in the plasma levels of Aβ.
In cross-sectional studies in patients without any known mutations, plasma Aβ levels were either higher (Mehta et al., 2000; Assini et al., 2004; Sobow et al., 2005) lower (Pesaresi et al., 2006) or unchanged (Tamaoka et al., 1996) in individuals with AD or MCI. Only three longitudinal studies have addressed this issue. Of these, one study (Mayeux et al., 2003) reported an association between high plasma Aβ1-42 levels and risk of AD, while the other studies (Van Oijen et al., 2006; Graff-Radford et al., 2007) reported an association between risk of AD and increased levels of Aβ1-40 and a low Aβ1-40: Aβ1-42 ratio, but not between AD risk and Aβ1-42 levels. Given the variability of the findings in previous studies and the potential implications of Aβ levels as a marker of disease risk, the present study investigated the relationship between plasma Aβ1-42 levels in probable/possible sporadic AD and age matched non demented healthy subjects.

4.3 Red blood cells (RBC’s) – Aβ1-42

Circulating blood cells (RBCs) are exposed to soluble Aβ1–40/Aβ1–42 with nanomolar levels of Aβ1–40/42 detected in blood (Seubert et al., 1992). RBCs have also been reported to sequester monomeric Aβ with binding detected even at physiological levels of 5 ng/ml (Kuo et al., 2000). Further, it has been shown that RBCs bind Aβ fibrils in vitro and Aβ uptake by RBCs causes swelling of cells, a decrease in cell deformability and increased oxidative stress (Jayakumar et al., 2003). These RBCs are rapidly removed, as expected, from the circulation with much of the amyloid fibrils ending up in spleen phagocytes (Ravi et al., 2005). Moreover, several studies have documented alterations in RBCs in AD, including perturbations in the physical state of membrane proteins (Mark et al., 1995), irregular distortion of RBCs (Goodall et al., 1994), and abnormal cellular aging with increased IgG binding and breakdown of band 3 protein (Bosman et al., 1991). Recent studies have demonstrated that circulating Aβ1-42 is subject to complement
C3b-dependent adherence to complement receptor 1 (CR1) on erythrocytes, a classical set of mechanisms by which pathogens and proteins recognized as foreign are cleared from the bloodstream (Rogers et al., 2006). Based on the strong red cell–Aβ interactions demonstrated by previous studies the present study was aimed to detect the levels of Aβ1–42 bound RBCs in probable/possible sporadic AD compared to age matched healthy control subjects and to hypothesize that clearance of Aβ1-42 in the blood can be used as a peripheral biochemical marker for sporadic AD.

4.4 Materials and Methods

4.4.1 Sources of reagents and fine chemicals

Sodium dodecyl sulphate (SDS), Bovine serum albumin (BSA), Sodium chloride (NaCl) was obtained from SRL laboratories, India. Nonidet P-40, sodium azide (NaN₃) was obtained from Sigma chemical Co., (St. Louis, USA). Aβ1–42 polyclonal antibodies were obtained from Calbiochem, USA. Fluorescein isothiocyanate (FITC) conjugation kit was obtained from Genei, Bangalore, India. All other chemicals were of analytical grade and double distilled water was used in all experiments.

4.4.2 Measurement of plasma Aβ1–42 by ELISA

Aβ1–42 was measured in EDTA plasma samples by sandwich ELISA as described by (Suzuki et al., 1994; Tamaoka et al., 1994, Tamaoka et al., 1995, Kosaka et al., 1997). In detail, Aβ1–42 synthetic peptide standards were diluted in casein buffer (0.1% casein in phosphate-buffered saline (PBS), 5mM EDTA, 1mM PMSF, and 0.8μg/ml pepstatin) to 2 pg/ml. In a NUNC flat bottom high-binding 96-well microtiter plates 5 ug/ml of Aβ1–42 antibodies were coated with 100 μl/well of coating buffer (10mM Tris, 10mM NaCl and 10mM NaN₃, pH 8.5) and incubated
overnight at 4°C. Following the initial binding the microtiter plate was incubated with 150µl/well of 0.1% casein in PBS for 30 minutes at 37°C to reduce non-specific binding. 100 µl of a standard peptide, plasma sample from each patient and controls were applied in coated ELISA plates and incubated at 4°C for 24 hours. The plates were washed with phosphate-buffered saline and incubated with 100 µl/well horseradish peroxidase (HRP)-labeled BC05 monoclonal antibodies for 3 hours at room temperature. After another wash, assays were developed with TMB/H₂O₂ substrate. The reaction was allowed to proceed for a maximum 15 minutes at room temperature, after which the color developed was stopped with 50 µl/well of 2N H₂SO₄ and the plates were read using microtiter plate reader (Bio-rad, USA) at 450 nM. Aliquots of pooled control samples with varying ranges of Aβ (low and high) were frozen and used as quality control samples.

4.4.3 Conversion of erythrocytes to spherocytes

Formalin fixed spherical erythrocytes were prepared by adding 0.1 ml of whole blood to 1.0 ml containing 50 µg/ml sodium dodecyl sulphate (SDS) and 1 mg/ml BSA. After one minute incubation the sphered cell mixture was added to a fixative solution containing 9.7 ml of saline, 0.3 ml of formalin (37% formaldehyde) and 10 µg/ml SDS. After 1 hour and 30 minutes an additional 0.8 ml of formalin was added to the mixture and the suspension was allowed to fix overnight. All fixation steps were performed at room temperature. The fluid cells were washed twice with staining buffer and resuspended and stored in 1 ml of staining buffer giving a final cell concentration of about 5x10⁸ cells/ml. Staining buffer contains the following; 10mM sodium phosphate (pH 7.2), 0.15 M NaCl, 5 mg/ml BSA, 0.01 % Nonidet P-40 and 100 µg/ml sodium azide (NaN₃). Fixed cells were stored at 2-4°C for upto two weeks with minimal effects on antibody binding. (Figure 4.2)
4.4.4 Fluorescein isothiocyanate (FITC) conjugation

Based on the F/P ratio required, FITC:Aβ1–42 polyclonal antibodies were prepared in a molar ratio of 10:1 in a 20 ml centrifuge tube covered with aluminum foil to protect from light. After the addition of FITC, the fluorescein-Aβ1–42 antibody conjugate was incubated for 2 hours at room temperature with gentle mixing. At the end of 2 hours 1/20 volume of 1M ammonium chloride solution was added to the fluorescein-Aβ1–42 antibody conjugate and incubated again for 1 hour at room temperature.

4.4.5 Separation of labeled Aβ1–42 antibody from the free dye

The fluorescein labeled Aβ1–42 polyclonal antibodies was purified from the unconjugated fluorescein using sephadex G-25 column. In brief, 1 ml of fluorescein labeled Aβ1–42 antibody was loaded to the top of column gel bed and eluted the column with 3 ml of 1X PBS. Monitored the absorbance of each fraction using low pressure liquid chromatography (Bio-rad, USA) (Figure 4.3). Based on the bands visible at 280 nm the fraction was collected and the F/P molar ratio was determined.
by measuring the absorbance at 280 nm and at 495 nm. 1 % BSA and 0.1% sodium azide was added to the conjugate and stored at 2-8°C.

![Absorbance of gel filtration of FITC conjugate](image)

**Figure 4.3 Absorbance of gel filtration of FITC conjugate**

### 4.4.6 Binding of FITC labeled Aβ1–42 antibody to RBCs

The fixed cells were resuspended in staining buffer and washed twice. To 100 µl of fixed cells 100 µl of FITC labeled Aβ1–42 antibody was added and the cells were incubated at room temperature for 3 hours in the dark for binding of Aβ1–42 on the red cell membrane. The cells were then washed twice with staining buffer to remove the unbound FITC labeled Aβ1–42 antibody. The quantification of the number of molecules of Aβ1–42 bound per erythrocyte was determined by flow cytometry. Results were expressed in arbitrary units as the mean cell fluorescence intensity.

### 4.4.7 Flow cytometry

The samples were analyzed on an FACSCAN flow cytometer (Becton Dickinson Immunocytometry Systems; San Jose, CA) equipped with a 15-mW argon laser. Red blood cell fluorescence was measured at an emission wavelength of
530 nm with excitation at 488 nm. A total of 50000 events were measured for each RBC sample. Both the fluorescence and light scattering properties of the treated cells were measured. The final events were recorded as histograms and dot-plots. The percentage of gated events and differentially stained cells were obtained using cell-quest software.

4.5 Data analysis and statistical evaluation

Results were expressed as mean ± SD. Clinical and laboratory results were assessed using paired t tests. Differences were considered statistically significant at $P<0.05$ (2 tailed). Fisher Protected Least Significant Difference (PLSD) test was applied to determine main effects within groups and determined the degree of linear correlation between age and each of the parameters. The source of variation was assessed by unpaired student’s t-test for normally distributed parameters. The association of variables was studied by Pearson correlation test. The mean plasma Aβ1–42 levels were determined for each individual from two duplications (SD among duplications, < 8%). Statistical analysis was performed using SPSS 14.0 version.

4.6 Results

In this study, we have evaluated a group of subjects including patients affected by sporadic AD at different stage of severity and age matched healthy control subjects matched with the other groups for demographic characteristics. Demographics and clinical characteristics of the study population is given in chapter I (Table1.4). Mean plasma concentration of Aβ1–42 in probable/possible AD and age matched healthy control subjects are presented in Table 4.1.
Table 4.1  Mean values of the plasma and RBC $\alpha\beta1$–42 peptides in probable/possible AD and age matched non demented healthy subjects.

<table>
<thead>
<tr>
<th>ANALYTE</th>
<th>Probable/Possible AD</th>
<th>Age matched healthy controls</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma $\alpha\beta1$–42 (pg/ml)</td>
<td>22.06 ± 9.81</td>
<td>19.23 ± 5.42</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>RBCs – $\alpha\beta1$–42 (%)</td>
<td>24.31 ± 4.63</td>
<td>27.94 ± 6.1</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
</table>

The plasma $\alpha\beta1$–42 levels did not statistically differ from probable/possible AD and age matched non demented healthy subjects ($p$>0.05). Mean plasma $\alpha\beta1$–42 levels were higher in probable/possible AD compared to age matched non demented healthy subjects but there was substantial individual variability and overlap between these groups. However, approximately 7% of the patients with probable/possible AD had increased concentrations of $\alpha\beta$ 1-42 in plasma (Figure 4.4). Correlation analysis between age and plasma $\alpha\beta1$–42 levels in probable/possible AD did not reveal any significance (Figure 4.5); the observed differences were also unrelated to subjects gender, but control subjects showed a positive correlation on plasma $\alpha\beta1$–42 levels with increase in age ($r = 0.34; \ p < 0.05$) (Figure 4.6).
Figure 4.4  Plasma Aβ1-42 levels in patients with probable/possible AD and age matched non demented healthy subjects.

Figure 4.5  Correlation curve between Age and plasma Aβ1-42 levels in probable/possible sporadic AD
Figure 4.6  Correlation curve between Age and plasma Aβ l-42 levels in age matched non demented healthy subjects.

Aβ1–42 adhered to RBC were analysed by flow cytometry (Figure 4.7; 4.8). The mean RBC bound Aβ1–42 levels in probable/possible AD and age matched healthy control subjects are presented in Table 4.1.

Figure 4.7  Flow cell distribution RBC’s reacted with FITC conjugate (Aβ1-42 antibody) in probable/possible AD patients.
On assessment of mean RBC bound Aβ1–42 levels in probable/possible AD with age matched healthy subjects found no significance ($p>0.05$) (Figure 4.9). Although when individual RBC bound Aβ1–42 values were compared there was minimal overlap among the probable/possible AD and age matched healthy controls. Correlation analysis between age and RBCs-Aβ1–42 levels in probable/possible AD and age matched non demented healthy subjects did not reveal any significance (Figure 4.10; 4.11); the observed differences were also unrelated to subject’s gender.
Figure 4.9  RBC Aβ 1-42 levels in patients with probable/possible AD and age matched non demented healthy subjects.

Figure 4.10  Correlation curve between Age and RBC Aβ 1-42 levels in probable/possible sporadic AD
4.7 Discussion

Since Aβ1-42 may be important in aggregation of Aβ (Jarrett et al., 1993; Tamaoka et al., 1994) and deposition of Aβ is one of the pathological hallmarks of AD, the present study measured Aβ 1-42 in plasma and RBCs as a biochemical marker for the diagnosis of sporadic AD. This study showed that the plasma Aβ1-42 concentrations did not significantly differ among the probable/possible AD and age matched healthy subjects. There was substantial individual variability and a considerable overlap among plasma values of Aβ1-42 in these groups. These results suggest that plasma Aβ may not be a diagnostic marker for sporadic AD. However, in a few probable/possible AD cases, the percentages of plasma Aβ1-42 were higher. Several lines of evidence have shown that the cross-sectional studies that previously examined the relation between plasma Aβ and dementia yielded inconsistent results (Iwatsubo 1998; Mehta et al., 2000; Assini et al., 2004). Since plasma Aβ concentrations are believed to change in the course of the dementia process, longitudinal observations are more useful to study whether Aβ concentrations are
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associated with an increased risk in asymptomatic people. Thus far only three longitudinal studies have been reported that assessed the association between plasma Aβ and risk of dementia. Of these, one study (Mayeux et al., 2003) reported an association between high plasma Aβ1-42 levels and risk of AD, while the other studies (Van Oijen et al., 2006; Graff-Radford et al., 2007) reported an association between risk of AD and increased levels of Aβ1-40 and a low Aβ1-42: Aβ 1-40 ratio, but not between AD risk and Aβ1-42 levels. Interestingly, plasma Aβ levels seem to be associated not only with AD but with other types of dementia. Currently only one prospective longitudinal study has reported a significant association between the plasma Aβ1-42: Aβ1-40 ratio and risk of VaD. This is in contrast with the Rotterdam Study, in which high levels of Aβ1-40 increased the risk of VaD. This association needs to be further investigated in other longitudinal studies.

Since there is evidence that plasma concentrations of Aβ change during the presymptomatic period of dementia, differences in timing of the Aβ measurements with respect to dementia diagnosis could be responsible for differences between studies. More prospective longitudinal population based studies with long follow-up of different types of dementia and repeated measurements of Aβ are needed to establish the value of plasma Aβ in identifying individuals at risk of early stage dementia and are needed to clarify the role of peripheral Aβ as a predictor of different types of dementia. However, the source of plasma Aβ is not known. APP is produced by various cells, including cells inside and outside the brain, such as platelets. Although it is still unclear whether the dementia disease process in the brain affects or is affected by plasma Aβ.

The present study also found no association between RBC bound Aβ1-42 levels in probable/possible AD and age matched healthy controls. However, this study suggests a dynamic relationship between Aβ in the plasma and erythrocyte. As
approximately 20% of the patients with probable/possible AD had decreased levels of Aβ1-42 in the erythrocytes. There was substantial individual variability and a considerable overlap among RBC Aβ1-42 levels in these groups. This might be due to individual variations in the capacity to transfer opsonized materials or immune complexes (i.e., pathogen–antibody complexes) to the erythrocyte compartment resulting in higher plasma levels (Schifferli et al., 1989).

The discrepancy between plasma and RBC Aβ1-42 values in these groups may also result from masking of the antibody epitopes of Aβ that will interfere with detection of true Aβ values in body fluids. It is reported that the amphoteric and amphipathic characteristics of the Aβ peptides endow these molecules with a capacity to interact with a large number of plasma proteins such as albumin, α2-macroglobulin, α1-antichymotrypsin, amyloid P component, complement proteins, transthyretin, apoferritin, apolipoproteins and lipoproteins (Biere et al., 1996; Kalaria et al., 1993; Strittmatter et al., 1993; Schwarzman et al., 1994; Matsubara et al., 1995; Webster et al., 1996; Matsubara et al., 1999; Kuo et al., 1999). The interaction of Aβ with heterologous proteins and lipids results in the masking of antigenic epitopes and interferes with detection of true Aβ values in body fluids using immunoassays. (Matsumoto et al., 1997; Kuo et al., 1999).

In conclusion, this study shows that there is a lack of definitive differences in plasma and RBC Aβ1-42 concentrations among the probable/possible AD and age matched healthy controls, indicating the lack of applicability of this assessment in the differential diagnosis of sporadic AD. Although we found no relationship between probable/possible AD and Aβ levels in the plasma and RBC, longitudinal studies will be necessary to determine conclusively whether there is a relationship between plasma Aβ and interaction of RBC- Aβ, apolipoproteins-Aβ and progression of AD. Such studies are particularly important to determine whether modulation of plasma Aβ may be a useful measure of disease modifying therapies.